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SOME OF THE EFFECTS OF CONCENTRATED
SPENT MEDIUM ON THE ACTIVITY OF
RESTING CELLS OF HYDROGENOMONAS EUTROPHA

by

WILLIAM SCOTT MOODY

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements for
the Degree of Master of Science
in the Department of Microbiology

State College, Mississippi

January 1970

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OF THE PAPERS OF COLLEGE
 AND THE UNIVERSITY OF
 THE UNIVERSITY OF THE STATE OF NEW YORK

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INTRODUCTION

Future manned space travel involves many problems related to closed ecological systems. One major problem is the provision of an adequate oxygen supply for the astronauts.

The electrolysis of water has been offered as a method of supplying oxygen in a closed ecological system. However, highly explosive hydrogen gas is produced by this method and the production of hydrogen gas raises many objections to its use. It is apparent that hydrogen gas must be converted into some non-explosive form in order for electrolysis to be considered as a source of oxygen.

Research with large batch cultures (Gentry, 1966) and continuous cultures (Foster, 1967; Schlegel and Lafferty, 1965) of Hydrogenomonas eutropha indicated that hydrogen gas can be converted successfully into cellular material. Hydrogenomonas eutropha seemed to be the ideal micro-organism for these studies since it can utilize hydrogen gas as its sole energy source for growth and reproduction. In addition, it can utilize carbon dioxide gas given off by the astronauts as its only source of carbon, thus eliminating another problem in closed ecological systems for future space flights.

Futhermore, H. eutropha has been found to be rich in protein (Canfield and Lechtman, 1964) which gives it potential value as human food. However, Waslien, Calloway,

and Margen (1969) found that feeding ruptured H. eutropha cells to human volunteers produced various gastrointestinal disturbances.

Continuous culture systems for growing H. eutropha are important to closed ecological systems if prolonged space flights are to be possible. Among the problems which arise with these systems is the accumulation of overflow culture. The term overflow culture refers to that portion of the continuous culture systems which is discarded as new nutrients are introduced.

Assuming that the cellular portion of the overflow culture can be converted into food, spent medium (the cell-free portion of the overflow culture) remains the major non-usable by-product of the regenerative life support system.

The spent medium will contain residual mineral salts, extracellular by-products from the organisms, (Brown, Cook, and Tischer; 1964) and water.

As pointed out by Proctor (1965), great savings can be made in life support systems by the recovery and reuse of water. It seems feasible that enough water could be recovered from the overflow culture to provide fresh nutrients for the continuous culture. It could also be electrolyzed to supply oxygen. Canfield and Lechtman (1964) attempted the electrolysis of water with the addition of H. eutropha cells, but failed in their attempts because of high sodium chloride concentrations.

This investigation was undertaken to show some of the effects of spent medium on the activity of resting cells of H. eutropha. Residual mineral salts and extracellular by-products will increase within limits, therefore the effects of concentrated spent medium were included in this study.

REVIEW OF THE LITERATURE

Recent advances in space exploration have stimulated increasing interest in the hydrogen-utilizing microorganisms, particularly in the genus Hydrogenomonas. These bacteria have been selected for possible use in bioregenerative life support systems because of their unique ability to utilize hydrogen gas as an energy source. As mentioned earlier, Hydrogenomonas species are capable of utilizing hydrogen gas as their sole source of energy and carbon dioxide gas as their sole source of carbon.

As early as 1839 de Saussure experimented with hydrogen-utilizing microorganisms. In searching for reasons for the low concentration of hydrogen gas in our atmosphere, de Saussure incubated soil cultures under conditions suitable for growth and demonstrated the disappearance of hydrogen and oxygen from the atmosphere.

About the same time, Immendorff (1892) reported similar results in his experiments. He carried the research a step further by proving that the disappearance of hydrogen gas was due to physiological occurrences. This was accomplished by introducing chloroform into one set of culture vessels at the beginning of an experiment. Hydrogen and oxygen consumption was prevented in the treated vessel. However, the uptake of hydrogen and oxygen did occur in untreated cultures.

Kaserer was credited (1906) with the first isolation of a hydrogen-utilizing microorganism. He incubated a soil sample in mineral salts medium under an atmosphere of hydrogen, oxygen, and carbon dioxide. He isolated an organism which he called Bacillus pantotropha (Hydrogenomonas pantotropha; (Breed, Murray, and Smith, 1957) from this enriched medium. In his investigations he found that the organism could also grow heterotrophically using a variety of organic compounds as carbon sources.

He also reported that the mechanism of hydrogen metabolism was unclear. He postulated that hydrogen gas was oxidized by carbon dioxide to a reduced organic compound.

Kiklewski (1906) is credited with the isolation of three new species of hydrogen-utilizing bacteria, Hydrogenomonas flava, Hydrogenomonas vitrea, and Hydrogenomonas agilis. Two of these organism, H. vitrea and H. flava, were sensitive to high oxygen tensions. He found that the growth of these two organisms was inhibited whenever the partial pressures of oxygen exceeded 0.1 atm. He also observed that all three organisms grew heterotrophically; but when transferred to autotrophic medium, they had a reduced capacity to oxidize hydrogen.

Another new species of hydrogen-utilizing bacteria, Hydrogenomonas eutropha, was isolated by Bovell (1957). He found that the oxy-hydrogen reaction of this bacterium was not greatly affected by oxygen tension. Hydrogen oxidation

occurred with oxygen pressures ranging from 0.5 to 0.6 atm. He also discovered that H. eutropha required ferrous iron in its growth medium in order to oxidize hydrogen.

Repaske (1962) described the nutritional requirements for H. eutropha. He observed that nitrogen could be supplied and used in the form of ammonia, nitrate, and urea. Optimum pH for growth was found to be from 6.4 to 6.7 and the optimum temperature for the growth of H. eutropha reported was 30° C. He obtained good growth with gas mixtures composed of 70% hydrogen, 20% oxygen and 10% carbon dioxide. However, he found that the rate of growth was dependent upon the oxidation state and the concentration of iron present. Ferrous iron was found to be superior to ferric iron in supporting growth.

Repaske's claim that H. eutropha possesses a definite iron requirement was substantiated by Lechtman, Goldner, and Canfield (1964).

Ruhland (1922) shed new light on the mechanism of hydrogen gas utilization by demonstrating experimentally that oxygen, not carbon dioxide, was the primary acceptor of hydrogen.

Kluyver and Manten (1942) conducted manometric experiments with resting cell suspensions of Hydrogenomonas flava. They found that hydrogen gas was oxidized only when the organisms were grown autotrophically. When grown heterotrophically the resting cells failed to use hydrogen, but hydrogen was consumed when these resting cells were given

organic substrates under an atmosphere of air. In addition, Kluyver and Manten reported that autotrophically grown cells of H. flava were unable to oxidize hydrogen gas with carbon dioxide as the sole hydrogen acceptor. However, these cells very efficiently oxidized hydrogen in the presence of oxygen. Thus Kaserer's theory that carbon dioxide was reduced by hydrogen was refuted. Kluyver and Manten concluded that hydrogen oxidation required a special catalytic system which was independent of the catalysts active in normal respiration processes. They also proclaimed that this system is present only when the organism is grown autotrophically, and that the ability to oxidize hydrogen is gradually lost by heterotrophically-grown cells.

The oxy-hydrogen reaction was not confined to the genus Hydrogenomonas as pointed out by Gest (1951). He conducted research with cell-free extracts of the photosynthetic bacterium Rhodospirillum rubrum in which he observed the oxidation of hydrogen in the dark.

Schatz and Bovell (1952) using manometric techniques showed that resting cells of a new hydrogen-utilizer, Hydrogenomonas facilis, reduced nitrate to nitrite with the subsequent oxidation of hydrogen to water.

Schatz (1952) undertook an independent study of the uptake of carbon dioxide, hydrogen, and oxygen by resting cells of H. facilis. The gas mixture for this manometric study was composed of 89% hydrogen, 1% carbon dioxide, and

10% air. The author determined oxygen uptake by alkaline pyrogallol absorption. Carbon dioxide uptake was determined from HCO_3^- by tipping in an acid solution and instantaneously halting metabolism. Hydrogen was determined directly from the manometer reading by multiplying by the flask constant. He used the oxygen constant K_{O_2} . The investigator justified this by showing that the solubility of both oxygen and hydrogen in water were very small and similar.

$$\alpha_{\text{O}_2}^{30^\circ} = 0.03 \qquad \alpha_{\text{H}_2}^{30^\circ} = 0.02$$

This term, α , is sometimes referred to as the "Bunsen Coefficient" and is expressed as ml of gas dissolved per ml of water.

In his investigation Schatz found that carbon dioxide fixation did not always occur.

Ultimately he determined that resting cells of H. facilis were unable to utilize the energy derived from the oxidation of hydrogen by nitrates for the fixation of carbon dioxide.

Wilson et al. (1953) confirmed the findings of Kluyver and Manton that in non-propagating cells of Hydrogenomonas, hydrogenase functions simultaneously with enzymes which bring about the oxidation of organic substrates. They cultured strains of H. facilis from a single organism autotrophically, heterotrophically, and in the presence of

both hydrogen and the organic substrate lactate. Heterotrophically grown cells did not oxidize hydrogen even though they were active on lactate.

Rittenberg and Goodman (1969) reported on the mixotrophic (i.e. combined autotrophic and heterotrophic substrates) growth of Hydrogenomonas eutropha. Mixotrophic conditions were established by the addition of lactate (4 to 8 μ moles/ml) to cultures growing autotrophically in an atmosphere of hydrogen, oxygen, and carbon dioxide (6:2:1). They found that specific growth rates of mixotrophic cultures were double that of autotrophic cultures, and that lactate disappearance paralleled growth. In addition, growth rates in mixotrophic cultures were significantly greater than those in heterotrophic cultures for equal lactate consumption. They concluded from these results that the enhanced growth was due to the simultaneous functioning of autotrophic and heterotrophic growth physiologies.

Atkinson and McFadden (1954) identified hydrogenase as the enzyme which activates molecular hydrogen. They stated that it is probably involved in all biological processes which liberate or consume hydrogen. The authors were particularly interested in the "Knallgas Reaction," or the reaction of hydrogen with oxygen which supplies energy for the reduction of carbon dioxide.

Atkinson (1955a), using manometric techniques, studied the adaptive oxidation of organic substrates by resting

cells of H. facilis. He found that autotrophically grown cells of H. facilis not only failed to use glucose, but also failed to adapt to it under air. However, cells which were harvested with little exposure to air, and were used immediately or stored in the cold, exhibited a typical adaptive pattern to glucose. This pattern consisted of a short lag period followed by a sharp increase in oxygen utilization in the presence of glucose. He concluded from this investigation that free glucose could not be formed in the autotrophic metabolism of H. facilis.

In another study, Atkinson (1955b) made an investigation into the effects of inorganic nitrogen compounds on the hydrogenase activity of H. facilis. He found ammonium chloride to be a suitable nitrogen source as it yielded a large increase in cells with a low cellular density. However, nitrate in a concentration of 0.03 to 0.05 M decreased the hydrogenase activity of autotrophically- and heterotrophically-grown cells. Autotrophic growth was further repressed at lower nitrate levels. The best growth on nitrate was obtained at a level of 0.003 M, while highest total hydrogenase activity was found either at this concentration or at 0.01 M. Thus, Atkinson concluded that nitrate was reduced to a strongly inhibitory compound. This compound is an intermediate in the conversion of nitrate to either an amino acid or to a side product.

Repaske (1962) reported that a 0.019 M concentration of ammonium chloride was the optimum for growth of H. eutropha.

Atkinson (1956) continued his research with the hydrogenase activity of hydrogen-utilizing microorganisms. He found that cyanide at a concentration of 2×10^{-2} M failed to inhibit the oxy-hydrogen reaction in intact cells of H. facilis, but the reaction was inhibited by cyanide at a concentration of 8×10^{-5} M.

At a constant partial pressure of hydrogen and constant levels of cyanide, gas uptake by cells of H. facilis was inhibited as the partial pressure of oxygen was increased. Moreover, he observed that under a hydrogen-oxygen atmosphere the rate of hydrogen uptake was almost independent of a cyanide concentration above 8×10^{-4} M. The rate of hydrogen uptake was completely independent of the hydrogen concentration and the time of preincubation with cyanide. The degree of inhibition was almost exclusively dependent upon the oxygen concentration.

Atkinson concluded from his research that hydrogenase forms an inactive compound or complex with oxygen, and that the compound is decomposed by a cyanide-sensitive enzyme. Furthermore, he assumed that the enzyme accumulated to detectable levels only in the presence of cyanide.

Wittenberger (1960) studied hydrogen oxidation and the electron transport system with an unidentified species of Hydrogenomonas and with Hydrogenomonas eutropha. Both of

these organisms were insensitive to high partial pressures of oxygen. Wittenberger obtained good growth with a gas mixture containing 70% hydrogen, 20% oxygen, and 10% carbon dioxide. Moreover, both organisms oxidized hydrogen with the subsequent reduction of methylene blue. He also reported that cell free extracts of both organisms contain a flavin and a cytochrome of the "C" type that were reduced under an atmosphere of hydrogen and reoxidized under vigorous aerobic conditions. The oxidation of cytochrome "C" suggests the presence of a cytochrome oxidase, but this author stated that he could not substantiate this speculation.

Wittenberger (1960) continued his investigation with the hydrogenases of an unidentified species of Hydrogenomonas and with H. eutropha. He found that H. eutropha produced a soluble hydrogenase, but it was associated with subcellular particles in Hydrogenomonas species. In addition, he reported that cell-free extracts of H. eutropha catalyze the reduction of diphosphopyridine nucleotide (DPN) in the presence of hydrogen, but not the reduction of triphosphopyridine nucleotide (TPN). Both resting cells and cell-free extracts of several Hydrogenomonas species were inactive with DPN or TPN. Furthermore, he found that the reduction of DPN by cell-free extracts of H. eutropha required the mediation of one or more unidentified compounds in addition to hydrogenase. Wittenberger surmised from his research that a metal or metal-enzyme was required for DPN

reduction along with the hydrogenase.

Repaske and Lizotte (1965) examined crude extracts of Hydrogenomonas eutropha for pyridine nucleotide coenzyme Q reductase activity. They found that these extracts contained menadione reductase that was specific for nicotinamide adenine dinucleotide (DPN).

Kuehn and McFadden (1968) were interested in the fate of ribulose-diphosphate carboxylase (RDPC) in facultative organisms growing heterotrophically. Other laboratories established that this enzyme catalyzed the primary fixation of carbon dioxide in autotrophic organisms. These workers found that fructose-grown cells of H. facilis and H. eutropha retained high levels of RDPC only when the following conditions were met: low aeration, the addition of FeCl_3 to the fructose medium, and cell harvest at or prior to the middle of the exponential phase of growth.

Cohen and Burris (1955) found that the addition of trace elements greatly reduced the generation time for H. facilis from 15.4 to 7.5 hours. They also reported one of the first large batch culture methods for growing Hydrogenomonas. The culture apparatus consisted of a ten-liter bottle containing eight liters of culture. This culture was vigorously aerated by a recirculating gas system. These authors obtained cellular yields up to 15 grams of cells for eight liters of medium in 16-18 hours.

Wittenberger and Repaske (1961) designed a mass-culture apparatus similar to the Cohen and Burris design in which

H. eutropha were grown in nine-liter quantities. The gas mixture, composed of 70% hydrogen, 20% oxygen, and 10% carbon dioxide, was constantly bubbled through the medium. With a one-percent inoculum, shake conditions, and 20-24 hours of incubation, they reported a cellular yield of 2.4-3.0 grams wet weight per liter of culture medium.

Foster and Litchfield (1964) initiated research on continuous cultures of H. eutropha. Their culture assembly, which followed the basic design of Ellsworth, Herbert, and Telling (1956), was fitted with sensors which monitored the artificial environment. Any deficiency in the environment was immediately relieved as the appropriate sensor signaled for the addition of a needed material.

Ammann, Reed, and Durichek (1968) conducted a feasibility study on the usefulness of Hydrogenomonas eutropha as a component of a regenerative life support system for manned space flights. Their results indicated that such a life-support system would require 60 liters of actively growing culture to balance the gas exchange for one man. A more detailed investigation revealed that the system demanded 18.7 ± 1.2 liters of fresh medium every 24 hours to maintain a constant predetermined cell population.

MATERIALS AND METHODS

Culture

The culture used in this study was obtained from Dr. David Wilson Cook¹. The culture has since been maintained in a mineral salts medium under an atmosphere of hydrogen, oxygen, and carbon dioxide.

Culture Media

The mineral salts medium used in this study is a modification of that described by Repaske (1962). Stock solutions were prepared as follows:

Solution A: Phosphate Buffer. The following salts were dissolved in about 80 ml of distilled water² in a volumetric flask. The volume was adjusted to 100 ml and the solution was autoclaved at 121° C for 15 min at 15 psi.

Na₂HPO₄ 21.690 g

KH₂PO₄ 13.260 g

Distilled water. 100 ml

When this solution was diluted 1:100 ml with distilled water, it yielded a buffer concentration of 0.025 M phosphate at pH 7.0.

¹Present location: Gulf Coast Research Laboratories, Ocean Springs, Mississippi.

²Distilled water was produced using a Barnstead Model EL-2 still and stored in polyethylene carboys until used. The water contained 2.5 mg/l total solids reported as NaCl.

Solution B: Ferrous Iron. Approximately 100 ml of distilled water were acidified with 1 N H_2SO_4 to a pH of 2.5 to 3.0. The ferrous ammonium sulfate was then dissolved in about 80 ml of this acid water and the solution was diluted to 100 ml with the acidified water in a volumetric flask.

$\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ 0.1104 g
 Acidified Distilled Water. 100 ml

Solution C: The following salts were dissolved in the order listed in about 80 ml of distilled water which had the pH adjusted to 6.8 with 1 N NaOH. The solution was diluted to 100 ml in a volumetric flask and then autoclaved at 121° C for 15 min at 15 psi.

NH_4Cl 10.0 g
 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.0 g
 NaCl 1.0 g
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0 g

Solution D: Microelements. The following salts were dissolved in about 800 ml of distilled water and diluted to one liter.

$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2 mg

$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 400.0 mg

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 2.0 mg

$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 10.0 mg

$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 10.0 mg

This solution was autoclaved at 121° C for 15 min at 15 psi.

The mineral salts medium was prepared by aseptically adding one ml of each of the sterile stock solutions to 96.0 ml of sterile distilled water with thorough mixing after each addition. Where larger volumes of culture were required, the amount of water was adjusted to correct for loss due to autoclave sterilization and for added stock solutions.

Mineral salts agar was prepared by adding 1.75 g of Bacto agar (Difco) per liter (final volume) of distilled water. The agar solution was autoclaved for 15 min at 121° C at 15 psi. Stock solutions were then aseptically added as previously described. The medium was dispensed as desired into sterile containers and allowed to harden.

Chemicals

All inorganic chemicals used in these investigations were of "Analytical Grade."

Gas Mixtures

All gases used in this investigation were of commercial grade purity.¹

The gas mixture used as the growth substrate was composed of 66% hydrogen, 22% oxygen, and 11% carbon dioxide.² This mixture was prepared in a type G-1 oxygen tank which was equipped with a pressure-vacuum gauge and a cutoff valve. The tank was evacuated and the appropriate amount of each gas added to obtain the desired mixture.

The gas mixture employed in manometric studies was prepared as before, in the following percentages:

Nitrogen.	80.0%
Hydrogen.	13.3%
Oxygen.	4.4%
Carbon Dioxide.	2.2%

With this gas mixture and the appropriate Pardee buffer (described in the Manometric Studies section), the 6:2:1 ratio of hydrogen to oxygen to carbon dioxide could be maintained while keeping the total level of carbon dioxide at 2%.

Culture Apparatus

The organism used in this study was grown autotrophically in a one-liter Erlenmeyer flask. The flasks were

¹Standard Welders Supply Company, Columbus, Mississippi

²Bannister Enterprises, Columbus, Mississippi

fitted with rubber stoppers containing a cotton-plugged gas filter (Figure 1).

Large batch cultures were grown in twenty-liter carboys in ten-liter quantities.

All liquid cultures were incubated on a New Brunswick rotatory shaker at 180 rpm at 30° C for 18-20 hours with continuous gassing.

The author is aware that the growth chamber described here does not account for some of the charges which might occur in the gas mixture during growth. Thus the true ratio of gases is unknown after incubation has started.

Inoculum

Cells used as inoculum were grown in 250 ml Erlenmeyer flasks containing 100 ml of mineral salts medium. For the large batch cultures, inoculum was grown in two-liter Erlenmeyer flasks containing one-liter of mineral salts medium.

Tests for Purity

The inoculum was checked for purity by streaking on nutrient agar plates and observing the colonial morphology of colonies which developed. The Gram reaction was observed by staining with the Gram stain. Cellular morphology was observed on simple stains of the cultures. The presence of short, Gram-negative rods constituted a pure culture. All cultures were checked for contamination immediately

after incubation. The stains were made in accordance with the Society of American Bacteriologist's Manual of Micro-Biological Methods (1957).

Optical Density Measurements

All optical density (O.D.) measurements in this study were obtained using a Bausch and Lomb Spectronic 20 Colorimeter. Unless otherwise stated, a wavelength of 655 mμ was used.

Temperature

The cultures used in this investigation were grown at $30 \pm 2^{\circ} \text{C}$.

pH

All pH measurements were carried out with a Beckman Zeromatic II pH meter using a combination electrode at ambient room temperature.

Dry Weight Determinations

Dry weight determinations were made by filtering 50 ml aliquots of five different cell suspensions with known optical densities through tared Millipore filter discs of 0.45 μ porosity. The filters were washed with five ml of distilled water, dried at 105° C for three hours, cooled to room temperature in a desiccator, and weighed on an analytical balance. A standard curve (Figure 2) was constructed

from these data (Table 1).

Harvest and Preparation of Cells for Manometric Studies

Cells from a liquid culture were centrifuged at 10,000 rpm for ten min in a refrigerated centrifuge and washed three times in physiological saline. The washed cells were then resuspended so that a 1:10 dilution gave an O.D. reading of 0.5 at 655 m μ .

Collection of Spent Medium

The culture was grown in ten-liter quantities as described previously. The cells were removed with a Sharples Super Centrifuge at 20,000 rpm. The spent medium was then filtered through a membrane filter of 0.45 μ porosity and immediately concentrated 1:16 as described below.

Concentration of Spent Medium

Concentration of the spent medium was accomplished with a continuous-flow Buchler Flash Evaporator. The temperature of the water bath was set at 62° C. The flow rate of spent medium into the evaporating flask was adjusted so that approximately 600 ml were concentrated per hour.

After the desired volume was obtained, the concentrated spent medium was pre-filtered through coarse filter paper to remove the precipitated salts. The elements of time and expense prevented the quantitative or qualitative

analysis of these precipitated salts, therefore they were discarded. Next, the concentrated spent medium was aseptically filtered through a membrane filter of 0.45 μ porosity and aseptically transferred to a sterile container. It was then stored at 5° C until needed.

Removal of the precipitated salts altered the medium such that the quantities of the different salts were unknown.

Concentration of Mineral Salts Medium

Normal mineral salts medium was prepared in sufficient quantities as before. Concentration was accomplished as described for spent medium.

Preparation of Fractions from Concentrated Spent Medium

Three portions of the concentrated spent medium were adjusted to pH 5, 7, and 9 respectively. Each was then distilled to approximately 1/2 volume in a Buchler Flash Evaporator using a two-liter evaporation flask and a two-liter condensing flask. Two fractions, a distillate and a residue, for each of the three samples were collected. After distillation, the pH of each fraction was readjusted to pH 7.0. The fractions were stored in 6-oz prescription bottles at 5° C until needed.

Preparation of Fractions from Concentrated Mineral Salts Medium

Fractions from concentrated mineral salts medium were obtained in a manner similar to that for concentrated spent medium.

Salt Concentration

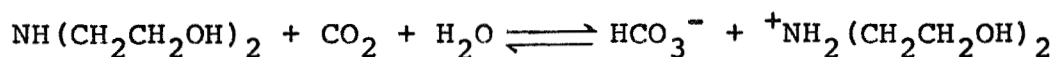
The salt concentration of samples tested in this investigation was determined as total solids. A known volume of each sample was evaporated to dryness at 105° C in a tared crucible, cooled in a desiccator to room temperature, and weighed with an analytical balance to constant weight. The weights were expressed in mg/ml.

Manometric Studies

Manometric studies for this investigation were carried out in accordance with those procedures described by Umbreit, Burris, and Stauffer (1964). Special emphasis was given to the manometric methods of Pardee (Umbreit, Burris, and Stauffer, 1964) whereby hydrogen-oxygen uptake was measured in the presence of carbon dioxide. Pardee's "CO₂-buffer" was used to maintain 2% carbon dioxide in the reaction vessels. This buffer was prepared with the following:

Diethanolamine. . . . 60 ml
 Thiourea. 150 mg
 KHCO₃ 30 g
 6 N HCl 35 ml
 Distilled water . . . 55 ml

The thiourea and potassium bicarbonate were dissolved separately in the diethanolamine. Distilled water and 6 N HCl were added next. The solution was stored over-night before use. It is capable of maintaining a virtually constant pressure of CO₂ in the gas phase by binding carbon dioxide reversibly as represented by the following equation:



Any carbon dioxide taken up by the cells is replaced by the reaction proceeding from right to left. The reference for this method contained no details concerning the precision with which the CO₂ atmosphere could be maintained.

All Warburg investigations were conducted at 30° C in either single or double side arm flasks containing a total liquid volume of 3.2 ml. These investigations employed a Precision Scientific 18-place Warburg Apparatus.

All ground glass joints were sealed with Dow Corning high vacuum silicone stopcock grease.

The center wells of blank or control flasks contained 0.6 ml of Pardee's buffer. The main body of blank flasks contained 1.3 ml of 0.15 M phosphate buffer at pH 7.0, 0.4 ml of the cell suspension, and 0.9 ml of distilled water.

Other flasks contained 0.6 ml of Pardee's buffer in the center well, 1.3 ml of 0.15 M phosphate buffer at pH 7.0, 0.4 ml of the cell suspension, 0.6 ml of the test material, and 0.3 ml of distilled water.

The flasks were placed on the manometer and flushed with the gas mixture. The flasks were then transferred to the water bath and a brief period of equilibration allowed. Readings were taken at 10 min intervals for 60 min.

The results were expressed either in graphic form or as $Q_{\text{(H}_2\text{-O}_2\text{-CO}_2\text{)}}^{\text{(H}_2\text{-O}_2\text{)}}$ (dry weight). This represents the μl of hydrogen-oxygen consumed in an atmosphere of hydrogen-oxygen-carbon dioxide per mg of dry weight of cells per hour.

Warburg data from these experiments were analyzed using an IBM 360 computer and a computer program designed to carry out the necessary calculations.

Statistical Analysis

The data were analyzed for linear regression resulting in an equation for each line:

$$\hat{Y} = b_1 + b_2X$$

Where appropriate, the homogeneity of regression coefficients between two regressions was tested. For this, a t value was calculated from the following formula and tested at the 0.05 and 0.01 level of probability:

$$t = \frac{b_1 - b_2}{\sqrt{b_1 - b_2}}$$

Homogeneity of regression coefficients indicates that the two lines have the same slope but not that they are the same line (Steel and Torrie, 1960).

EXPERIMENTAL RESULTS AND DISCUSSION

Preliminary Investigations

Preliminary manometric studies were conducted in order to determine some of the effects of spent medium on resting cell suspensions of Hydrogenomonas eutropha and to develop experimental techniques for use in future experiments.

The cultures used in this project were grown autotrophically. The term "resting" cells used in this manuscript denotes cells which have been washed free of any extracellular nitrogen source. The author realizes that the test materials used in this project contained nitrogen in small amounts. These materials were used in small quantities (0.6 ml/flask) and the experiments were conducted over short time periods (60 min). Therefore, the growth of H. eutropha cells which may have taken place due to this added nitrogen was neglected.

Cell suspensions which were not given the test materials (hereafter referred to as blank samples) and cell suspensions which were given the test materials (hereafter referred to as experimental samples) were run in triplicate. The data were averaged and corrected for gas controls and endogenous respiration. Endogenous respiration was determined from blank samples in an air atmosphere.

The Effects of Mineral Salts Medium

Normal mineral salts medium was used as the test substrate in the first series of experiments, the results of

which are shown in Figure 3. The regression coefficient for blank cells (line No. 1) was 7.41. Cells which were given the mineral salts medium had a regression coefficient of 6.72 (line No. 2). A larger regression coefficient by the blank cells indicates that these cells consumed the gas mixture at a faster rate than the cells which were given mineral salts medium.

Table 2 shows the results of the t test in which the regression lines in Figure 3 were examined for difference. The calculated t value 8.767 is seen to be significant at the 0.01 level of probability when compared to a tabular t of 3.355. This means that the regression coefficients or the slopes of the lines pictured in Figure 3 are statistically different. Thus, the mineral salts medium appears to have reduced the rate of gas consumption by H. eutropha.

It is noted that the lines in Figure 3 intercept the y axis at different points. However, these lines converge, cross, and diverge which indicates that as time increases, the lines become farther apart. Therefore, the data indicate that under the conditions of this experiment the blank samples consume the gas mixture more efficiently than the cells given mineral salts medium.

It should be noted that a statistical difference between any regression lines does not necessarily indicate a practical difference.

The Effects of Concentrated Mineral Salts Medium

Cell suspensions were exposed to concentrated mineral salts medium and their activity was measured in terms of hydrogen-oxygen gas consumption as seen in Figure 4. Blank samples (line No. 1) with the larger regression coefficient of 6.30 as compared with 5.68 for line No. 2, seemed to be more active than the cells tested with concentrated mineral salts medium. These regression coefficients appear to be statistically different in Table 2. The calculated t value of 10.065 was significant at the 0.01 level of probability.

The results of these experiments indicate that the rate of gas consumption by cell suspensions of H. eutropha is reduced in concentrated mineral salts medium.

The Effects of Spent Mineral Salts Medium

Cell suspensions of H. eutropha were also subjected to treatment with spent medium. Figure 5 shows the results of these experiments. Blank cells (line No. 1) consumed more of the hydrogen-oxygen gas mixture than cells exposed to spent medium (line No. 2). The difference between these two regression coefficients is statistically significant at the 0.01 level of probability by the t test as seen in Table 2, row 3.

It is interesting to note that the regression coefficients (Table 2) of the blank and experimental samples in mineral salts medium are nearly 1 1/2 times greater than

in spent medium. Even so, the value b_1-b_2 for spent medium is only 1/2 as great as b_1-b_2 for normal mineral salts medium.

The larger value of b_1-b_2 for spent medium indicates that either the extracellular products of H. eutropha are non-toxic to new cells or that any toxic products present are in very low concentrations. The ability of cells given spent medium to utilize the hydrogen-oxygen gas mixture does not appear to be inhibited when compared with cells treated with normal mineral salts medium. Thus, it can be stated that any toxic compounds in spent medium are too dilute to affect the activity of H. eutropha.

The Effects of Concentrated Spent Medium

The investigations were broadened to include concentrated spent medium. The first experiment with concentrated spent medium was directed toward solving the toxicity question. Manometric procedures were employed using an air atmosphere. Readings were taken at 10 min intervals and the μ l of oxygen consumed and carbon dioxide produced were calculated. The gas exchange data for this experiment are shown in Table 3 and Table 4.

Table 3 shows that endogenous oxygen consumption for resting cells of H. eutropha in an air atmosphere is very low. Respiration by resting cells given concentrated spent medium was observed to be similar to the endogenous respiration in direction and magnitude. The data in Table

4 show that carbon dioxide production for endogenous cells and cells given concentrated spent medium resembles the results in Table 3.

In both the uptake of oxygen and the production of carbon dioxide, the organism failed to show any signs of inhibition in respiration. Therefore, it may be stated that concentrated spent medium does not contain materials which are toxic to new cells of H. eutropha.

The data from these experiments also indicate that resting cells of H. eutropha are unable to utilize concentrated spent medium as a substrate. If concentrated spent medium were a suitable substrate for these cells the data in Tables 3 and 4 would show increasing amounts of oxygen consumed and carbon dioxide produced while the respective endogenous respirations would remain small.

Manometric experiments with concentrated spent medium were expected to show a large difference between the regression lines for blank and treated cells. It was shown previously (Table 2) that mineral salts medium and concentrated mineral salts medium exhibited a difference ($b_1 - b_2$) between their respective blank samples and treated samples. It was reasoned that concentrated spent medium, having residual mineral salts plus any extracellular products from the organism, would show even greater differences between blank and treated cells than did concentrated mineral salts medium.

However, Figure 6 shows very little difference between the regression lines for blank cells and treated cells. The t test in Table 2 indicates that the two regression coefficients are not statistically different at the 0.01 level of probability. Figure 6 reveals that lines 1 and 2 are parallel. This would indicate that concentrated spent medium had no adverse affect on the ability of resting cells of H. eutropha to utilize the hydrogen-oxygen gas mixture.

Cells of H. eutropha have previously been shown to produce extracellular products (Brown, Cook, and Tischer; 1964). Thus, it is postulated that organic compounds which were increased as a result of concentration, enhanced the uptake of oxygen from the hydrogen-oxygen gas mixture. This enhancement in oxygen consumption is reflected in the total volume of the gas mixture consumed by cells given concentrated spent medium. This results in a larger regression coefficient for the treated cells, thus a smaller value for $b_1 - b_2$.

pH Studies

It was decided to further study the effects of concentrated spent medium since it would incorporate any effects due to spent medium alone. Samples were distilled at pH 5, 7, and 9. Two fractions, termed the distillate and the residue were collected per sample. The results of experiments with the fractions will be discussed later in this

manuscript.

The present study deals with the pH of concentrated spent medium and its effect on the ability of H. eutropha to utilize the hydrogen-oxygen gas mixture. Cell suspensions of the organism were exposed to samples of concentrated spent medium at pH 5, 7, and 9. Manometric techniques were employed to measure gas consumption. The results from these experiments are shown in Figure 7.

The regression lines are grouped into two pairs of lines: 1 and 3; and 2 and 4. The large differences between the pairs of lines were not tested for significance whereas the differences between regression coefficients within a pair were examined by the t test.

The data in Figure 7 indicate that concentrated spent medium at pH 5 and 9 adversely affects the organism's ability to utilize the hydrogen-oxygen gas mixture. The blank samples, represented by line No. 1, exhibit a regression coefficient of 6.06 as compared to 3.67 for the pH 5 sample (line No. 2) and 3.51 for the pH 9 sample (line No. 4). The regression coefficients of line 2 and line 4 appeared to be very close. The t test (Table 5, row 1) shows that the difference in regression coefficients of these two lines is statistically significant at the 0.05 level of probability. The adverse effects of exposing the organism to very acidic and to very basic media will be explored in later discussions.

A comparison of regression lines for blanks and cells treated with concentrated spent medium at pH 7 shows that the rates of gas consumption are similar. However, the t test of Table 5 reveals that the regression coefficient for treated cells is statistically larger than for blank cells at the 0.01 level of probability.

The pH of concentrated spent medium, prior to adjustment, was found to be 6.0. As the pH was increased to 7.0, ammonia was liberated and could be detected by odor. At pH 9 ammonia was liberated and a precipitate was formed. The addition of acid neither caused the formation of a precipitate nor allowed the liberation of ammonia.

It seemed likely that the precipitates were formed from mineral salts present in the medium. Therefore, samples of concentrated mineral salts medium were adjusted to pH 5, 7, and 9. Both the liberation of ammonia and the formation of a precipitate occurred as the pH was increased. Stock solution C, containing ammonium chloride, was thought to be the source of the ammonia. Indeed, this was the case for when the pH of stock solution C (diluted to approximate the concentrations found in the concentrated medium) was increased ammonia gas was detected. Thus, the evolution of ammonia gas can be attributed to the release of ammonia from ammonium ions present in stock solution C.

To further evaluate the effects of pH, experiments were conducted with samples of concentrated mineral salts

medium at pH 5, 7, and 9. The results are shown in Figure 8. The regression lines are seen to group according to the following pattern: lines 1 and 3; lines 2 and 4. The difference between pairs of lines was much less in Figure 8. Moreover, the regression coefficients of lines 1 and 3 are seen to be statistically different at the 0.01 level of probability (Table 5, row 2). In addition, the difference in regression coefficients for pH 5 (line No. 2) and pH 9 (line No. 4) samples are found to be statistically non-significant at the 0.05 level of probability.

A characteristic pattern can be seen in the data in Figures 7 and 8. The regression coefficients for blanks were statistically different from all other treatments in both experiments. The regression lines for pH 5 and pH 9 treatments were not statistically different at the 0.01 level of probability in either experiment.

The reduced gas consumption by resting cells of H. eutropha were exposed to concentrated spent medium or concentrated mineral salts medium at pH 5 or pH 9 may be explained in terms of phosphorous, an element required by all microorganisms. At alkaline conditions the common PO_4^- ion forms an insoluble complex with calcium. As the pH decreases phosphorous takes the form of the HPO_4^- ion ($\text{pK}_a = 6.23 \times 10^{-8}$) and the H_2PO_4^- ion ($\text{pK}_a = 2.2 \times 10^{-13}$). Normally, slightly acid to neutral conditions provide a mixture of these two ions. The concentration of each ion will depend upon the desired pH of the medium.

At very acid pH's phosphate forms complexes with other elements. Thus, it seems that the species of phosphate ion present depends upon the pH of the medium. Furthermore, phosphates are available within a very narrow pH range.

The reduction in gas consumption may also be explained by the fact that the enzyme systems are adversely affected by either alkaline or acidic conditions. In high concentrations of salt, enzymes may be precipitated. In addition the net charge on the enzyme molecule may be changed, due to salt adsorption by the protein ions, which may modify the enzyme activity.

Studies with Fractions of Concentrated Spent Medium

This part of the investigation was undertaken in an attempt to further explain the effects of concentrated spent medium on resting cells of H. eutropha. These fractions were prepared at the pH indicated, but were adjusted to pH 7.0 to remove any pH effect.

pH 5 Fractions

Figure 9 shows the results of manometric experiments with the pH 5 distillate and pH 5 residue. It is readily seen that the residue seriously impaired the organism's ability to utilize the hydrogen-oxygen gas mixture. When compared with blank samples the residue effected a 50% reduction in gas consumption. The residue was yellow in

color and contained a heavy precipitate probably of inorganic mineral salts which seemed to be responsible for the reduction in growth.

This reduction in growth was anticipated since the amount of material present in the residue was greatly increased by the concentration procedures.

The distillate on the other hand, seemed to enhance the gas consumption of the resting cell suspension as may be seen in Figure 9. This distillate essentially contained distilled water and perhaps a few volatile organic acids.

The regression coefficients of lines 1 and 2 were analyzed for difference by the t test (Table 6). The blank cells exhibited a regression coefficient of 5.55 as compared with 6.18 for distillate treated cells. These results indicate that the regression coefficients are statistically different between cells treated with the distillate and blank cells at the 0.01 level of probability.

pH 7 Fractions

The data in Figure 10 show the effects of pH 7 fractions on resting cells of Hydrogenomonas eutropha. The pH 7 fractions were similar in appearance to the pH 5 fractions, i.e., the distillate was light yellow in color and the residue was a darker yellow with a heavy precipitate. The appearance of this yellow color was also noted by Cook (1966), however the compound or compounds were

never identified.

Again the residue seemed to prevent the normal uptake of the hydrogen-oxygen gas mixture by the organism. The total gas consumption of cells treated with the residue was approximately 50% less than that for the blank. These data seem to substantiate the results obtained with the pH 5 fractions in that the neutral distillation apparently contained distilled water and a few volatile neutral compounds. Also, if any neutral compounds were present in the neutral distillate they had no ill effect on the system.

Gas consumption by cells treated with the pH 7 distillate was approximately equal to that of the blank cells. Table 6 reveals that statistically there is no significant difference between the regressions of lines 1 and 2 of Figure 10. In addition, Figure 10 reveals that lines 1 and 2 are parallel which indicates that the distillate had no adverse effect on the organism's ability to utilize the hydrogen-oxygen gas mixture.

pH 9 Fractions

The pH 9 fractions gave results similar to the pH 7 fractions. The distillate, being light yellow in color, did not alter the cell's ability to utilize the hydrogen-oxygen gas mixture. This is substantiated by the t test in Table 6, which shows no significant difference between the regression coefficients of lines 1 and 2, and by the

data in Figure 11. These data show that lines 1 and 2 are parallel. The residue, which was dark yellow in color and possessed a precipitate, caused a 50% reduction in gas consumption by the resting cells of H. eutropha.

Studies with Fractions of Concentrated Mineral Salts Medium

The residues for each fraction contained large amounts of precipitated salts. It was suspected that this high salt content could account for the adverse effect of the residues rather than the presence of unknown compounds. It was in response to this query that resting cells of H. eutropha were treated with fractions of concentrated mineral salts medium prepared at pH 5, 7, and 9.

pH 5 Fractions

The pH 5 fractions lacked the yellow color found in the same fractions of concentrated spent medium. As expected, the residue exhibited a very heavy precipitate.

The data in Figure 12 show that the residue reduced the total gas consumption by about 30%. In addition, it is noted that distillate treated cells increased the amount of gas mixture consumed. Table 6 shows that the regression coefficient of line 2 is statistically larger at the 0.01 level of probability than line 1.

The results, which are identical to the results illustrated in Figure 9, indicate that the loss of activity by the residue-treated cells is due solely to an increased

salt concentration. The increased activity of cells treated with the distillate is probably due to a decrease in the salt concentration.

Since the concentrated mineral salts medium contained no organic compounds, the distillate prepared from it would contain only distilled water.

These results are similar to the results obtained with fractions of concentrated spent medium at pH 5 in which the distillate enhanced gas consumption.

pH 7 Fractions

Figure 13 shows the results of pH 7 fractions on the ability of resting cell suspensions of H. eutropha to utilize the hydrogen-oxygen gas mixtures. Gas consumption by distillate-treated cells (line 2) was comparable to the blank cells (line 1). These lines are observed to be parallel. The difference in regression coefficients of these two lines was non-significant as seen in Table 6. Residue-treated cells revealed a 44% reduction in gas consumption which appears to be due to the high salt content.

The pH 7 distillate of concentrated mineral salts medium which contained only distilled water was comparable in effect to the corresponding fraction of concentrated spent medium. This seems to substantiate the assumption that neutral distillates of concentrated spent medium contain mainly distilled water.

pH 9 Fractions

The pH 9 fractions affected resting cell activity in much the same manner as the pH 5 fractions. These results are shown in Figure 14. It may be observed that the characteristic reduction in gas consumption is present when resting cells are given pH 9 residue. However, the distillate, which consisted of distilled water, increased the gas consumption of the organism. The difference between regression coefficients for line 1 and line 2 is seen to be statistically significant at the 0.01 level of probability (Table 6). The pH 9 distillate of concentrated spent medium, on the other hand, reduced gas consumption by the organism. This indicates that some volatile alkaline compounds were present in this fraction and that these compounds were harmful to resting cells of H. eutropha.

The results of studies with concentrated spent medium and concentrated mineral salts medium strongly suggest that the loss of ability to utilize the hydrogen-oxygen gas mixture with residue-treated cells is due to the increase in mineral salts.

The distillates seem to exert no inhibitory effects on the normal uptake of hydrogen-oxygen gas mixtures by cells of H. eutropha. In fact, they seem to stimulate the uptake of the gas mixture.

The Effects of Salt Concentration

The effects of high salt concentration on the ability of resting cells of H. eutropha to utilize a hydrogen-oxygen gas mixture were studied. The usual manometric techniques were employed for this investigation.

The salt content of samples used in the research project was determined by evaporating triplicate samples to dryness. The results are recorded in Table 7. It is observed from this table that the distillates are very low in salts as would be expected. The residues, however, contain several hundred times more salt than the respective distillates.

It is interesting to note that concentrated spent medium (C.S.M.) contains about 30% more salts than concentrated mineral salts medium (C.M.M.), but the distillates of C.S.M. are lower in salts than the distillates of C.M.M.

Table 7 also compares the salt content of the samples used in this project with the "Q" values for blanks and tests obtained from Warburg experiments. It can be seen that a substantial increase in the salt concentration is accompanied by a drop in the "Q" term for treated cells.

Warburg experiments were conducted to evaluate the effects of increased salt concentrations on resting cells of H. eutropha. The Warburg flasks contained 0.6 ml of six different salt solutions. The experiments were conducted over a 60 min period after which the total volume of gas mixture consumed by the organism was recorded. Figure 15

shows the results of these experiments.

Examination of Figure 15 reveals that gas consumption drops very rapidly as the salt content of the reaction vessels exceeds 130 mg/ml. Above 200 mg/ml salt, the decrease in gas consumption appears to be linear.

This gives conclusive evidence that increased salt concentrations cause a reduction in the volume of gas mixture consumed by resting cells of Hydrogenomonas eutropha -- thus a loss in the organism's ability to utilize the hydrogen-oxygen gas mixture.

SUMMARY

Resting cell suspensions were subjected to various forms of spent medium and uninoculated mineral salts medium under an atmosphere of hydrogen, oxygen, and carbon dioxide to determine their effects on the utilization of a hydrogen-oxygen gas mixture.

Preliminary experiments were carried out with normal mineral salts medium, concentrated mineral salts medium, spent medium, and concentrated spent medium. Blank cells, when analyzed by the t test, were found to be more active than cells treated with normal mineral salts medium, concentrated mineral salts medium, or spent medium. Concentrated spent medium appears to have no effect on the amount of gas mixture consumed by the organism.

Studies on the inhibition of gas consumption with concentrated spent medium were undertaken to determine any toxic effects of H. eutropha. It was found that concentrated spent medium was non-toxic to resting cells of H. eutropha. In addition, it was discovered that the cells were unable to utilize concentrated spent medium as a substrate under an atmosphere of air.

Other cell suspensions were treated with concentrated mineral salts medium and concentrated spent medium at pH 5, 7, and 9. It was observed that pH has a definite effect on the uptake of a hydrogen-oxygen gas mixture by H. eutropha. Concentrated spent medium at pH 7.0 did not significantly increase the amount of gas consumed. At pH 5 and 9 the

total amount of gas mixture consumed was reduced almost 40%. It was also noted that concentrated spent medium at pH 7 gave off ammonia gas. At pH 9 ammonia gas was liberated followed by the formation of a precipitate. Similar results were obtained when concentrated mineral salts medium was used instead of concentrated spent medium. Therefore, it was surmized that very acid and very basic pH's were detrimental to the normal uptake of hydrogen-oxygen gas mixtures by resting cells of H. eutropha because of the availability of phosphate ions to the organism.

Fractionation of concentrated spent medium at pH 5, 7, and 9 yielded a distillate and a residue. Resting cells of the organism were treated with these samples and their effects on gas consumption were observed. Cells which were treated with the distillates suffered no ill-effects. Cells which were subjected to the pH 5 distillates were significantly different from the blank samples at the 0.01 level of probability. The residue treated cells showed a marked loss of activity.

These results were also observed when resting cells were treated with fractions of concentrated mineral salts medium. The loss in activity accompanying residue treatment was felt to be due to the increased salt concentration.

A comparison of salt concentration and "Q" values was made. The amount of gas mixture consumed appeared to be inversely proportional to the salt concentration. In every instance where the salt content was very high, the "Q" value

was small. Final proof that increased salt concentrations interfered with the oxy-hydrogen reaction of H. eutropha was obtained from manometric experiments in which resting cell suspensions were given increasing amounts of salts in solution. The results show dramatically that increased amounts of inorganic salts are accompanied by a reduction in gas consumption.

ABSTRACT

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Major: Microbiology, Department of Microbiology
Mississippi State University

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Concentrated Spent Medium on the Activity
of Resting Cells of Hydrogenomonas
eutropha"

Directed by: Dr. Robert George Tischer

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Abstract

Experiments were performed to determine some of the effects of concentrated spent medium on the ability of resting cells of Hydrogenomonas eutropha to utilize a hydrogen-oxygen gas mixture.

Preliminary experiments showed that normal mineral salts medium, concentrated mineral salts medium, and spent medium reduced the ability of resting cells of H. eutropha to utilize a hydrogen-oxygen gas mixture. Concentrated spent medium appeared to have no effect on the gas consumption by the organism.

Concentrated spent medium was found to be non-toxic to cells of H. eutropha. It was also discovered that these cells were unable to utilize concentrated spent medium as a substrate.

Other experiments showed that resting cells were affected by a change in pH. At pH 7.0 gas consumption was

increased while at pH 5.0 and 9.0, the total amount of gas consumed was reduced by about 40%. It was assumed that the availability of phosphate ion to the organism was responsible for the interference of normal gas consumption.

Studies with fractions of concentrated spent medium indicated that an increase in mineral salt concentration resulted in a reduction of gas consumption.

A comparison of salt concentration and "Q" values showed that as the salt concentration increased, gas consumption decreased.

A P P E N D I X

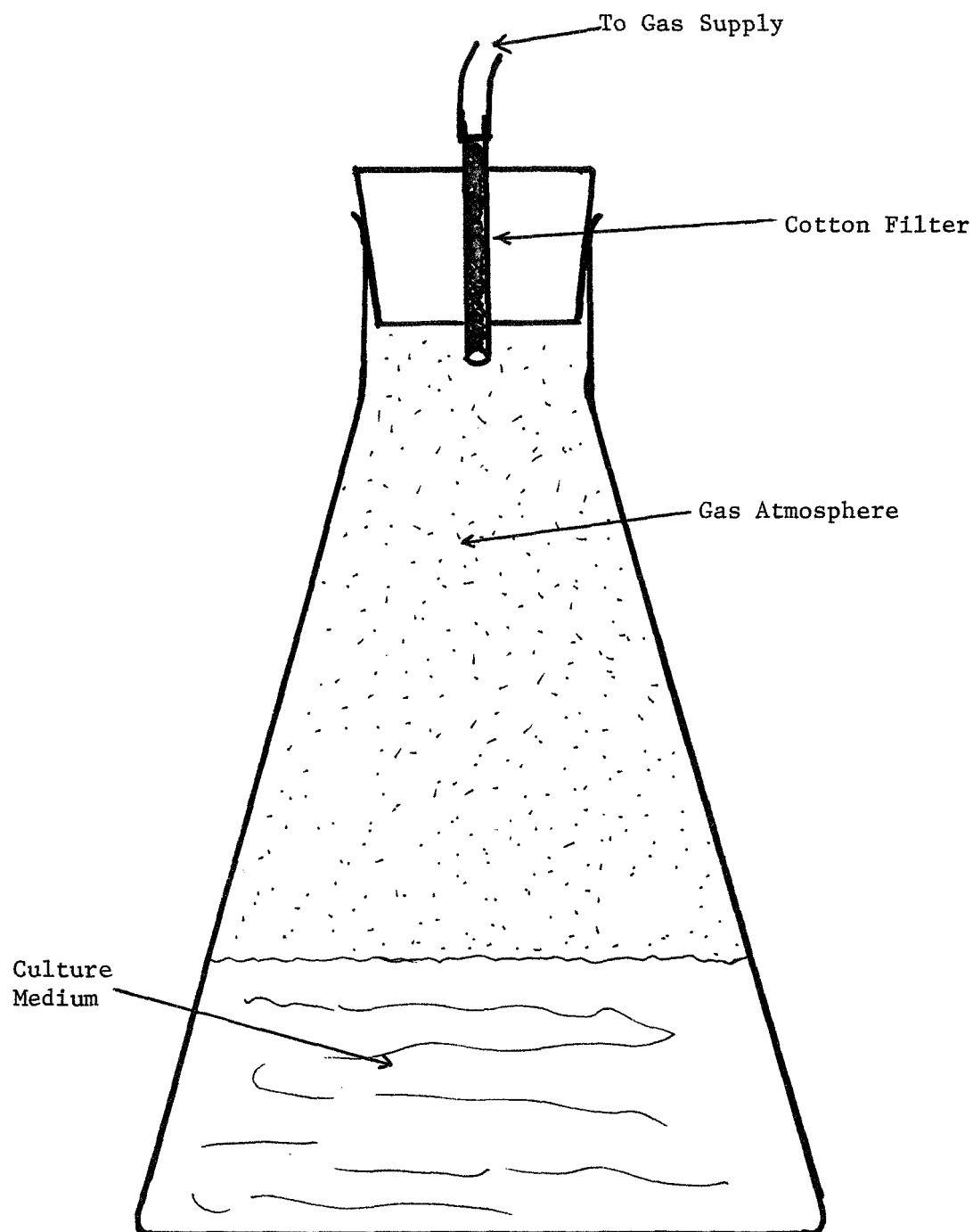


Figure 1. Culture vessel for the growth of Hydrogenomonas eutropha.

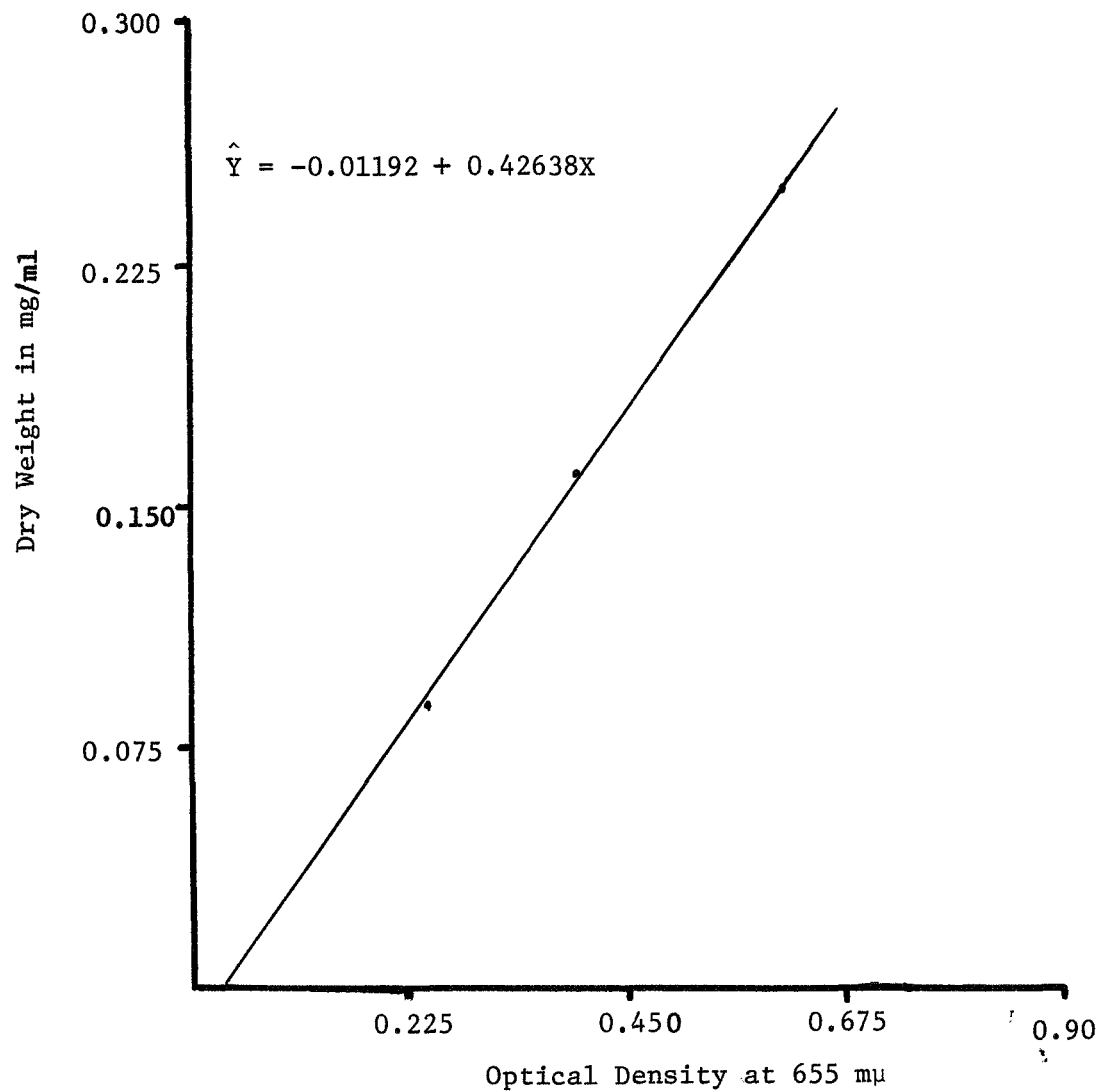


Figure 2. Standard curve for the determination of dry weight of cell suspensions.

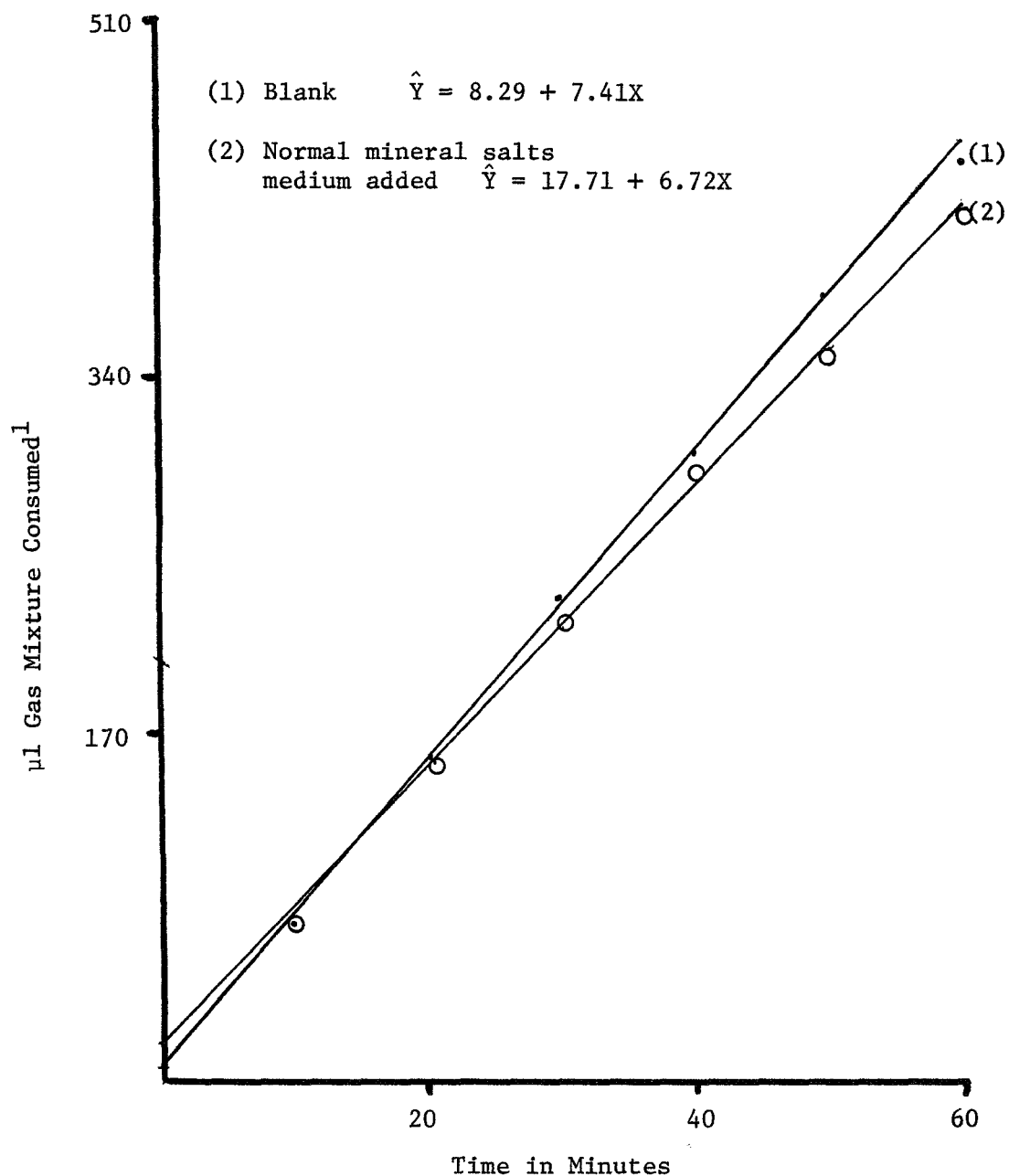


Figure 3. The effects of normal mineral salts medium on the gas consumption by resting cells of Hydrogenomonas eutropha.

¹Unless otherwise stated the μl Gas Mixture Consumed refers to the amount of hydrogen-oxygen gas consumed from a mixture containing 80.0% nitrogen, 13.3% hydrogen, 4.4% oxygen, and 2.2% carbon dioxide.

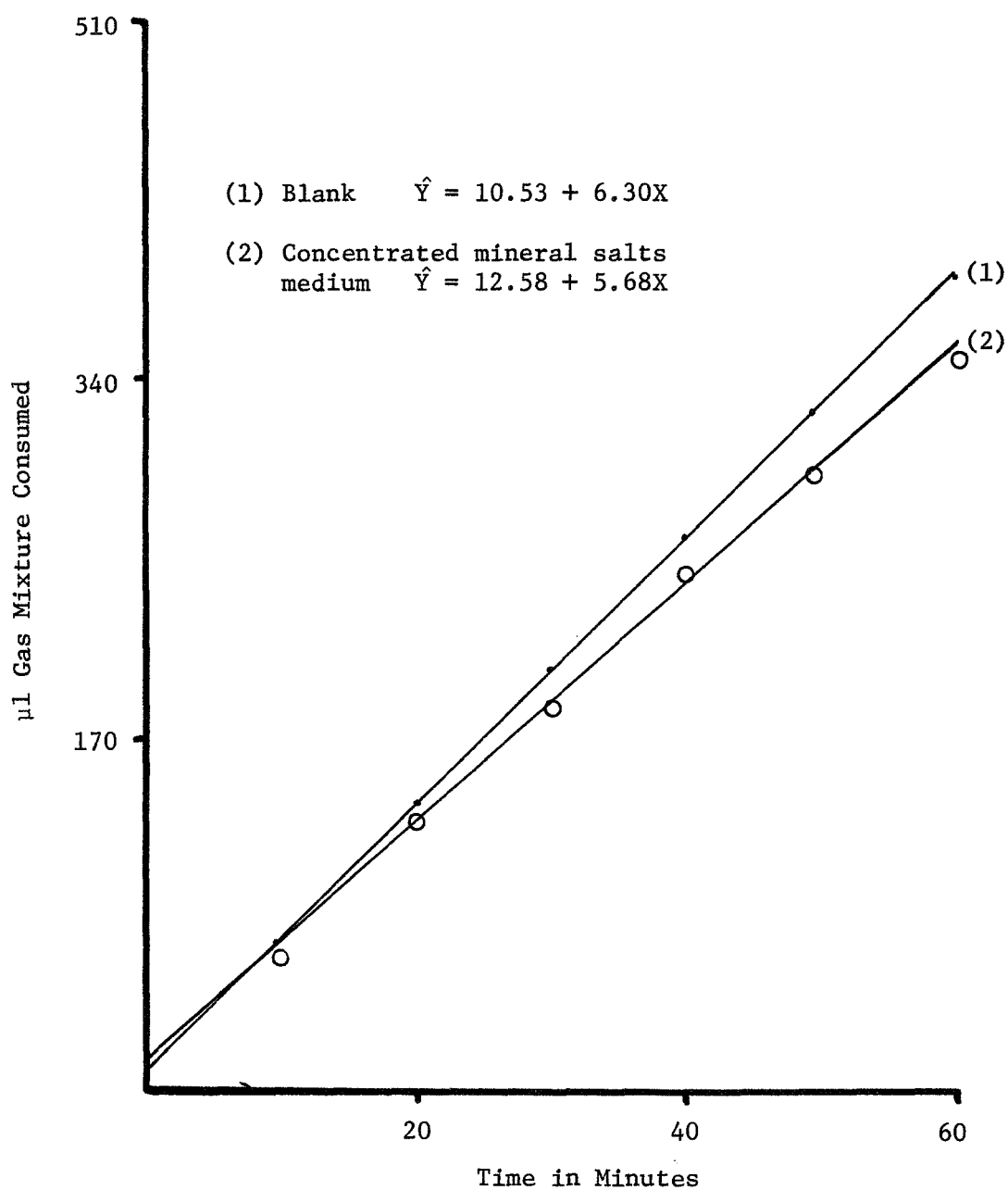


Figure 4. Gas consumption by resting cells of Hydrogenomonas eutropha as affected by concentrated mineral salts medium.

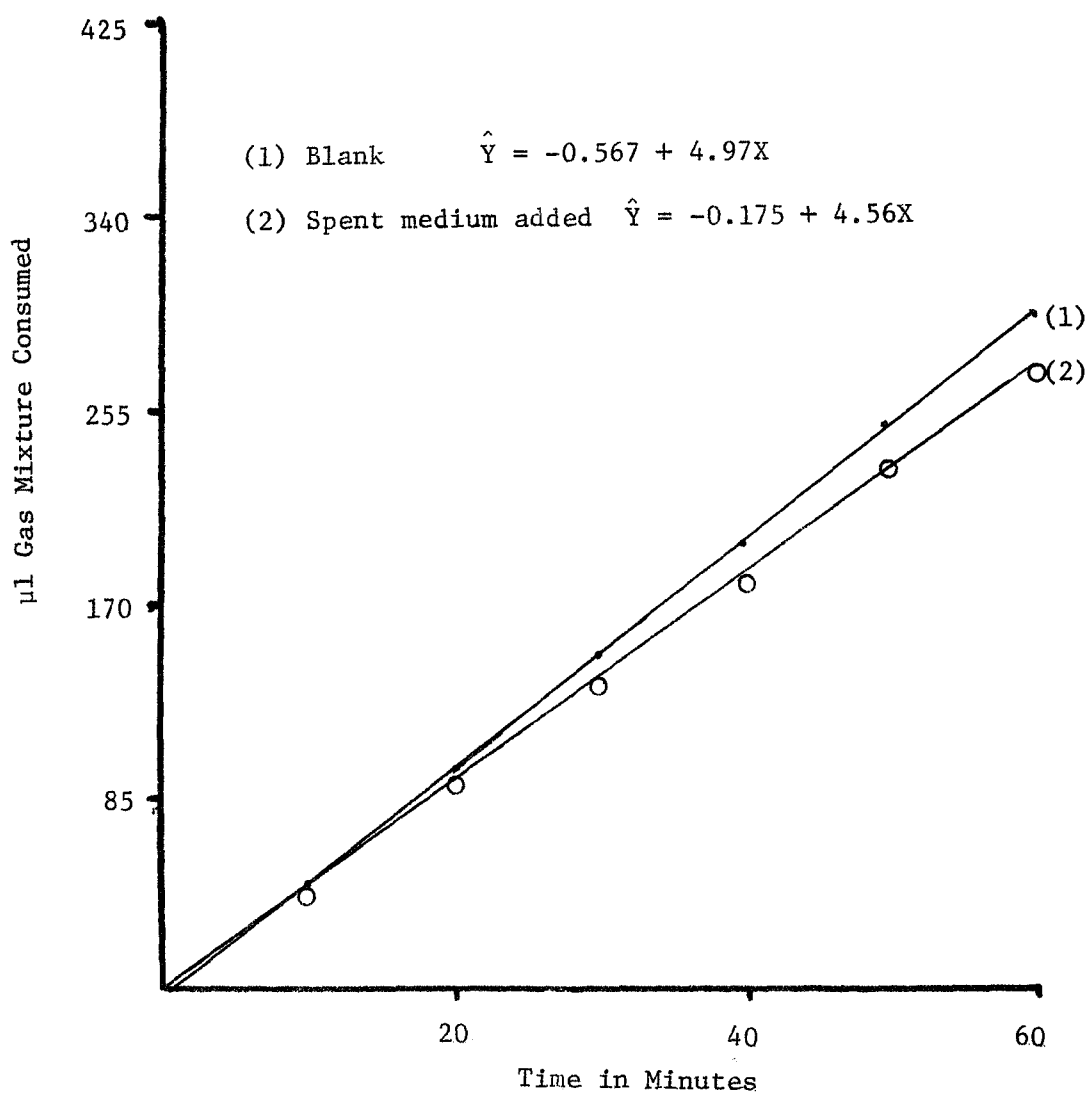


Figure 5. The effects of spent medium on the gas consumption by resting cells of Hydrogenomonas eutropha.

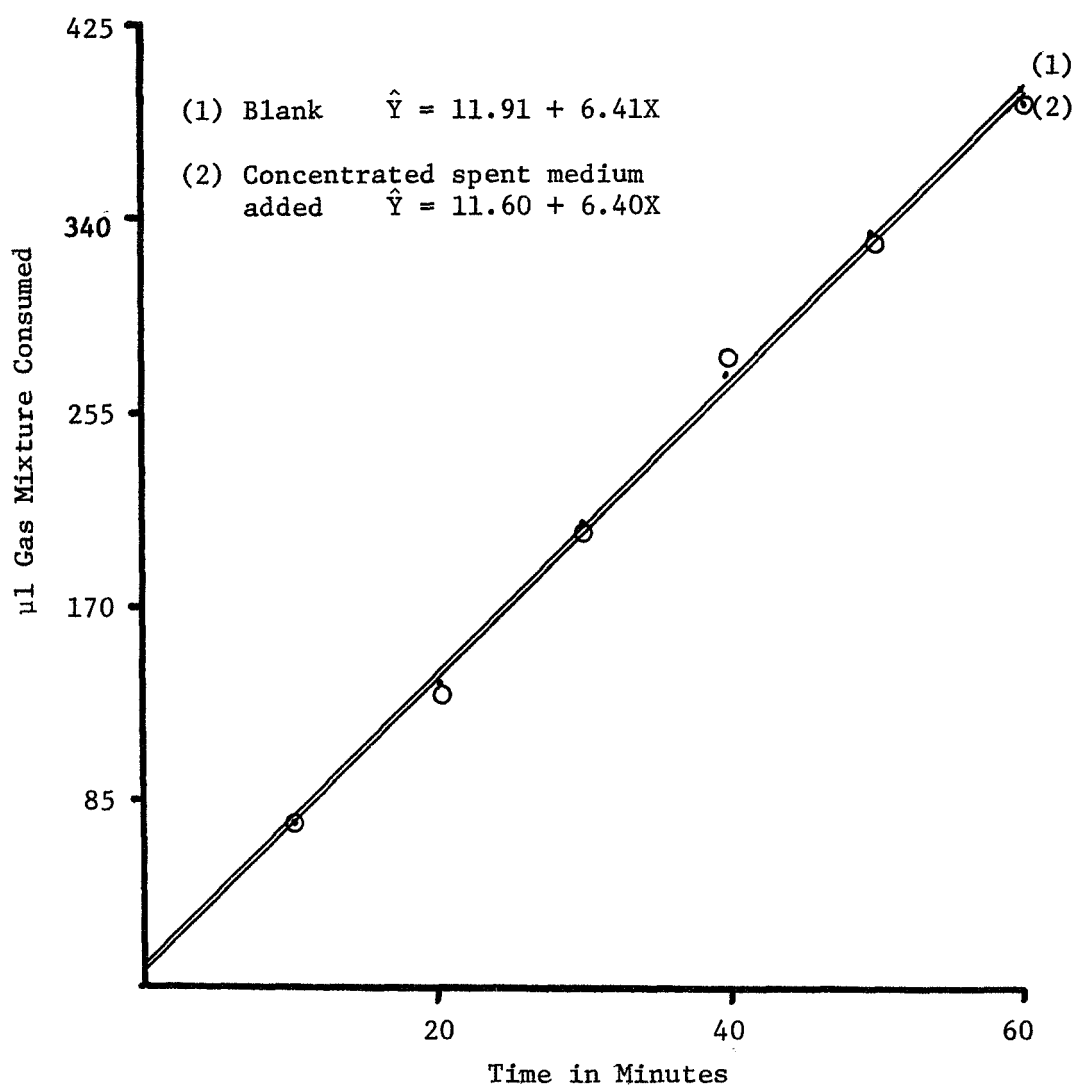


Figure 6. The effects of concentrated spent medium on the gas consumption of resting cells of Hydrogenomonas eutropha.

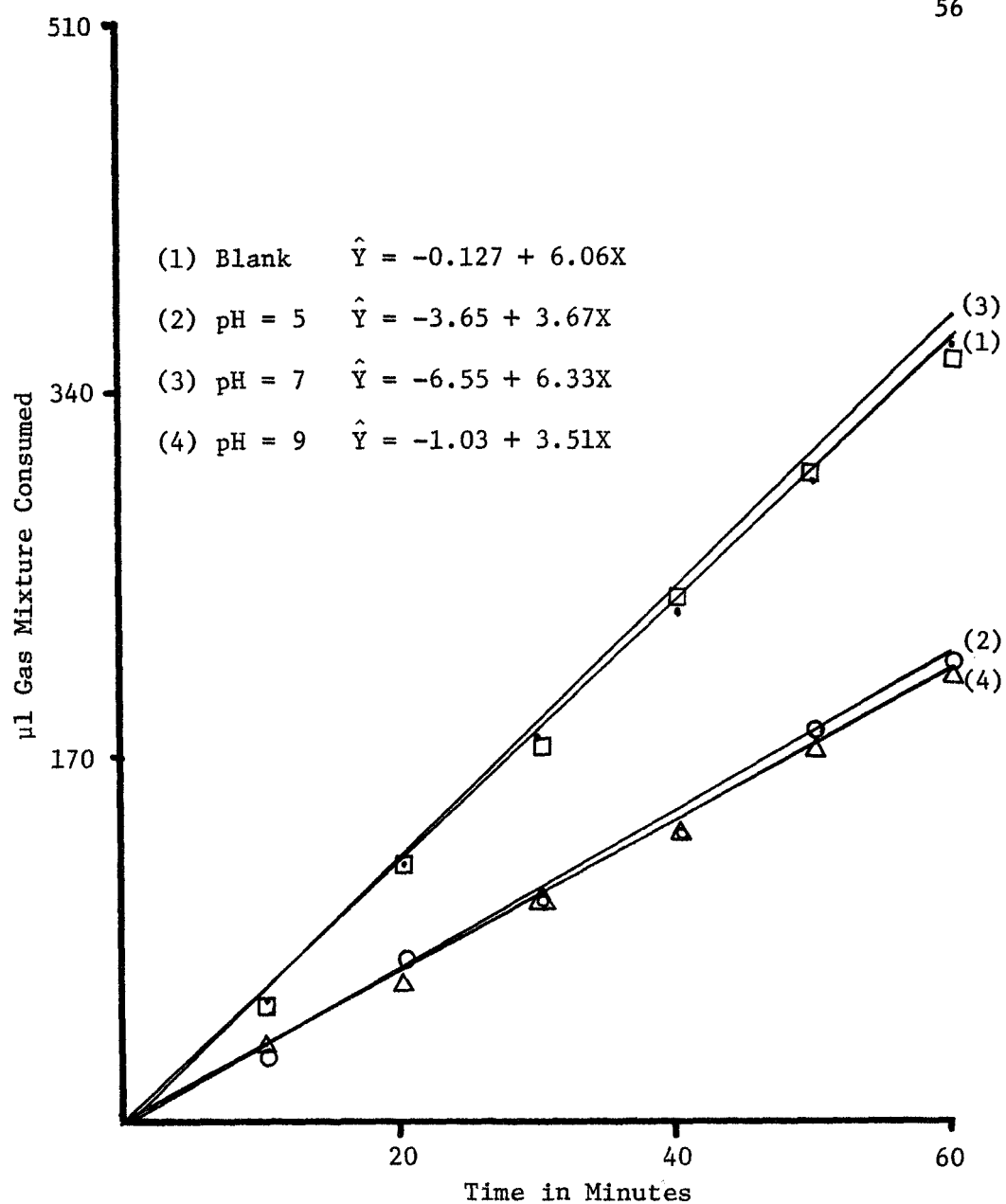


Figure 7. A comparison of the gas consumption by resting cells of *Hydrogenomonas eutropha* as affected by the pH of concentrated spent medium.

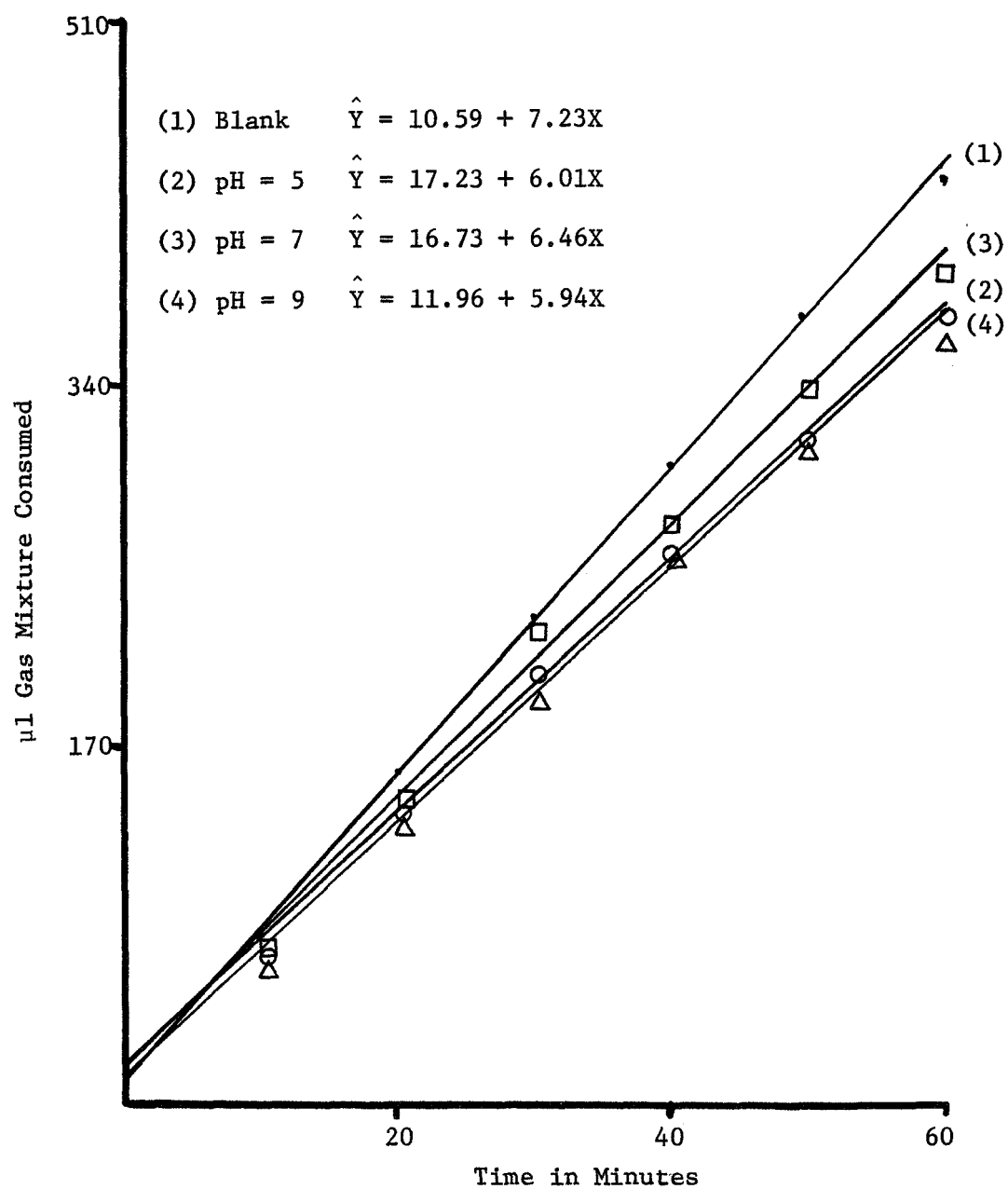


Figure 8. A comparison of the gas consumption of resting cells of *Hydrogenomonas eutropha* as affected by the pH of concentrated mineral salts medium.

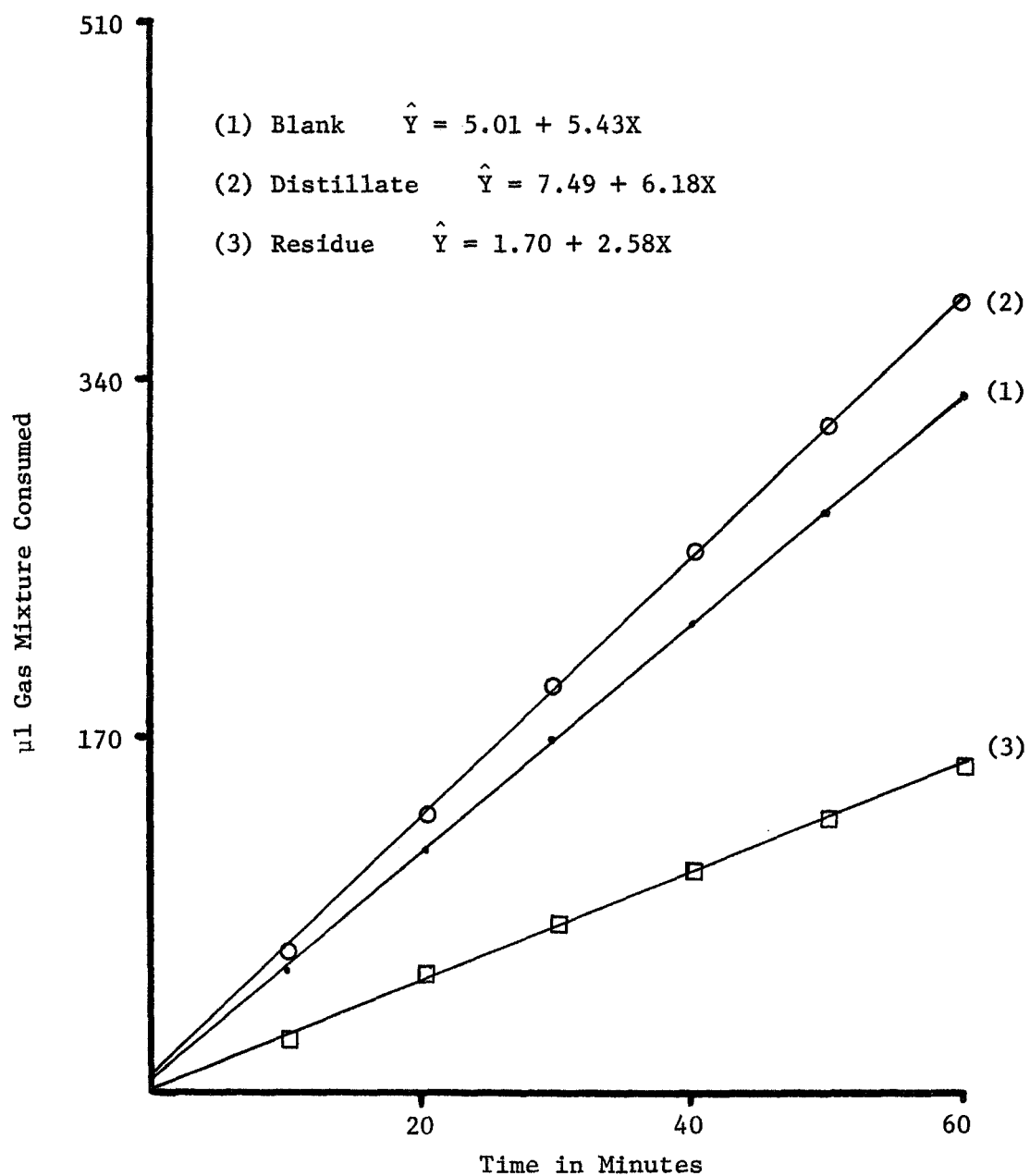


Figure 9. Gas consumption by resting cells of Hydrogenomonas eutropha as affected by the pH 5 fractions of concentrated spent medium.

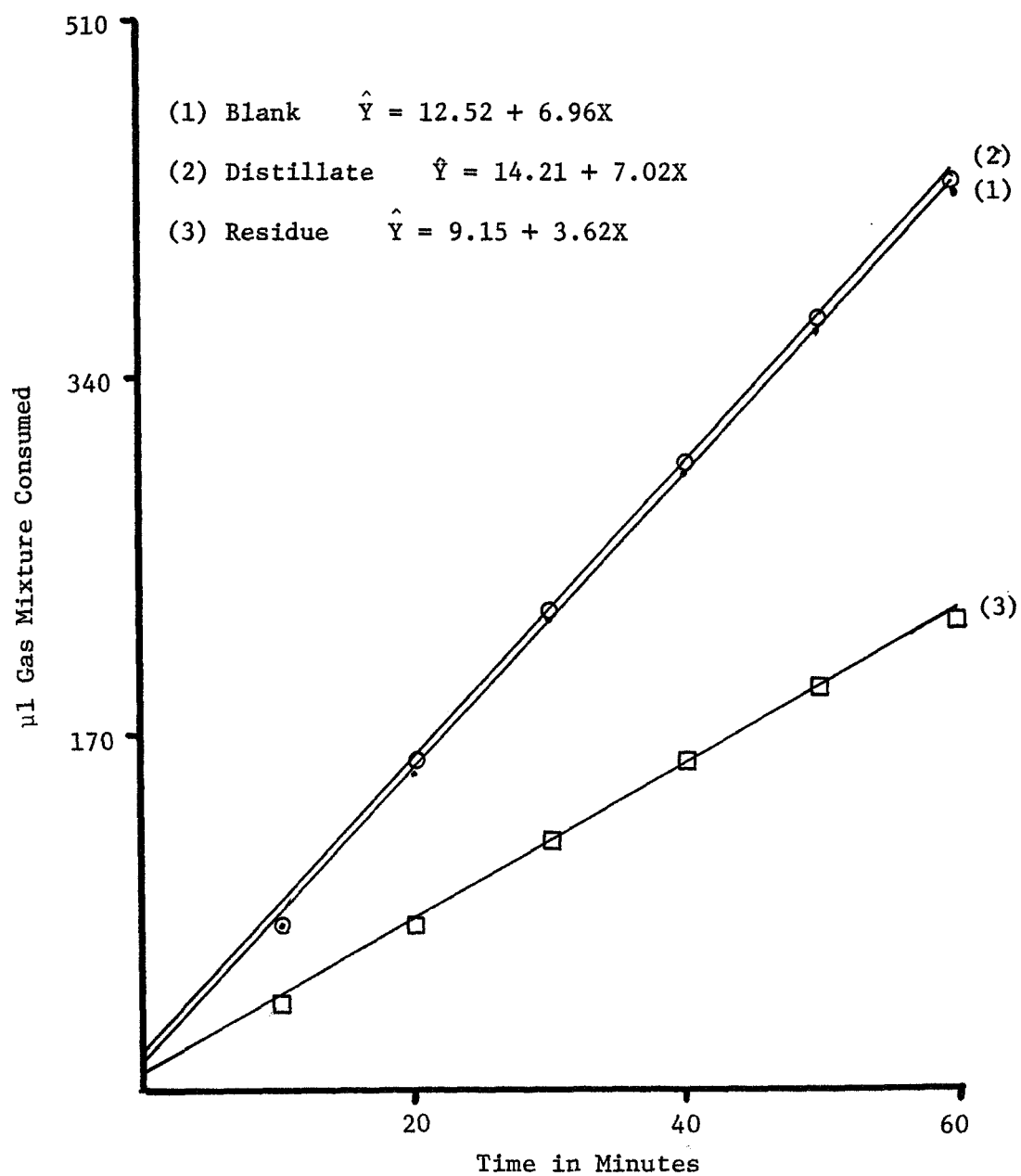


Figure 10. Gas consumption by resting cells of Hydrogenomonas eutropha as affected by the pH 7 fractions of concentrated spent medium.

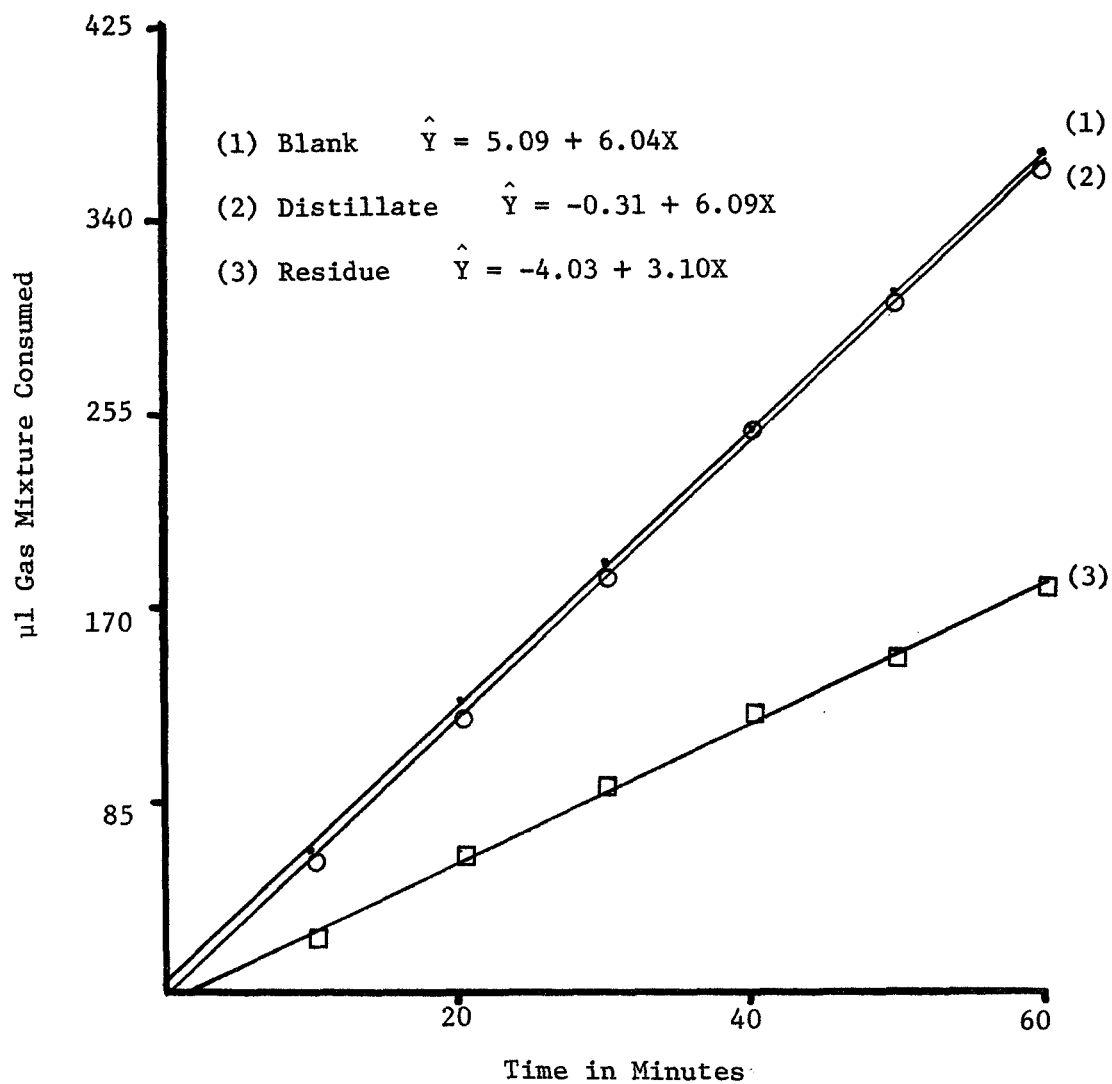


Figure 11. Gas consumption by resting cells of Hydrogenomonas eutropha as affected by the pH 9 fractions of concentrated spent medium.

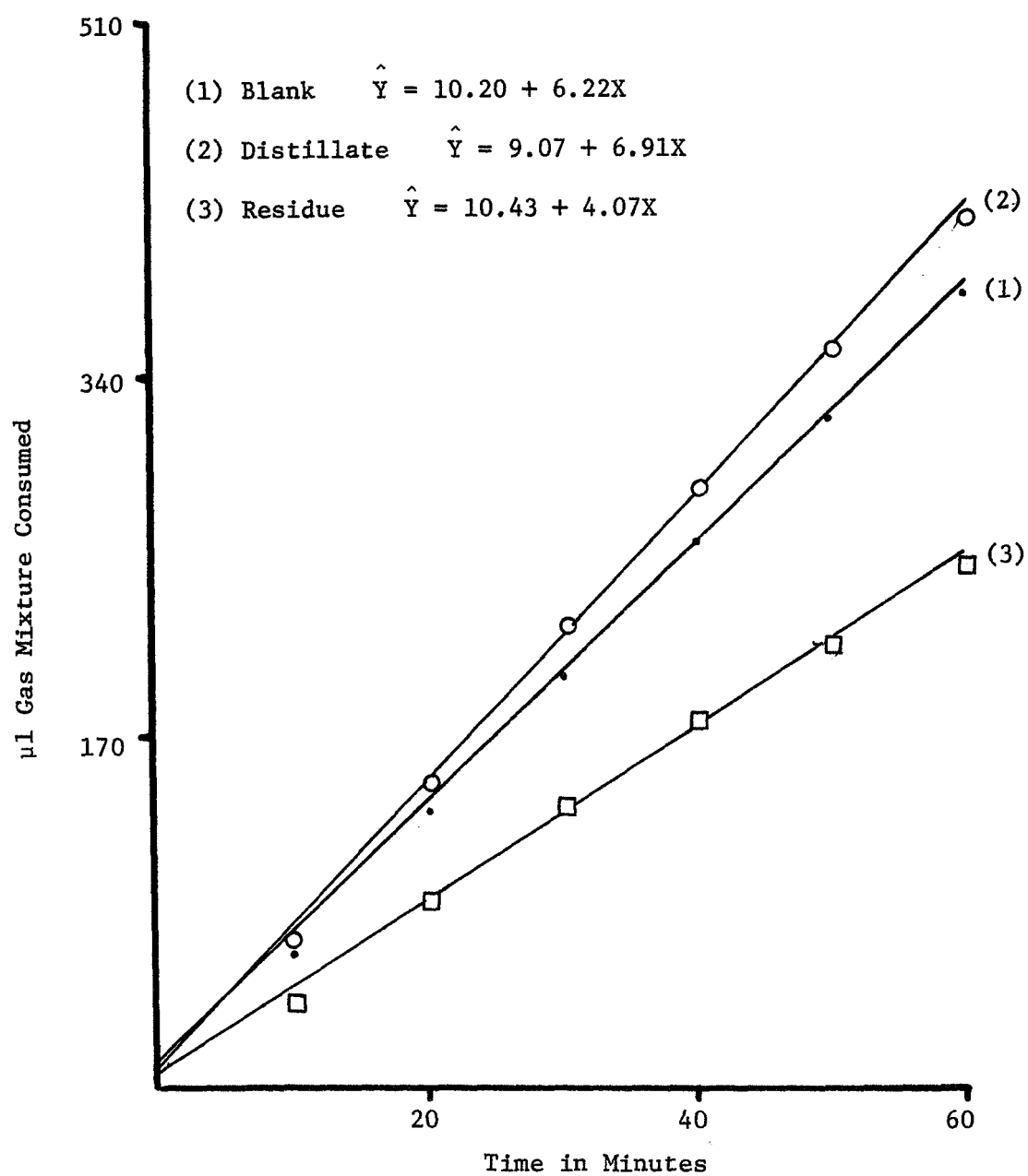


Figure 12. Gas consumption by resting cells of Hydrogenomonas eutropha as affected by the pH 5 fractions of concentrated mineral salts medium.

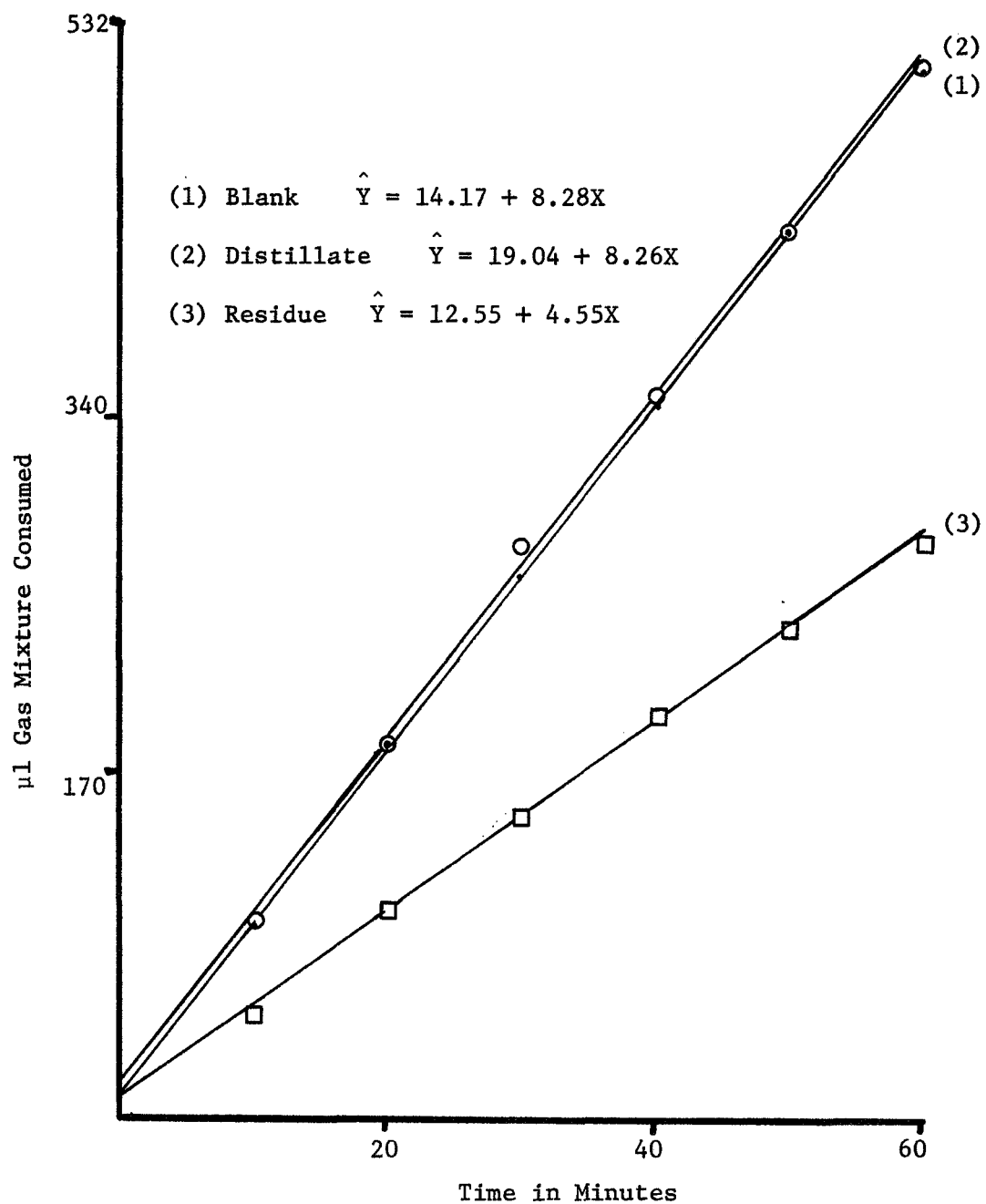


Figure 13. Gas consumption by resting cells of Hydrogenomonas eutropha as affected by the pH 7 fractions of concentrated mineral salts medium.

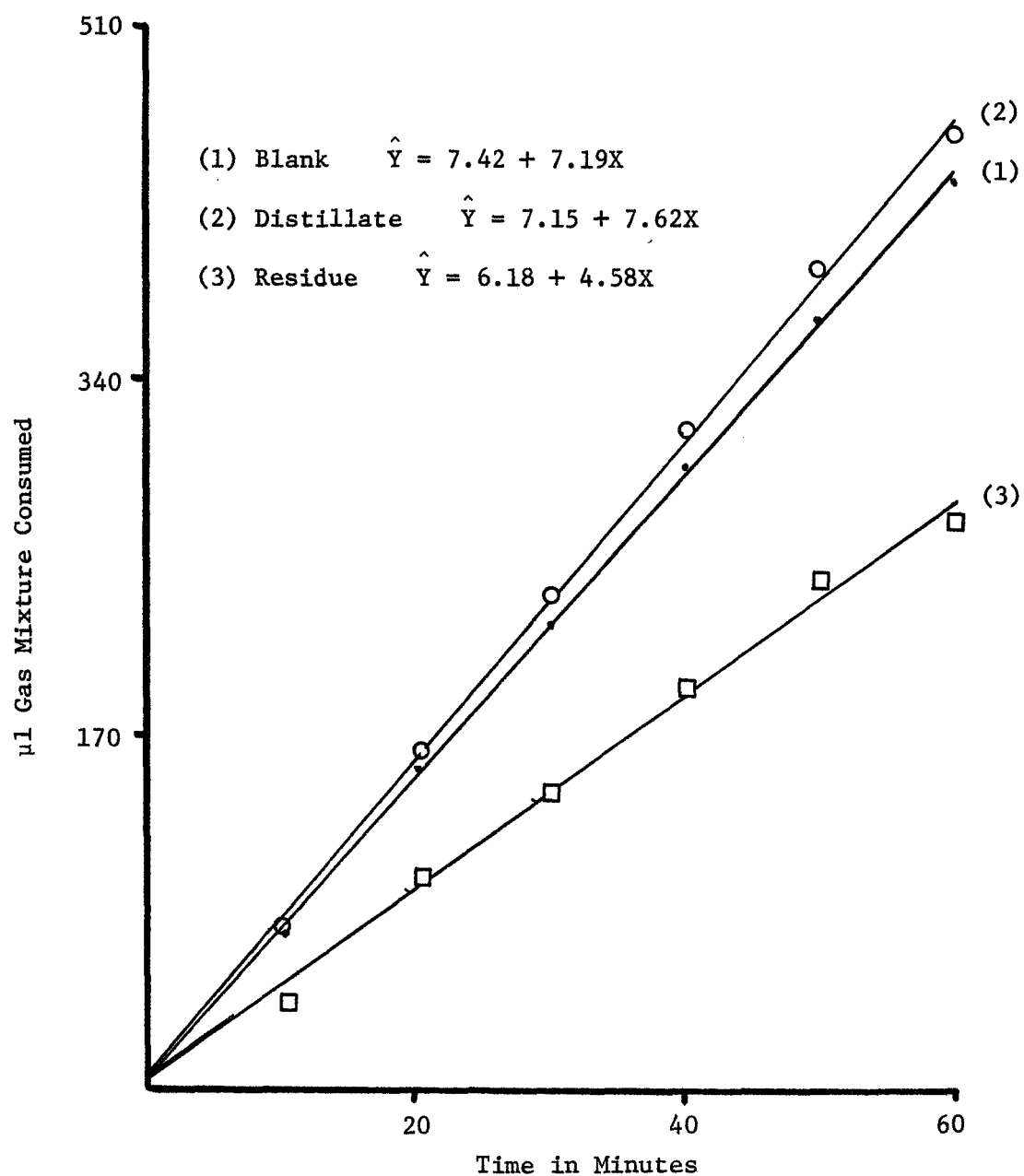


Figure 14. Gas consumption by resting cells of Hydrogenomonas eutropha as affected by the pH 9 fractions of concentrated mineral salts medium.

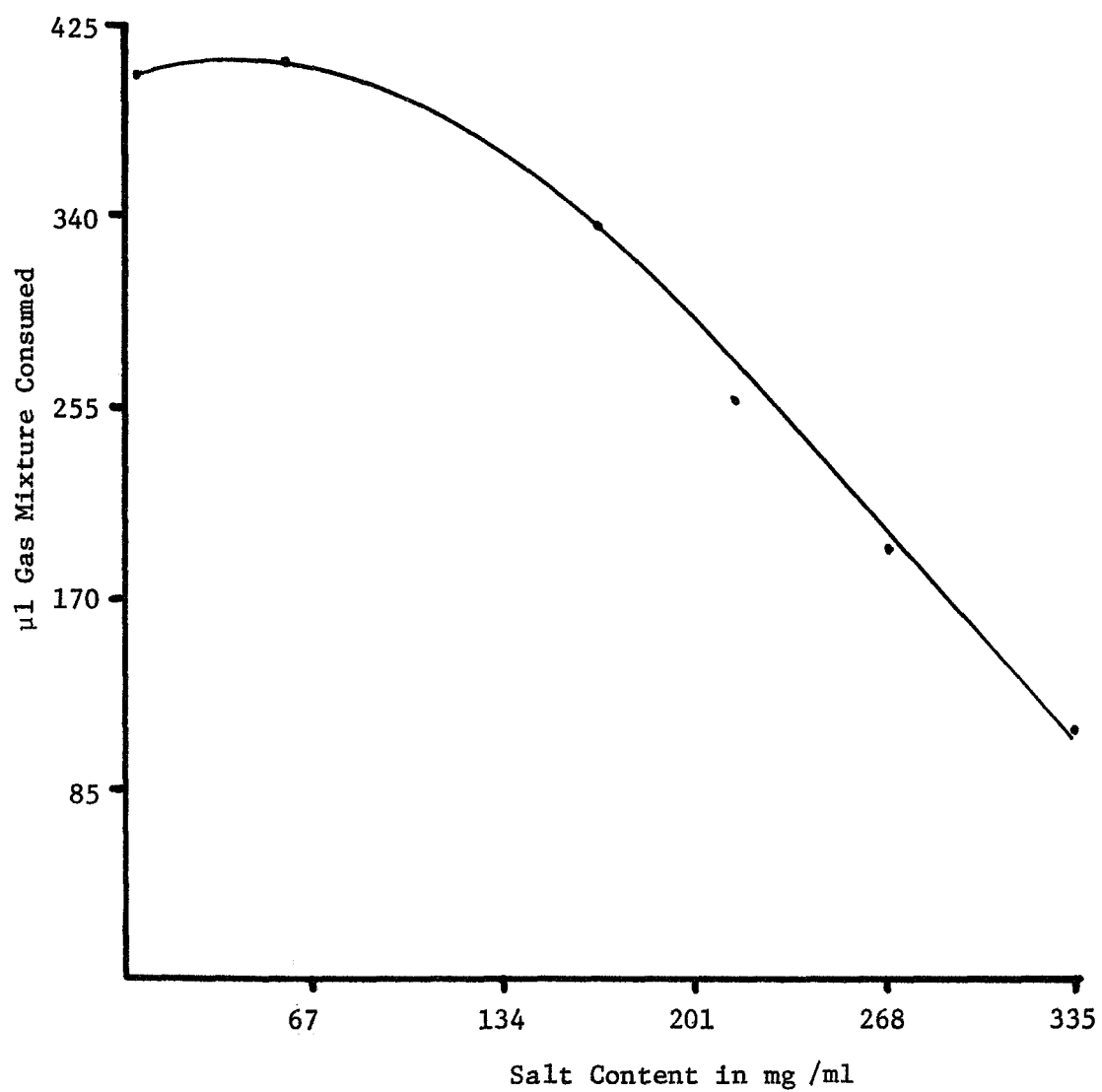


Figure 15. Gas consumption by resting cells of Hydrogenomonas eutropha as affected by salt concentration.

Table 1. The dry weight of cell suspensions of Hydrogenomonas eutropha at various optical densities.*

O.D.	gm/ml	$\bar{x}$ gm/ml
0.240	0.0780	0.0894
	0.0940	
	0.0960	
0.319	0.1200	0.1250
	0.1260	
	0.1300	
0.400	0.1820	0.1635
	0.1560	
	0.1520	
0.500	0.1900	0.1925
	0.1980	
	0.1900	
0.610	0.2626	0.2510
	0.2206	
	0.2726	

*Optical densities were determined at 655 m μ using a Bausch and Lomb Spectronic 20 Colorimeter.

Table 2. Student's t test for difference between regressions of four test materials.¹

Sample	Regression Coefficient		Difference $b_1 - b_2$ ²	Calculated t value
	Blank	Treatment		
1. Normal Mineral Salts Medium	7.41	6.72	0.69	8.767**
2. Concentrated Mineral Salts Medium	6.30	5.68	0.62	10.065**
3. Spent Medium	4.97	4.56	0.41	18.386**
4. Concentrated Spent Medium	6.41	6.40	0.01	0.114 ns ³

¹ t is distributed as Student's t with $n_1 - 2 + n_2 - 2 = 8$ degrees of freedom

$$\begin{array}{l} t_{05} \\ 01 \end{array} \quad 8 \text{ df} = \begin{array}{l} 2.306 \\ 3.355 \end{array}$$

²The value for $b_1 - b_2$ used in this project was selected to be a positive number. If "treatment" regression coefficient is larger than "blank," then "treatment" is taken as b_1 .

³ns = non-significant

Table 3. The effects of concentrated spent medium on oxygen consumption by resting cells of Hydrogenomonas eutropha in an air atmosphere.

Time in Minutes	Endogenous (μ l O ₂ Consumed)	Conc. Spent Medium Added (μ l O ₂ Consumed)	Difference
0	0	0	0
10	1.83	1.32	-0.51
20	2.70	2.60	-0.10
30	5.21	4.00	-1.12
40	5.50	4.00	-1.50
50	4.10	2.00	-2.10
60	4.60	3.30	-1.30
90	6.70	6.50	-0.20
150	2.50	1.26	-1.24

Table 4. The effects of concentrated spent medium on carbon dioxide production by resting cells of Hydrogenomonas eutropha in an air atmosphere.

Time in Minutes	: Endogenous :(μ l CO ₂ Produced)	: Conc. Spent Medium Added :(μ l CO ₂ Produced)	: Difference
0	0	0	0
10	0.97	0	+0.97
20	2.78	0.57	-1.21
30	1.80	2.53	+0.73
40	1.40	3.03	+1.63
50	5.98	5.58	-0.04
60	7.03	5.90	-1.03
90	9.54	2.22	-7.32
150	11.90	5.65	-6.25

Table 5. The t test for difference between pairs of regression lines for two different samples at various pH levels.

Sample	: Regression Coefficient	: Difference $b_1 - b_2^1$	: Calculated t value	
1. Concentrated Spent Medium	<u>Blank</u> 6.06	<u>pH 7</u> 6.33	0.27	4.631**
	<u>pH 5</u> 3.67	<u>pH 9</u> 3.51	0.16	3.024*
2. Concentrated Mineral Salts Medium	<u>Blank</u> 7.23	<u>pH 7</u> 6.46	0.77	8.780**
	<u>pH 5</u> 6.01	<u>pH 9</u> 5.94	0.07	0.719 ns

¹ The number $b_1 - b_2$ is always positive.

t_{05} 8 df = 2.306
 t_{01} 8 df = 3.355

Table 6. The t test for difference between regressions for fractions of two different samples.

Sample	: Regression Coefficient		: Calculated
	: Blank	: Distillate	: t value
Concentrated Spent Medium			
pH 5 Fraction	5.43	6.18	26.596**
pH 7 Fraction	7.02	6.96	1.097ns
pH 9 Fraction	6.04	6.09	0.982ns
Concentrated Mineral Salts Medium			
pH 5 Fraction	6.22	6.91	12.410**
pH 7 Fraction	8.28	8.26	0.237ns
pH 9 Fraction	7.19	7.62	5.964**

$$t_{05} \quad 8 \text{ df} = 2.306$$

$$t_{01} \quad 8 \text{ df} = 3.355$$

Table 7. A comparison of salt concentration and gas utilization by resting cells of Hydrogenomonas eutropha.

Sample	Salt Concentration in Warburg Flask (mg/ml)	"Q" Gas ¹	
		Blank	Treatment
Normal Mineral Salts Medium	1.019	558	516
Spent Medium	1.119	367	339
Concentrated Spent Medium	47.858	487	484
pH 5 Distillate	0.231	410	468
pH 5 Residue	118.369	410	193
pH 7 Distillate	0.062	531	535
pH 7 Residue	128.077	531	277
pH 9 Distillate	0.427	453	449
pH 9 Residue	137.258	354	223
Concentrated Normal Mineral Salts Medium	33.454	482	435
pH 5 Distillate	0.240	473	521
pH 5 Residue	95.231	473	312
pH 7 Distillate	0.252	631	632
pH 7 Residue	74.615	631	349
pH 9 Distillate	3.515	539	568
pH 9 Residue	59.762	539	337

¹"Q" is a term representing the rate of gas mixture consumed:

$$Q \frac{(H_2-O_2-CO_2)}{(H_2-O_2)} \quad (mg)$$

This represents the μ l of hydrogen-oxygen gas consumed in an atmosphere of hydrogen, oxygen, and carbon dioxide per mg of dry weight tissue per hour.

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