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SOME OF THE EFFECTS OF THREE AMINO ACIDS ON THE GROWTH OF HYDROGENOMONAS EUTROPHA

By

EVA ELLIOTT BLAKE

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

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APPROVED:

Chairman of Supervisory Committee and Director of Thesis	
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Member of Committee	Dean of the College of Arts and Sciences
Member of Committee	Dean of the Graduate School

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E.E.B.

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INTRODUCTION

Among the many problems presented by a spaceflight of long duration is the limited area in which to carry supplies. To alleviate this situation scientists have searched intensively for a biological system to create a closed ecology. The goal of these investigations is a microcosm in which microbes will break down human waste, regenerate the atmosphere, and produce useable foodstuff.

Many different approaches to this problem have been examined. The electrolysis of water has been considered a suitable source of oxygen, but, with each mole of oxygen, there will be liberated two moles of the highly explosive gas hydrogen. Two microorganisms which can use the energy obtained from the oxidation of molecular hydrogen to reduce carbon dioxide have been considered. Clostridium aceticum uses hydrogen and carbon dioxide according to the following equation:

$$4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$$

There are many disadvantages to the use of this bacterium for the purpose of a regenerative system in a closed ecology and the use of its byproducts for human nutrition. There is a need for strict anaerobic conditions, the microbe has a slow growth rate, there is a slight production of proteins, and there is the acidic end product of doubtful nutritional

value (Schlegel, 1964).

The use of an organism of the genus Hydrogenomonas appears more favorable (Lechtman, Goldner, and Canfield, 1963; Schlegel, 1964).

At present the facultative autotroph Hydrogenomonas eutropha is being investigated to determine its behavior in a system to remove hydrogen and carbon dioxide and to form cellular material of possible nutritional value (Lechtman, Goldner, and Canfield, 1963; Bongers, 1963; Schlegel, 1964; and Brown, Cook, and Tischer, 1964). The ability of this organism to use urea as a sole source of nitrogen (Repaske, 1962a, Lechtman, Goldner, and Canfield, 1963; and Bongers, 1963) and its ability to grow rapidly and to form cells rich in poly-β-hydroxybutyric acid (Lechtman, Goldner, and Canfield, 1963; Schlegel, 1964) enhance its potential usefulness in spaceflight.

A possible disadvantage which this microorganism may present is that it grows well on heterotrophic media (Bovell, 1957). When growth is terminated during the late log phase, among the products found in the depleted medium after the removal of the cells are the amino acids tyrosine, alanine, and glutamic acid (Brown, Cook, and Tischer, 1964).

It is anticipated that the growth medium to be used by biological systems in these spacecrafts must be suitable for recycling after removal of the bacterial cell mass and replenishment of the basic minerals. For this reason

experiments to determine what effects the presence of these organic compounds in the basic medium will have on the growth and utilization of hydrogen, carbon dioxide, and oxygen by Hydrogenomonas eutropha were begun in this laboratory.

REVIEW OF LITERATURE

As facultative chemolithoautotrophs the genus Hydrogenomonas occupies a key position in the biological world. The simplicity of the reaction which gives the necessary driving energy for their autotrophic growth has been responsible for their popularity in research directed toward understanding this mode of life. Possibly for this reason their history seems to divide itself into four parts:

1. Their initial isolation.
2. The recognition and naming of the enzyme hydrogenase.
3. The studies on the pathway of carbon during heterotrophic growth conditions, autotrophic growth conditions, and storage conditions.
4. The possible role of a species of Hydrogenomonas in a biological atmosphere regenerating system.

It is generally known by microbiologists today that hydrogen-oxidizing bacteria can be isolated from soil by enrichment techniques. A report that soil possesses some characteristics that caused the consumption of a mixture of hydrogen and oxygen antedated the establishment of the germ theory by several decades. De Saussure (1839) sought means to explain the rarity of hydrogen in our atmosphere. The belief that the decomposition of organic substances should continually add hydrogen to the atmosphere prompted de Saussure to incubate a number of different substances

under a mixture of one volume of oxygen and two volumes of hydrogen. Among the different substances that he used were leaf-mold and arable soil. From these experiments he found that the two gases disappeared and carbonic acid was formed. This action did not occur if sulfuric acid or salty water was added to the incubation flask at the beginning of the experiment. De Saussure concluded that the oxygen that did not go into the formation of carbonic acid combined with the hydrogen to form water.

While conducting experiments concerning another problem Immendorff (1892) found that a mixture of hydrogen and oxygen disappeared when incubated with moist soil. To determine if this phenomenon were due to some physical and chemical property of soil or if it were due to microorganisms Immendorff conducted several experiments. When he incubated the soil with the mixture of hydrogen and oxygen, there was a slight production of carbonic acid followed in a few weeks by the disappearance of the gases. If, at the onset of the experiment, a few drops of chloroform were introduced into his incubation apparatus, there was no change in the gas mixture except for a slight production of carbonic acid. From these results, he felt there was a great possibility that microorganisms were the causative agents. He likened this process to those studied by Winogradsky, namely the microbial oxidation of ferrous iron, hydrogen sulfide, and ammonia.

The first report of the isolation of a hydrogen-oxidizing bacterium was made by Kaserer (1906). The preceding year he had published an article discussing the oxidation of hydrogen by microbes (1905).

Kaserer recognized the autotrophic nature of his microorganism and proposed that his isolate, Bacillus pantotrophus, used the energy it obtained from the oxidation of hydrogen to assimilate carbon dioxide in a scheme similar to that used by green plants. Contrary to the behavior of the known autotrophic microorganisms of that time, Bacillus pantotrophus was able to grow heterotrophically on ordinary culture media (1906).

The appearance of Kaserer's announcement of this newly recognized type of bacterial species apparently inspired more investigation in this area, for several articles concerning the utilization of hydrogen by microorganisms were soon published.

Nabokisch and Lebedeff (1906) were highly critical of Kaserer in that they felt his experimental method was inadequate. By using a closed culture flask connected to a manometer they were able to confirm the disappearance of the gas mixture, and by gas analyses they were able to establish that hydrogen had been oxidized. Later Lebedeff (1908) reported the isolation of an autotrophic hydrogen-oxidizing bacterium.

Nikitinsky (1907) had observed, in studies of

microorganisms in sewage, that when hydrogen was used to produce anaerobic conditions a decrease in pressure resulted. He confirmed that this was caused by microbes in a series of experiments using inocula obtained from sewage treatment plants. The control vessels contained a disinfectant. The hydrogen was always consumed after several weeks except in the flasks containing the disinfectant.

What might be considered the first review article concerning hydrogen-oxidizing bacteria was written by Niklewski (1906). Using a mineral salts medium in a closed flask containing a mixture of hydrogen, oxygen, and carbon dioxide he was able to cultivate a rod-shaped bacterium that could form a pellicle with carbon dioxide as a carbon source using the energy it derived from the oxidation of hydrogen. He continued his studies and was able to isolate two distinct species: Hydrogenomonas vitrea and Hydrogenomonas flava. Both species were sensitive to high tensions of oxygen (1910).

Later Niklewski (1914) published a thorough report of his investigations on Hydrogenomonas agilis. He found this bacterium was able to grow anaerobically under an atmosphere of hydrogen when nitrate was present in the medium. Under an atmosphere of hydrogen all three of his isolates were able to grow in a sugar solution in the presence of nitrate.

Further investigations were published by Ruhland (1922; 1924), stating the nature of hydrogen-oxidizing bacteria in

general and of Bacillus pycnoticus in particular. Ruhland especially emphasized the importance of maintaining the proper hydrogen ion concentration and of having iron available to the microorganism in the ferrous state. He concluded that the presence of organic compounds in the medium, when autotrophic growth conditions were imposed on the microorganism, was detrimental to the growth of Bacillus pycnoticus only if the organic compound brought about the production of acids. The knallgas reaction (oxidation of hydrogen) was retarded at first under these conditions, but later large amounts of the gas mixture were consumed. It may be of interest to mention that these experiments concerning gas consumption lasted from about one to four weeks.

There was considerable interest in the energy efficiency of hydrogen-oxydizing bacteria. It was the opinion of Kaserer (1906) that the primary reaction was the reduction of carbon dioxide by hydrogen. This view was supported by Baas-Becking and Parks (1927) and by Burks (1931) who gave, respectively, values of 26.4 percent and 28.4 percent for the free energy efficiency of the knallgas reaction as brought about by hydrogen-oxidizing bacteria. Kluyver and Manten (1942) refuted this theory by experimentation. By enrichment techniques these men isolated a microorganism which they believed to be Hydrogenomonas flava. Manometric studies were performed on resting cell suspensions of this bacterium grown under autotrophic conditions. It was found

that no gas was consumed when the atmosphere consisted only of hydrogen and carbon dioxide.

Stephenson and Stickland (1931) gave the name hydrogenase to the bacterial enzyme capable of activating molecular hydrogen to bring about the reduction of molecular oxygen, methylene blue, nitrate, and fumarate. According to them, hydrogenase catalyzes the following reaction:

$$H_2 \rightleftharpoons 2\,[H]$$

The fact that hydrogenase is present in many microbial species was early recognized (Grohmann, 1924; Stephenson and Stickland, 1931; Dworkin and Foster, 1958).

A species of the green alga Scenedesmus, after anaerobic adaptation in the dark under an atmosphere of hydrogen, was shown to be able to absorb hydrogen. Oxygen was reduced when present in small quantities. In the light the carbon dioxide was reduced to form cellular substances.

$$CO_2 + 2\,H_2 \rightarrow H_2O + \langle CH_2O \rangle$$

The rates of carbon dioxide reduction by hydrogen were proportional to and limited by the light intensity (Gaffron, 1939; 1940; 1942; Gest, 1954).

Korkes suggested that a common pathway exists between hydrogen utilization and photosynthesis because some algae oxidize hydrogen while others produce hydrogen for

reductive synthetic reactions (1955).

It was the opinion of Krasna and Rittenberg (1956) that the term hydrogenase should be limited to enzymes which reversibly activate molecular hydrogen. Their studies on the exchange reaction (the ability to interconvert ortho- and para-hydrogen) with different microorganisms showed that activation of molecular hydrogen appeared to be relatively simple, but certain cofactors or additional enzyme systems were necessary for the utilization of the activated hydrogen for chemical reduction.

Schatz and Bovell (1952) isolated the hydrogenomonad, Hydrogenomonas facilis, which has been the subject of the greatest amount of investigation. By a manometric procedure Schatz (1952) showed that the overall reaction effected by this bacterium is as follows:

$$6\ H_2 + 2\ O_2 + CO_2 \rightarrow \langle CH_2O \rangle + 5\ H_2O$$

The studies of Schatz and Bovell (1952) showed that with carbon dioxide or glucose as a carbon source nitrate was reduced to nitrite. The hydrogenase of Hydrogenomonas facilis appeared to be constitutive since cells grown on an organic medium used hydrogen regardless of the composition of the medium, incubation temperature, oxygen tension, or number of transfers. Atkinson and McFadden (1954) found that Hydrogenomonas facilis cells which had been cultivated heterotrophically could not reduce methylene blue in an

atmosphere of hydrogen. Wilson, Stout, Powelson, and Koffler (1953) found heterotrophically grown cells would show hydrogenase activity if they were grown on heterotrophic medium under an atmosphere of ninety percent nitrogen, five percent oxygen, and five percent carbon dioxide. Linday and Syrett (1958) exposed cells that had not shown hydrogenase activity under an atmosphere of ninety-five percent hydrogen and five percent air. Hydrogenase activity was developed, but the cells showed no increase in weight. When 2,4-dinitrophenol (DNP) or chloramphenicol was present in the medium, hydrogenase activity failed to develop.

Since nitrate in the growth medium decreases the hydrogenase activity of Hydrogenomonas facilis, Atkinson (1955b) tested the inorganic nitrogen compounds intermediate in oxidation between nitrate and ammonia for hydrogenase inhibition by the methylene blue assay. Nitric oxide was found to exert fifty percent inhibition at a molarity of about 3×10^{-5}.

Atkinson and McFadden (1954) found the hydrogenase of Hydrogenomonas facilis retained its activity when heated to sixty degrees centigrade for five minutes.

For their studies on hydrogenase Packer and Vishniac (1955a) isolated a species of a hydrogen-oxidizing bacterium, Hydrogenomonas ruhlandii. The sensitiveness of its hydrogenase to high tensions of oxygen was shown by the failure of the microbes to grow autotrophically when the

inoculum came from a nutrient broth culture grown under air. When a nutrient broth culture was grown under ninety percent nitrogen and ten percent oxygen and then transferred to autotrophic conditions, rapid growth occurred.

Later Packer and Vishniac (1955b) purified a diphosphate nucleotide- (DPN) linked hydrogenase from Hydrogenomonas ruhlandii. The ability of this hydrogenase to reduce DPN with molecular hydrogen was dependent on catalytic amounts of manganous chloride, cysteine, and flavin mononucleotide (FMN), and was stimulated by inorganic phosphate. It was reported by Peck and Gest (1955) and Korkes (1955) that hydrogenase from species of Clostridium appeared to reduce DPN. Packer (1958) continued his studies with Hydrogenomonas ruhlandii and found, in the intact cells, such respiratory carriers as flavins, cytochromes of the "b" and "c" type, and two carbon monoxide-binding pigments.

Schlegel (1953) isolated a species of Hydrogenomonas for physiological studies. Nitrate was reduced by this bacterium, and the hydrogenase was thermostable.

Bovell (1957) isolated a hydrogen-oxidizing bacterium containing a soluble hydrogenase. Repaske (1962a) states that this microbe was later named Hydrogenomonas eutropha. Bovell (1957) found that this bacterium, regardless of substrate, would oxidize hydrogen, and the rate of this reaction was not greatly affected by pressures of oxygen ranging between 0.05 and 0.6 atmosphere. The intact cells were

able to evolve hydrogen from reduced methylviologen. Cyanide was found to be inhibitory, and the inhibition due to the presence of cyanide on the fixation of carbon dioxide and the oxidation of hydrogen was about the same.

Fractional centrifugation of the disrupted cells revealed the hydrogenase activity, as measured by methylene blue assay, to be in the supernatant following centrifugation at 100,000 times gravity. This fraction was designated as 100,000-S. The pellet resulting from this procedure contained the components of the electron transport system to oxygen, and it would oxidize reduced diphosphate nucleotide (DPNH) and could reduce oxygen with hydrogen.

A soluble cytochrome was contained in the 100,000-S fraction. This fraction was found capable of reducing substrate quantities of DPN with hydrogen, but he could not demonstrate a DPNH-oxidase.

Using the same microorganism similar observations were made on the products of fractional centrifugation by Wittenberger (1960), Wittenberger and Repaske (1961), and Repaske (1962b). These workers found that the supernatant resulting from centrifugation at 144,000 times gravity (144,000-S) coupled, under anaerobic conditions, oxidation of DPNH to the reduction of endogenous flavin and cytochrome "c". They also found a cyanide sensitive pathway for the anaerobic oxidation of DPNH (Wittenberger, 1960; Wittenberger and Repaske, 1961). Repaske (1962b) further

reported that the DPN reducing system required FMN and a reducing agent for activity. The reduction of DPN was accelerated by the presence of ferrous ion and inhibited by ammonium and sodium ions.

Bovell (1957) found that Hydrogenomonas eutropha would use a number of organic compounds, including glutamate, for a source of carbon and energy. He observed that the capacity to oxidize hydrogen is lost unless iron is present in the ferrous state. He suggested that the loss of hydrogenase activity experienced by others with hydrogenomonads perhaps was due to autoclaving the growth medium. In experiments with other microbial species, Hyndman, Burris, and Wilson (1953), and Peck, San Pietro, and Gest (1956), concluded that iron is essential for hydrogenase activity.

Bartha (1962) isolated a species of Hydrogenomonas for investigations similar to those already discussed. He found that after growth in peptone medium hydrogenase activity was induced with more difficulty than following growth in organic acids. He made the interesting observation that the rate of oxidation of hydrogen is greater in the presence of carbon dioxide than in its absence.

That hydrogen-oxidizing bacteria fix carbon dioxide by a system similar to that in green plants had been suggested by Kaserer (1906) and Lebedeff (1908). The work by Calvin elucidating the path of carbon in photosynthesis (1962) apparently inspired investigation with other autotrophic

forms. Aubert, Milhaud, and Millet (1957) found an assimilatory pattern for carbon dioxide in Desulfovibrio desulfuricans analogous to that of the ribulose diphosphate cycle of Calvin.

McFadden and Atkinson (1957) studied factors affecting the autotrophic fixation of carbon dioxide by Hydrogenomonas facilis. They found a number of inhibitors retarded carbon dioxide fixation without affecting the oxidation of hydrogen. They felt their results concerning inhibitors were in general agreement with the results found by workers with photosynthesis using isolated chloroplasts.

Orgel, Dewar, and Koffler (1956) and Orgel (1958) found formic acid and doubly-labeled acetic acid among the early labeled products following $C^{14}O_2$ incorporation by Hydrogenomonas facilis cells growing under autotrophic conditions. These results could not be duplicated by McFadden (1959), Bergmann, Towne, and Burris (1958), and Dewar (1962). The early products found following similar treatment by these workers were phosphoglyceric acid and some phosphorylated sugars.

While studying the formation of labeled amino acids from $C^{14}O_2$ assimilation by autotrophically growing cells of Hydrogenomonas facilis, Faust (1958) found that the amino acids which were labeled early were glutamic acid, glycine, alanine, serine, and aspartic acid. Valine and leucine were weakly labeled. Since glutamic acid was found at high

levels after longer exposure times, it was considered that this amino acid was formed as a result of carboxylation of a C_4 compound to yield α-ketoglutarate. That glycine was found to be labeled predominantly in the carboxyl group indicates the operation of the ribulose diphosphate cycle.

In a discussion of these findings by Lees (1960) he concluded that most of the carbon dioxide fixed by hydrogenomads appears to follow a pathway of fixation similar to that of green plants. Quayle (1961) stated that the finding of formic and acetic acids required more investigation for proper assessment.

McFadden (1959) found that under heterotrophic conditions Hydrogenomonas facilis assimilated $C^{14}O_2$ in a pattern similar to that of autotrophic conditions but to a lesser extent. Later studies of heterotrophic fixation of carbon dioxide by this bacterium by McFadden and Homann (1963) showed that fixation is curtained by DNP.

Kanai, Miyachi, and Takamiya (1961) found that lactate accelerates the fixation of carbon dioxide in air by either autotrophically or heterotrophically grown cells of Hydrogenomonas facilis. Heterotrophic fixation of carbon dioxide was 1/11 that of autotrophic fixation. Rather interestingly, they found that pretreatment of autotrophically grown cells of Hydrogenomonas facilis with an atmosphere of oxygen and hydrogen caused the cells to acquire a capacity to fix carbon dioxide from an atmosphere of carbon

dioxide and nitrogen. Soulen (1963) found that crude extracts obtained from Hydrogenomonas facilis carried out an esterification of radioactive inorganic phosphate. This reaction was linked to the oxidation of molecular hydrogen. These results bring to mind those of Vogler (1942) and Vogler and Umbreit (1942) who found that Thiobacillus thiooxidans was capable of storing energy derived from the oxidation of sulfur in a form available to the microbe when sulfur was removed from the medium. When sulfur was no longer available, the microbe could fix carbon dioxide with this stored energy. The data of Vogler and Umbreit (1942) showed that the storage of energy by the cells was accompanied by the removal of inorganic phosphate from the medium by the cells.

McFadden and Howes (1962) found the optimal conditions for the catalysis of isocitrate lyase (D_S-isocitrate glyoxylate-lyase, EC 4.1.3.1) and malate synthase (L-malate glyoxylate lyase, EC 4.1.3.2) through their work with a soluble preparation from Hydrogenomonas facilis.

Bartha (1962) found that in a medium limited in a nitrogen source his species of Hydrogenomonas when under autotrophic conditions would synthesize poly-β-hydroxybutyric acid (PBHA). Hirsch (1963), Hirsch and Schlegel (1963), and Hirsch, Georgiev, and Schlegel (1963) sought means to determine the path of carbon dioxide fixation under these conditions which they termed storage conditions. Following

incorporation of $C^{14}O_2$ the bulk of the radioactivity was found contained in hexose monophosphates. When C^{14}-acetate was offered under nitrogen-deficient conditions PBHA was found to be labeled. Evidence suggested that the stored PBHA could be metabolized and used for protein synthesis when cells containing this polymer are brought in contact with a nitrogen source (Schlegel, Gottschalk, and Bartha, 1962).

Until the last few years, the species of Hydrogenomonas had received attention only in basic research aimed at resolving problems of comparative biochemistry. The mounting interest in space travel brought with it the realization of the many difficulties to be encountered in such an endeavor and spurred investigations to meet these problems. From this was conceived the idea of a biological atmosphere regenerating system and the desire for a closed ecology based on biological systems.

It was the opinion of Lechtmann, Goldner, and Canfield (1963) that an anaerobic hydrogen utilizing bacterium would be unsuitable. They confined their investigations to Hydrogenomonas eutropha and Hydrogenomonas ruhlandii. Their results showed Hydrogenomonas eutropha, when compared to Hydrogenomonas ruhlandii, had a shorter generation time, consumed more of the gas mixture, and appeared to grow better in a static culture.

Bongers (1963) found that when Hydrogenomonas eutropha

was growing under autotrophic conditions and when urea replaced ammonium chloride as the nitrogen source in the culture medium, the pH was not lowered during the growth period and more gas was consumed. His findings showed that a cell density of between ten and twelve grams of cells per liter consumed enough of the carbon dioxide in the gas mixture to balance the metabolic needs of one man.

Schlegel (1964) compared the use of hydrogenomonads with Clostridium aceticum for use in an atmosphere regenerating system. The latter has the advantage of being anaerobic, and the use of this microorganism would allow all of the oxygen produced by electrolysis of water to replenish the atmosphere of the space craft. The disadvantages of this microorganism were discussed in the introduction. Schlegel calculated that to support one man 0.1 kilogram of bacterial mass must be formed within three hours.

That Hydrogenomonas eutropha can grow under an atmosphere of hydrogen, oxygen, and carbon dioxide in a one to one mixture of urine and fecal extract was established by Lechtmann, Canfield, and Goldner (1963). Under these conditions the ratio of hydrogen, oxygen, and carbon dioxide shifts from about 6-2-1 to about 18-7-1. These figures seem to indicate that the presence of organic material in the culture medium diminishes the amount of carbon dioxide fixed by the microorganism. An important point that needs to be established is whether autotrophically grown cells can

oxidize organic compounds. Schatz and Bovell (1952) reported that autotrophically grown cells of Hydrogenomonas facilis grow well in air on a number of organic compounds and also are capable of oxidatively assimilating a number of organic compounds without a period of adaptation. Atkinson (1955a) investigated the ability of Hydrogenomonas facilis to adapt to organic substrates. Employing the Warburg respirometer he found that Hydrogenomonas facilis cells grown on a complex medium containing certain metabolic acids were adapted to all of the acids without a period of adaptation. These cells would not oxidize glucose; glucose was oxidized only by Hydrogenomonas facilis cells which had been grown on glucose.

Cells grown under autotrophic conditions and harvested with little or no exposure to air showed a typical adaptive oxidative pattern. There was no adaptation to glucose by cells grown under autotrophic conditions. Somewhat similar observations concerning oxidative assimilation were made by Marino and Clifton (1955), but Crouch and Ramsey (1962) and Crouch (1963) gave evidence that Hydrogenomonas facilis cells grown under autotrophic conditions do oxidize glucose.

Wilson, Stout, Powelson, and Koffler (1953) found that greater amounts of the hydrogen mixture are utilized in the presence of lactate than in its absence. Using another species of Hydrogenomonas, Wilde (1962) found that carbon dioxide fixation occurs in the presence of organic substrates.

CULTURE AND CULTURE MEDIA

Culture

Hydrogenomonas eutropha

This microorganism was originally obtained from Dr. L. Bongers of the Space Science Division of the Martin Marietta Company and was maintained under strict autotrophic conditions by Mr. David W. Cook, in the Microbiology Department, Mississippi State University.

Culture Media

A. Nutrient Agar

yeast extract	3.0 grams
peptone	5.0 grams
agar	20.0 grams
Distilled water	1000.0 milliliters

B. Cook's modification of Repaske's medium (Personal Communication, 1965)

Solution A

NH_4Cl	10.0 grams
$CaCl_2 \cdot 2H_2O$	1.0 gram
NaCl	1.0 gram
$MgSO_4 \cdot 7H_2O$	1.0 gram
Distilled water	100.0 milliliters

The pH was adjusted to 6.8 with 1N NaOH.

Solution B

Na_2HPO_4	21.690	grams
KH_2PO_4	13.260	grams
Distilled water	100.0	milliliters

Solution C

Approximately eighty milliliters of distilled water was acidified to a pH of 3.0 with 1N H_2SO_4. To this solution 110.4 milligrams of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was added and dissolved. The solution was then diluted to a volume of one hundred milliliters with distilled water. The pH was measured to determine if the range was between 2.5 and 3.0 since the salt precipitates at a lower hydrogen ion concentration.

Solution D

$CoCl_2 \cdot 6H_2O$	0.2	milligram
$MnCl_2 \cdot 4H_2O$	400.0	milligrams
$CuSO_4 \cdot 5H_2O$	2.0	milligrams
$Na_2MoO_4 \cdot 2H_2O$	10.0	milligrams
$ZnSO_4 \cdot 7H_2O$	10.0	milligrams
Distilled water	1000.0	milliliters

Solutions A and C were filter-sterilized with an ultrafine sintered glass filter or with a millipore filter. Solutions B and D, distilled water, the nutrient agar, and equipment were sterilized in the autoclave at 121 degrees centigrade for fifteen minutes at fifteen pounds pressure.

All distilled water was obtained from a Barnstead Water Still. Analyticalgrade inorganic salts were used.

One milliliter each of sterile solutions A, B, C, and D was added aseptically, in this order, to ninety-six milliliters of sterile distilled water with thorough mixing after each addition. Unless otherwise noted, this medium will hereafter be referred to in this text as Repaske's medium.

C. Repaske's medium containing the amino acids.

An aqueous solution of twice the desired strength was prepared by adding the weighed amount of the amino acid to approximately fifty milliliters of distilled water. If necessary the solution was neutralized with sodium hydroxide, and the solution was diluted with distilled water to a final volume of one hundred milliliters. This solution of the amino acid was sterilized in the autoclave at 121 degrees centigrade for fifteen minutes at fifteen pounds pressure. Comparative paper chromatograms showed that this treatment did not change the Rf values of the amino acids.

Two milliliters each of sterile solutions A, B, C, and D were added aseptically, in this order, to ninety-six milliliters of sterile distilled water with thorough mixing after each addition. The amino acid solution and the concentrate of Repaske's medium were combined.

The three amino acids used were L-alanine, L-tyrosine, and L-glutamic acid which were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

D. Control Repaske's medium for amino acids requiring neutralization.

To approximately fifty milliliters of distilled water two milliliters of Solution B were added. That amount of sodium hydroxide required to neutralize the amino acid was then added. The resulting solution was neutralized with hydrochloric acid. The volume was then diluted to 194 milliliters with distilled water. The medium was completed after sterilizing in the autoclave by adding two milliliters each of Solutions A, C, and D aseptically,in this order, with thorough mixing after each addition.

E. Gas mixture

The gas mixture was prepared in a steel bomb fitted with a Bourdon gauge. The bomb was evacuated to thirty inches of mercury by water aspiration. The gases were admitted to the bomb in these amounts and in this order: one part carbon dioxide, two parts oxygen, and six parts hydrogen. Ideally, the percentages were 11.11, carbon dioxide; 22.22, oxygen; and 66.67, hydrogen. This gas mixture will hereafter be referred to as the gas mixture.

The bomb was connected to a manifold with five outlets. The manifold was connected to a gauge which was connected to a water aspirator. The rubber rubing on the manifold was connected to the filters on the culture bottles, the valve on the gauge was opened, and the culture bottles were evacuated to thirty inches of mercury by water aspiration.

The valve was closed, the gas mixture was admitted to the culture bottle to atmospheric pressure, and the tubing below the filters was clamped.

EXPERIMENTAL PROCEDURE

Procedure Used to Maintain Inoculum for Growth Studies

The original culture was on a Repaske agar slant. A transfer was made to Repaske's medium, and the culture was allowed to grow on a reciprocal shaker at thirty degrees centigrade under an atmosphere of the gas mixture until a dilution of one part in five had an optical density of about 0.15 at a wavelength of 655 mμ. This bacterial suspension was allowed to remain in the culture medium and, after being refrigerated for two days, was used as an inoculum.

A standard operating procedure for maintaining an inoculum under autotrophic conditions was established: All cultures for the inoculum were grown in six-ounce Owens Oval prescription bottles closed with a rubber stopper containing a glass tube to which was connected a filter of Fiberglas (Figure 1). Each culture bottle contained twenty-five milliliters of Repaske's medium inoculated with 0.2 milliliter of the inoculum. The bottles were evacuated through the filter and were filled with the gas mixture. The cultures were incubated at approximately thirty degrees centigrade on either the rotary or reciprocal shaker for forty-four to forty-eight hours.

At the end of the growth period the culture bottles were evacuated through the filter by water aspiration to remove any residual gas. The culture bottles were allowed

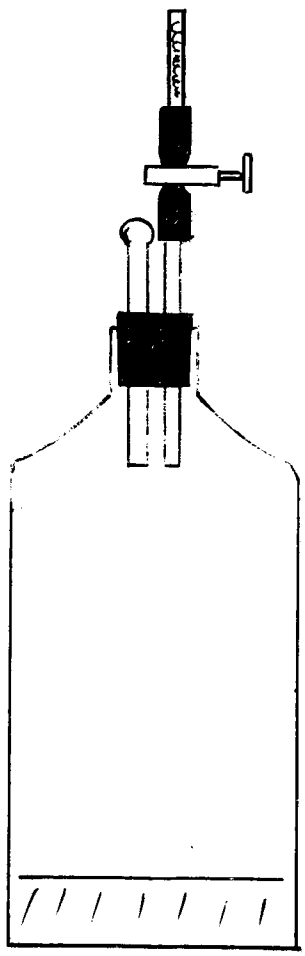

Figure 1. Culture bottle used to grow the inoculum. A six-ounce Owens Oval prescription bottle was fitted with a number 0 rubber stopper with two holes. One hole was sealed. The second hole was fitted with a two inch glass tube with an internal diameter of 0.25 centimeter. This tube was connected to a Fiberglas filter. The stopper and the bottle were sterilized separately in the autoclave at 121 degrees centigrade for fifteen minutes at fifteen pounds pressure.

to fill with air through the filter. The bacterial suspension was then subjected to the standard operating procedure for determining the purity of the cultures. After the streak plate had been examined, any bacterial suspension free of contaminants and having an optical density of one part in five of 0.1 to 0.2 at a wavelength of 655mμ was used as an inoculum for the comparative growth studies. This inoculum was discarded four days after the termination of the growth period.

Procedure Used to Determine Purity of Culture

A standard operating procedure for determining purity of the culture was outlined and is as follows: A simple stain with crystal violet and a gram stain were made from the cell suspension in Repaske's medium. A streak plate on nutrient agar was made. At the end of forty-eight hours the streak plate was observed under the dissecting microscope with oblique lighting for colony morphological characteristics after the method of Braun (1953). A typical colony was picked from the streak plate, and a simple stain with crystal violet and a gram stain were made. The culture was discarded if any atypical colonies were noted or if the microscopic examinations disclosed any microorganisms other than the short, plump, gram negative rods of Hydrogenomonas eutropha.

Procedure Used for Comparative Growth Studies

With each study Repaske's medium, as a control, and Repaske's medium containing the amino acid were inoculated with one percent by volume of the inoculum. The cultures were grown under four different conditions, and five cultures of each were examined at the end of certain growth periods of various lengths of time for the effects of growth. Any culture showing contamination was discarded.

A. Comparative Growth Study with an Atmosphere of the Gas Mixture.

Since one of the main objectives of these investigations was to determine the effects of the presence of the amino acids on the volume of the gas mixture consumed a unit of three bottles was adapted to fit this purpose (Figure 2).

The culture bottle contained five milliliters of the inoculated medium. Before the gas mixture was admitted, the tubing connecting the second and the third bottle was clamped. The first and the second bottles were evacuated by water aspiration through the filter connected to the first bottle. When the gauge indicated evacuation to thirty inches of mercury, the two bottles were flushed with the gas mixture. To insure that the atmosphere over the culture medium was that of the bomb, the unit was evacuated and

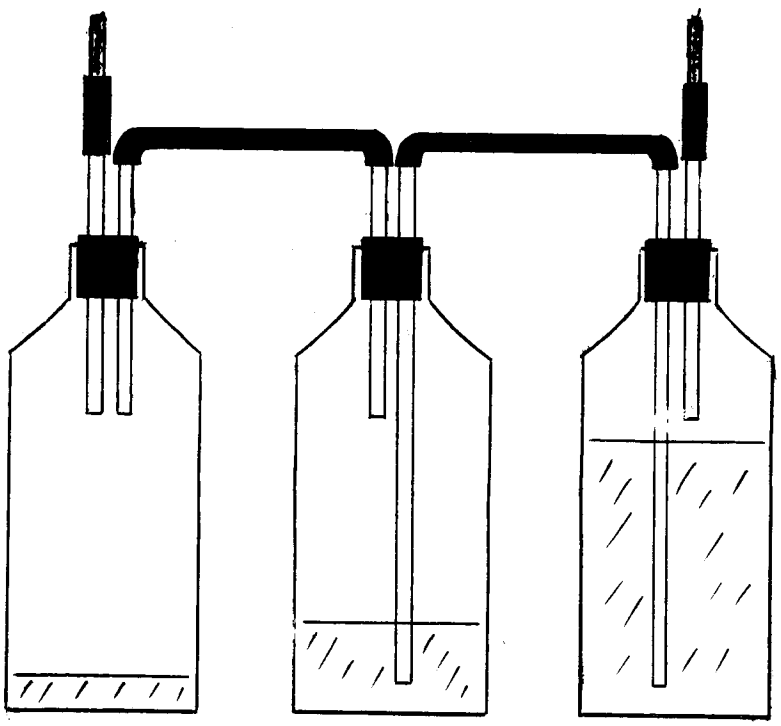

Figure 2. Unit for growth studies.
This unit was used for growth studies under an atmosphere of the gas mixture. The unit consisted of three six-ounce Owens Oval prescription bottles fitted with number 0 rubber stoppers with two holes. The internal diamter of the gas tubing was 0.25 centimeter. The bottles were connected by neoprene tubing with a wall thickness of 0.0625 inch and an internal diamter of 0.125 inch. The bottle on the left is the culture bottle. The middle bottle and the bottle on the right contained water acidified to a pH of approximately 2.5 with phosphoric acid. A siphon existed between these two bottles, and, to facilitate the observation of the siphon, the water solution was colored with methylene blue. The culture bottle and the bottle on the right were fitted with a Fiberglas filter. The various parts of the unit were individually sterilized in the autoclave at 121 degrees centigrade for fifteen minutes at fifteen pounds pressure.

flushed with the gas mixture three times.

After the system had been flushed for the third time, the tubing below the filter was clamped. The clamp between the second and the third bottle was removed. The unit was placed in an incubator at thirty degrees centigrade under stationary conditions. After an equilibration period of ten to thirty minutes, the unit was checked for a siphon between the second and the third bottle. If a siphon was not present the experimenter blew through the filter of the third bottle and momentarily released the clamp on the first bottle.

The units were allowed to equilibrate for a total of one hour. The level of the acidified water in the second bottle was then marked.

At the end of the growth period the level of the acidified water in the second bottle was marked.

As a check for contamination streak plates were made from all culture bottles. At the end of forty-eight hours the streak plates were observed under the dissecting microscope with oblique lighting for colony morphological characteristics after the method of Braun (1953).

B. Comparative Growth Study with an Atmosphere of Air.

To a sterile six-ounce Owens Oval prescription bottle, five milliliters of the inoculated medium used

for the growth studies under an atmosphere of the gas mixture were added. These cultures were incubated under stationary conditions at thirty degrees centigrade with an atmosphere of air.

At the end of the growth period all cultures were examined for contamination following the procedure described under Part A of this section.

Procedures Used to Measure the Effects of Growth

A. Turbidity

The optical density was measured on a Coleman Model 9 Nepho-Colorimeter employing a wavelength of 655 mμ. A one part in five dilution of all culture media was used with the exception of those culture media containing tyrosine. Because of its low solubility, any medium containing tyrosine was a suspension. It was found necessary to dilute those media containing one percent tyrosine one part in thirty to bring the tyrosine into solution for making turbidity measurements. The diluent and the blank were distilled water. All values for turbidity were multiplied by the appropriate dilution factor and were reported as optical density.

B. Hydrogen Ion Concentration

The pH measurements were determined on a Beckman Zeromatic pH meter.

C. Gas Analysis

A Beckman GC-2 gas chromatograph was used to analyze the gas mixtures. A four-foot silica gel column and a six-foot molecular sieve column were used, respectively, to measure the carbon dioxide and the oxygen and nitrogen finally in the culture units. With each experiment a unit containing sterile Repaske's medium was analyzed to obtain the initial percentages of the gases. The percentage of hydrogen was determined by the difference, assuming the two columns detected all gases present.

D. Gas Consumption

The volume of the change of the gas mixture was approximated by the amount of gas displacement in the second bottle of the culture unit.

Cellular Dry Weight Determinations

The cell mass for dry weight determinations was cultivated as described for the inoculum. The units were regassed three or four times during the growth period. The cells were collected by centrifugation and were resuspended in distilled water. The dry weights of one milliliter of the cell suspension were obtained by filtering through a tared millipore filter followed by drying at 100 degrees centigrade. These weights were obtained with a five place balance. Triplicate determinations of each sample were made.

The optical density of a one to five dilution at a wavelength of 655 mμ was obtained on each cell suspension. Dry weights are expressed as milligrams per milliliter.

Nitrogen Determinations

Nitrogen determinations were made by the semi-micro Kjeldahl method on one to five milliliters samples of the cell suspension described for dry weight determination. The method employed selenized Hengar granules as the catalyst as described by Davis (1965). Triplicate determinations of each sample were made. Total nitrogen values are expressed as milligrams per milliliter.

Manometric Studies

Manometric studies were conducted using double side-arm flasks with a conventional Warburg respirometer at thirty degrees centigrade.

The cells were cultivated as described for the dry-weight determinations and were centrifuged and washed three times in a refrigerated centrifuge with 0.85 percent sodium chloride. The cells were resuspended in the sodium chloride solution.

The optical density of each cell suspension was obtained on a one to five dilution at a wavelength of 655 mμ and was referred to the standard curves to obtain the cellular dry weight and total nitrogen corresponding to that

value. These cell suspensions were used within one day after termination of the growth period.

As substrate, a stock solution of each of the amino acids was prepared which, upon dilution with the contents of the main compartment of the flask, would give a one percent solution. The alanine and the glutamic acid used in these stock solutions were neutralized before diluting to volume.

The main compartment of the flasks contained M/15 phosphate buffer with a pH of 6.7 and the cell suspension. The total volume of the liquid phase in the flasks was 3.2 milliliters.

For the absorption of carbon dioxide the center well contained 0.2 milliliter of ten percent potassium hydroxide. A pleated wick of one by two centimeters of filter paper was placed in the center well to provide greater surface area.

For the absorption of any ammonia that might be formed due to deamination, one side-arm contained 0.4 milliliter of two percent boric acid with Tashiro's indicator (0.25 gram methylene blue, 0.375 gram methyl red in 300 milliliters of ninety-five percent ethanol). A pleated wick of one by two centimeters of filter paper was placed in this side-arm to provide greater surface area.

The other side-arm contained 0.2 milliliter of the stock solution of the substrate or a dilution of this stock solution. The flask was constructed so that additions to the main compartment could be made by tipping either

side-arm.

For those experiments using air as an atmosphere, vaseline jelly was used to seal all ground glass joints and Brodie's solution was used as the manometer fluid.

For those experiments using an atmosphere of oxygen and hydrogen, silicone stopcock grease was used to seal all ground glass joints and mercury was used as the manometer fluid.

To obtain an atmosphere of hydrogen and oxygen the flasks were flushed with a gas mixture for three minutes. As hydrogen is lighter than air, it is difficult to replace air with a mixture of hydrogen and another gas. To insure that the flasks would contain sufficient hydrogen, it was elected to use a mixture of ninety percent hydrogen and ten percent oxygen. This mixture was prepared by admitting to an evacuated steel bomb in this order one part of oxygen and nine parts of hydrogen.

The data from the manometric studies are expressed as microliters of the gas consumed per milligram of cellular dry weight or as follows:

Q_{O_2}[1] = microliters of oxygen consumed in an atmosphere of air per milligram of cellular dry weight per hour

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[1]Umbreit, W. W., R. H. Burris, and J. F. Stauffer, Manometric Techniques (Burgess Publishing Company, Minneapolis, 1964), p. 14.

$Q_{O_2(N)}$[2] = microliters of oxygen consumed in an atmosphere of air per milligram of tissue nitrogen per hour.

[2] Ibid.

EXPERIMENTAL RESULTS AND DISCUSSION

Preliminary Experiments

Preliminary experiments were done to determine if any of the three amino acids inhibited the growth of Hydrogenomonas eutropha. The pH of the medium containing glutamic acid was very low, and, as might be expected, there was no growth. It was then decided that during the preparation of the culture medium, the pH of the amino acid solution would be adjusted to seven whenever necessary (see Culture and Culture Media). The data discussed in this work were obtained using media that had received this treatment.

Further preliminary experiments were done employing an atmosphere of the gas mixture under shake conditions using a reciprocal shaker. All of the preliminary experiments employing an atmosphere of air were under stationary conditions. The incubation temperature was thirty degrees centigrade. A three percent by volume inoculum was used.

A composite of the results of these experiments is graphically represented in Figures 3, 4, 5, 6, and 7.

In examining the results obtained when using an atmosphere of the gas mixture (Figure 3) one observes that there is a better growth response, as indicated by turbidity measurements, in the presence of the amino acids than in Repaske's medium. When one percent each of tyrosine, glutamic acid, and alanine were added together to Repaske's

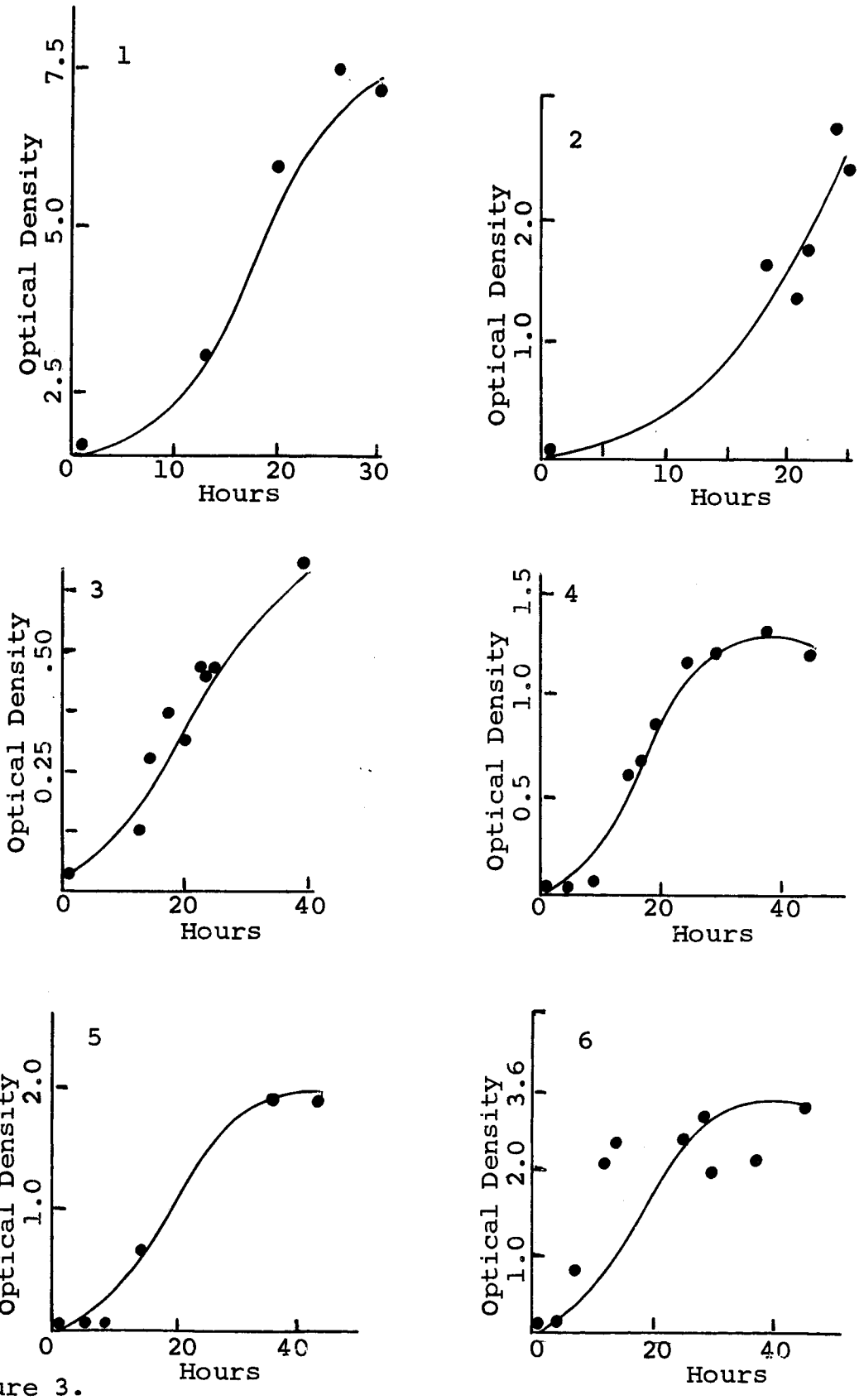

1
Optical Density
2.5
5.0
7.5
0
10
20
30
Hours
2
Optical Density
1.0
2.0
0
10
20
Hours
3
Optical Density
0.25
.50
0
20
40
Hours
4
Optical Density
0.5
1.0
1.5
0
20
40
Hours
5
Optical Density
1.0
2.0
0
20
40
Hours
6
Optical Density
1.0
2.0
3.6
0
20
40
Hours

Figure 3.

Figure 3. Composites of the results of turbidity changes (as measured by optical density) effected by the presence of the three amino acids during growth of Hydrogenomonas eutropha in Repaske's medium under shake conditions at thirty degrees centigrade with an atmosphere of hydrogen, oxygen, and carbon dioxide. Each point represents between two and five determinations. Please observe that the axes are of different scales.

1. 1% tyrosine, 1% glutamic acid, and 1% alanine in Repaske's medium.
2. 1% glutamic acid in Repaske's medium.
3. Repaske's medium as a control.
4. 0.1% glutamic acid in Repaske's medium.
5. 0.1% alanine in Repaske's medium.
6. 0.1% tyrosine in Repaske's medium.

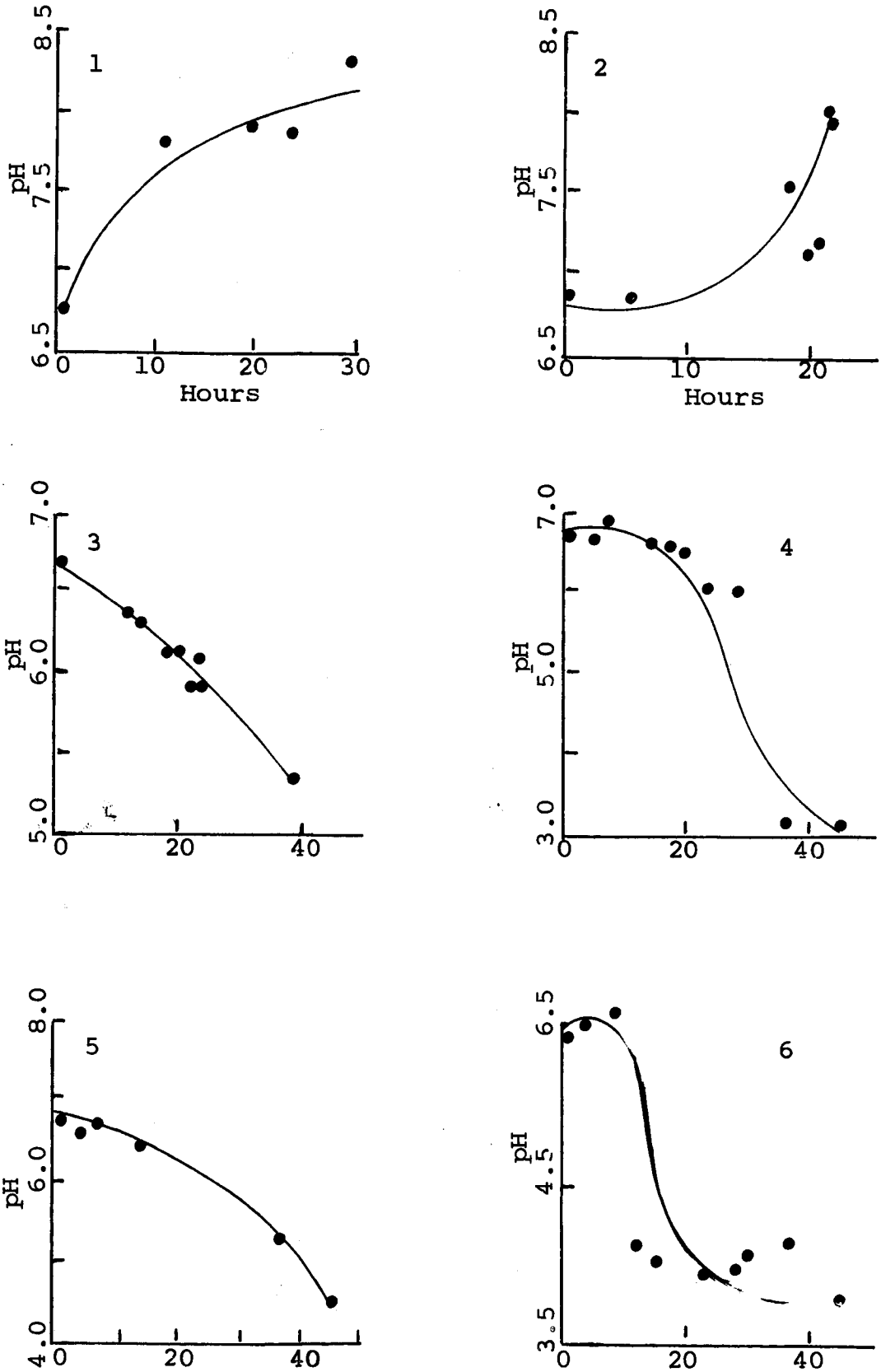
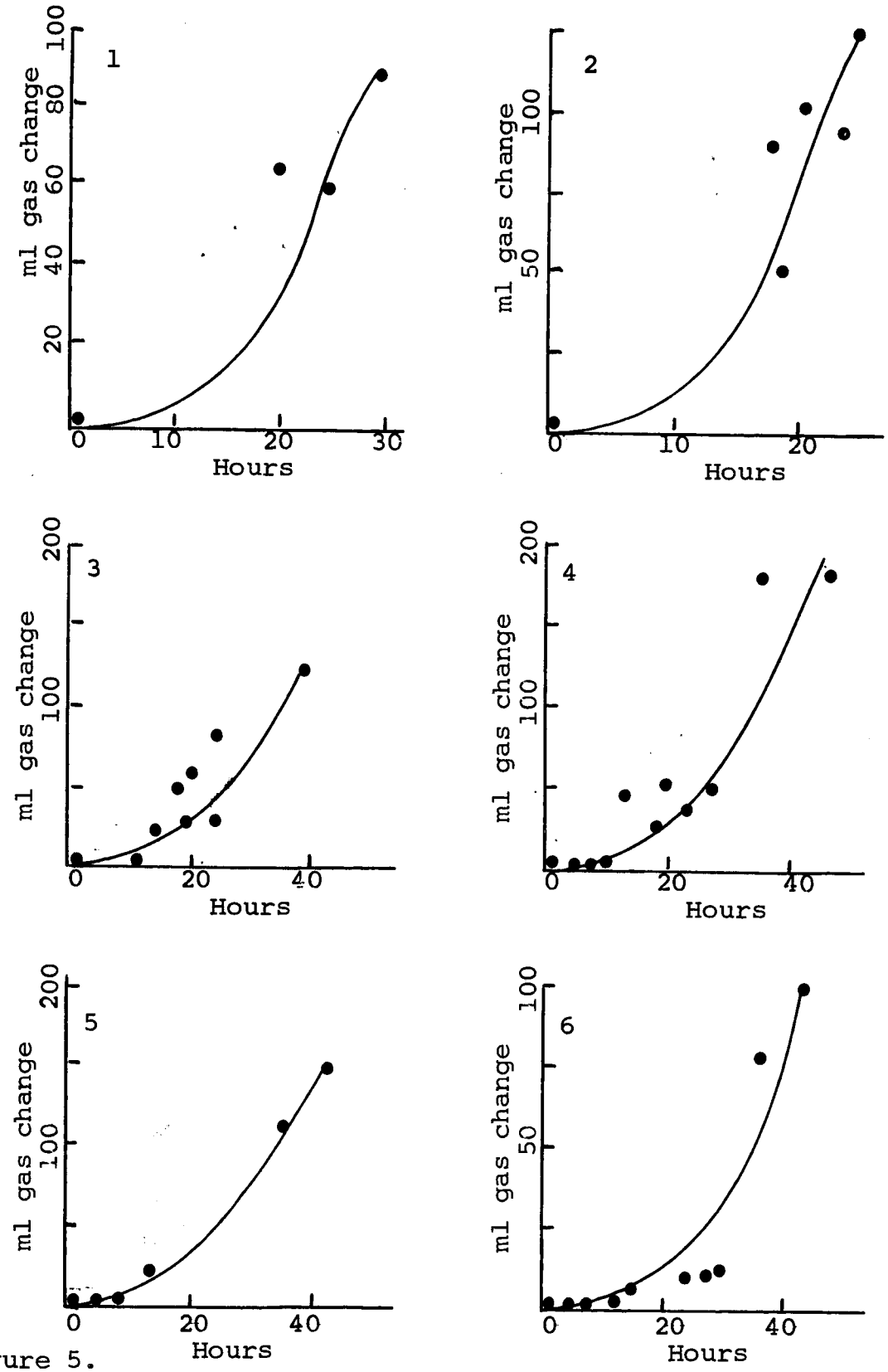

1
pH
8.5
7.5
6.5
0
10
20
30
Hours
2
pH
8.5
7.5
6.5
0
10
20
Hours
3
pH
7.0
6.0
5.0
0
20
40
4
pH
7.0
5.0
3.0
0
20
40
5
pH
8.0
6.0
4.0
0
20
40
6
pH
6.5
4.5
3.5
0
20
40

Figure 4.

Figure 4. Composites of the results of change in pH effected by the presence of the three amino acids during growth of Hydrogenomonas eutropha in Repaske's medium under shake conditions at thirty degrees centigrade with an atmosphere of hydrogen, oxygen, and carbon dioxide. Each point represents between two and five determinations. Please observe that the axes are of different scales.

1. 1% tyrosine, 1% glutamic acid, and 1% alanine in Repaske's medium.
2. 1% glutamic acid in Repaske's medium.
3. Repaske's medium as a control.
4. 0.1% glutamic acid in Repaske's medium.
5. 0.1% alanine in Repaske's medium.
6. 0.1% tyrosine in Repaske's medium.


1
ml gas change
20
40
60
80
100
0
10
20
30
Hours
2
ml gas change
50
100
0
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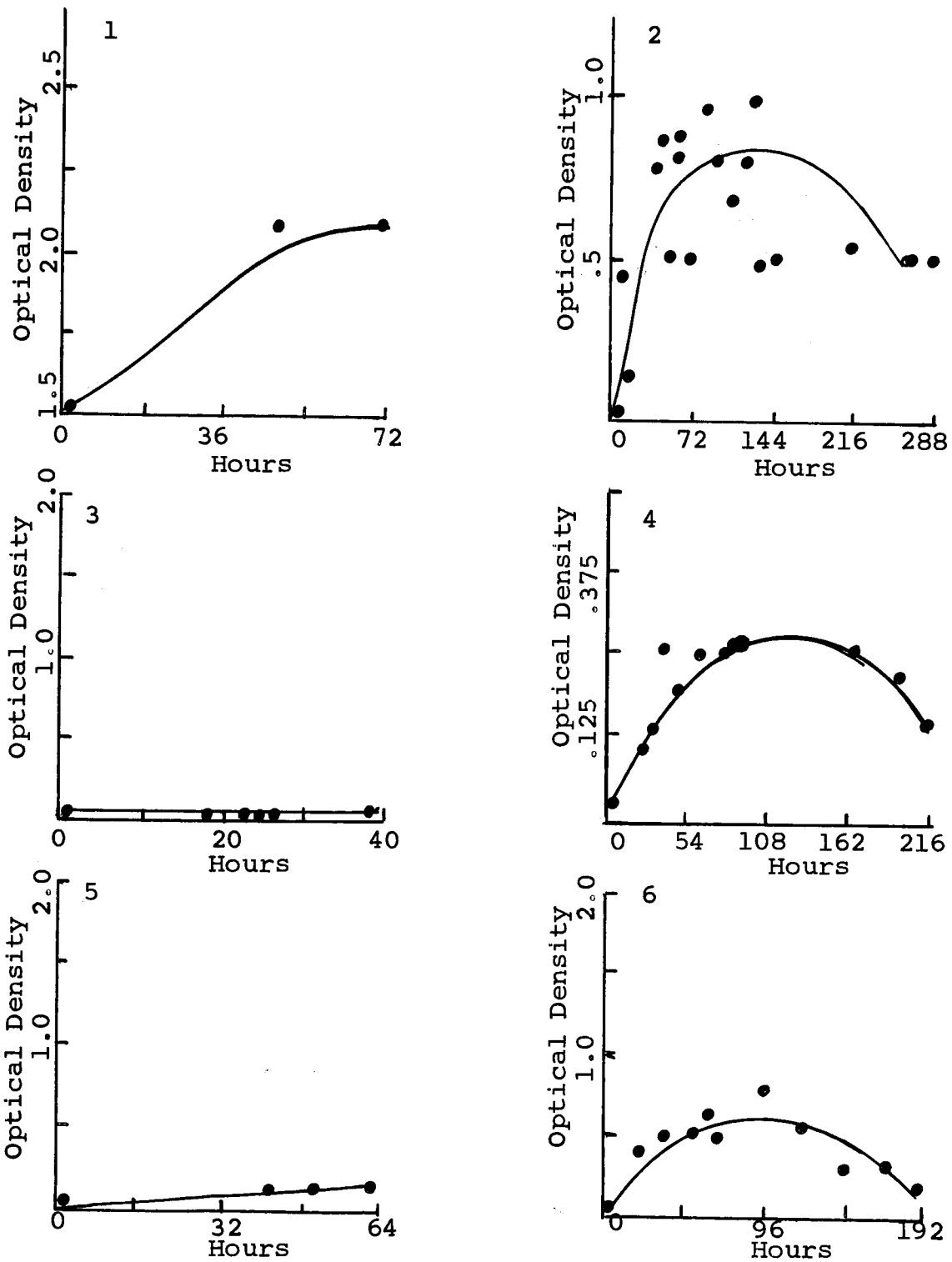
20
Hours
3
ml gas change
100
200
0
20
40
Hours
4
ml gas change
100
200
0
20
40
Hours
5
ml gas change
100
200
0
20
40
Hours
6
ml gas change
50
100
0
20
40
Hours

Figure 5.

Figure 5. Composite of the results of gas change effected by the presence of the three amino acids during growth of Hydrogenomonas eutropha in Repaske's medium under shake conditions at thirty degrees centigrade with an atmosphere of hydrogen, oxygen, and carbon dioxide. Each point represents between two and five determinations. Please observe that the axes are of different scales.

1. 1% tyrosine, 1% glutamic acid, and 1% alanine in Repaske's medium.
2. 1% glutamic acid in Repaske's medium.
3. Repaske's medium as a control.
4. 0.1% glutamic acid in Repaske's medium.
5. 0.1% alanine in Repaske's medium.
6. 0.1% tyrosine in Repaske's medium.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2.0
1.5
0
36
72
Hours
2
Optical Density
1.0
.5
0
72
144
216
288
Hours
3
Optical Density
2.0
1.0
0
20
40
Hours
4
Optical Density
.375
.125
0
54
108
162
216
Hours
5
Optical Density
2.0
1.0
0
32
64
Hours
6
Optical Density
2.0
1.0
0
96
192
Hours

Figure 6.

Figure 6. Composites of the results of turbidity changes (as measured by optical density) effected by the presence of the three amino acids during growth of Hydrogenomonas eutropha in Repaske's medium under stationary conditions with an atmosphere of air. The incubation temperature was thirty degrees centigrade. Each point represents between two and three determinations. Please observe that the axes are of different scales.

1. 1% tyrosine, 1% glutamic acid, and 1% alanine in Repaske's medium.
2. 1% glutamic acid in Repaske's medium.
3. Repaske's medium as a control.
4. 0.1% glutamic acid in Repaske's medium.
5. 0.1% alanine in Repaske's medium.
6. 0.1% tyrosine in Repaske's medium.

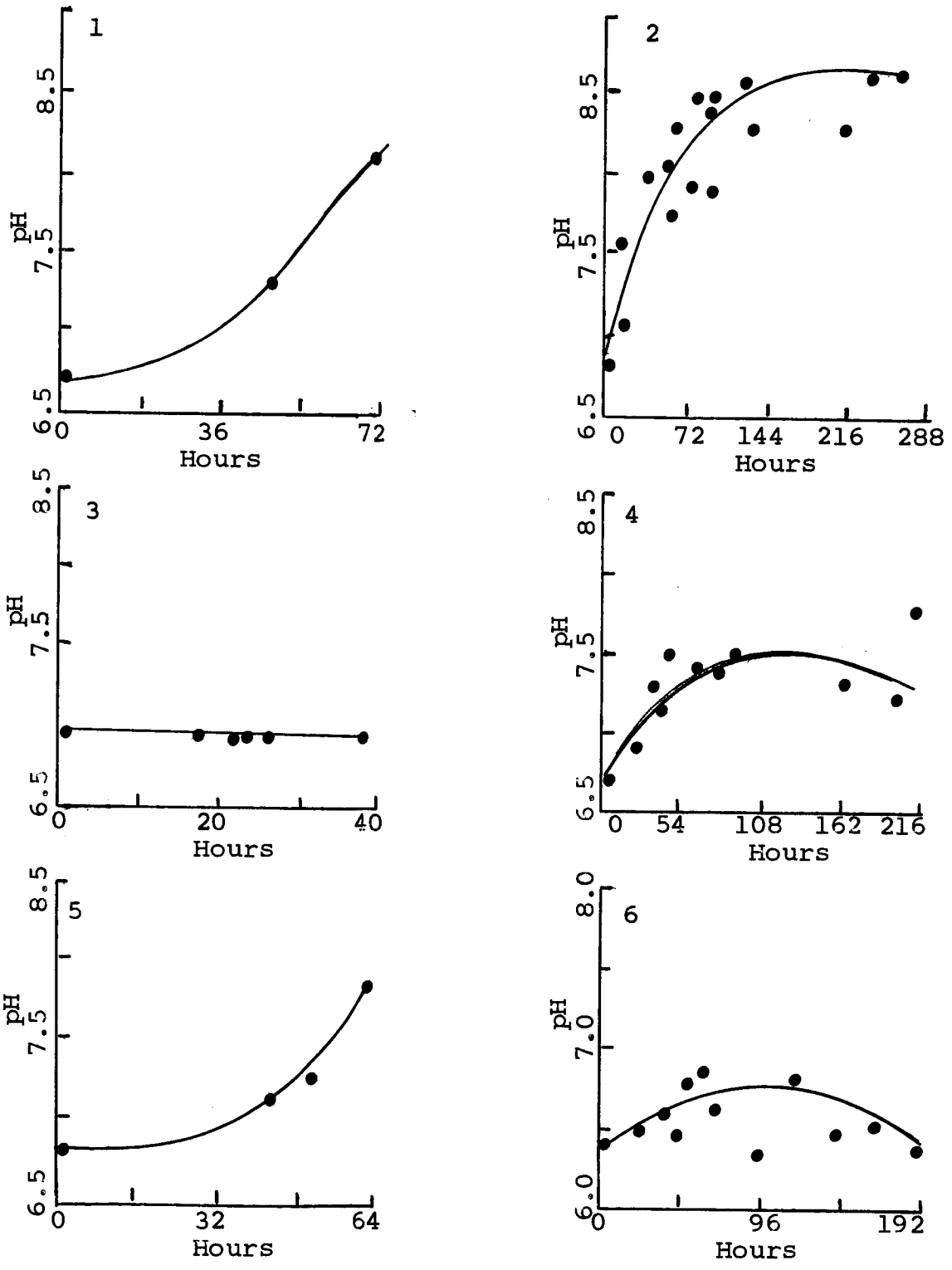

1
pH
8.5
7.5
6.5
0
36
72
Hours
2
pH
8.5
7.5
6.5
0
72
144
216
288
Hours
3
pH
8.5
7.5
6.5
0
20
40
Hours
4
pH
8.5
7.5
6.5
0
54
108
162
216
Hours
5
pH
8.5
7.5
6.5
0
32
64
Hours
6
pH
8.0
7.0
6.0
0
96
192
Hours

Figure 7.

Figure 7. Composites of the results of change in pH effected by the presence of the three amino acids during growth of Hydrogenomonas eutropha in Repaske's medium under stationary conditions with an atmosphere of air. The incubation temperature was thirty degrees centigrade. Each point represents between two and three determinations. Please observe that the axes are of different scales.

1. 1% tyrosine, 1% glutamic acid, and 1% alanine in Repaske's medium.

2. 1% glutamic acid in Repaske's medium.

3. Repaske's medium as a control.

4. 0.1% glutamic acid in Repaske's medium.

5. 0.1% alanine in Repaske's medium.

6. 0.1% tyrosine in Repaske's medium.

medium (Figure 3, 1) there appeared to be no toxicity, and there was an increase in turbidity which was greater than for any of the other media used in these experiments. This medium, the one containing one percent glutamic acid, and the one containing 0.1 percent tyrosine, all showed a marked increase in turbidity within twenty-four hours.

As the incubation time increased, there was a rise in pH for the medium containing all three of the amino acids and for the medium containing one percent glutamic acid (Figure 4). A thorough investigation of the nutritional requirements for Hydrogenomonas eutropha by Repaske (1962a) indicated that the pH optimum is 6.4 and 6.9. These same investigations also emphasized the importance of ferrous iron. In the description of the preparation of Repaske's medium (see Culture and Culture Media) it was mentioned that the ferrous salt will not go into solution at a high pH. This fact was mentioned by Ruhland (1924). Bovell (1957) found that the hydrogenase activity of this microorganism is impaired by the absence of ferrous iron. It would seem possible that less of the gas mixture would be consumed and there would be less growth under these conditions since less ferrous iron would be available to the microorganism.

In further examining the results obtained using the media containing the amino acids, it was found that the gas change was low considering the apparent increase in cell mass (Figure 5). Since organic substances were present in

these media and the control medium contained only inorganic salts, these results are difficult to interpret. When either one percent glutamic acid or all three of the amino acids was present in a concentration of one percent each, a rise in pH occurred as growth progressed. These results suggest that perhaps Hydrogenomonas eutropha has an enzyme system capable of oxidative deamination. If this is true, ammonia would be added to the atmosphere over the culture medium, as well as carbon dioxide from the metabolism of organic matter. The addition of these gases to the atmosphere would complicate any measurements on gas exchange or utilization.

A study of the turbidity changes of the six treatments under an atmosphere of air (Figure 6) indicates a fair growth response in the media containing one percent glutamic acid and the medium containing one percent each of the three amino acids. A leveling-off of growth, as indicated by turbidity measurements, begins to appear within three days. After three days, the pH of these media (Figure 7) was considerably higher than the reported optimum.

All media containing any of the amino acids in the concentration of 0.1 percent showed a poor growth response as indicated by turbidity measurements. Since Hydrogenomonas eutrophia grew well in the presence of any of the three amino acids under an atmosphere of the gas mixture, and since this bacterium also grew well in one percent

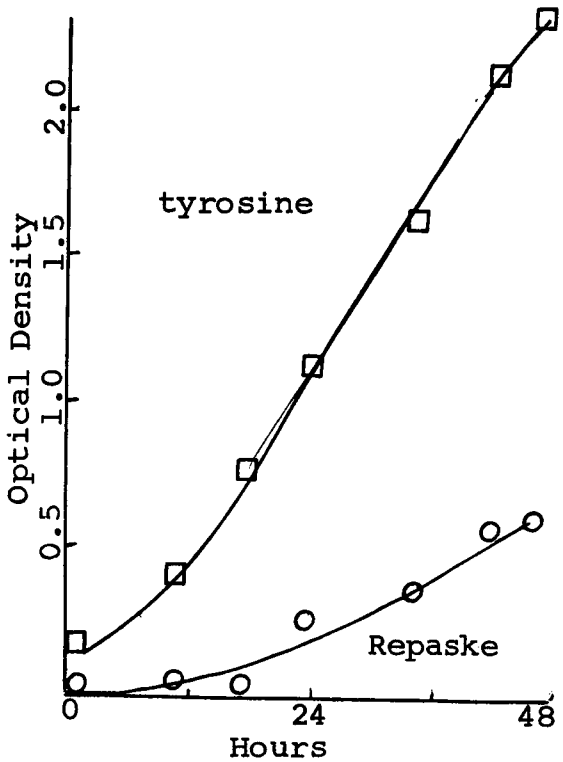
concentrations of all three of the amino acids and a one percent concentration of glutamic acid under air, it is possible that the poor growth response under these other conditions was due to a lack of a source of energy and/or nutrients.

Preliminary results of analyses by gas chromatography of the gas mixture remaining in the units following growth were disappointing. The percentage of nitrogen in the units varied from one percent to as much as fifty percent. To obtain uniformity of atmosphere in the culture bottles all stoppers and neoprene tubing were replaced and reassembled. It was then found that after growth the units contained about five percent nitrogen.

Another problem was insufficient space on the shaking apparatus for the culture units. It was felt that all of the cultures should be grown under the same conditions before valid comparisons could be made. Since five replicates of each treatment were desired, it was elected to grow all cultures under stationary conditions.

The Effects of the Presence of the Three Amino Acids on the Growth of Hydrogenomonas eutropha in Repaske's Medium Under Stationary Conditions with an Atmosphere of Hydrogen, Oxygen, and Carbon Dioxide.

When a comparison is made of the graphic representations of the results of turbidity measurements under stationary conditions (Figures 8, 9, 10, 11, and 12) with those



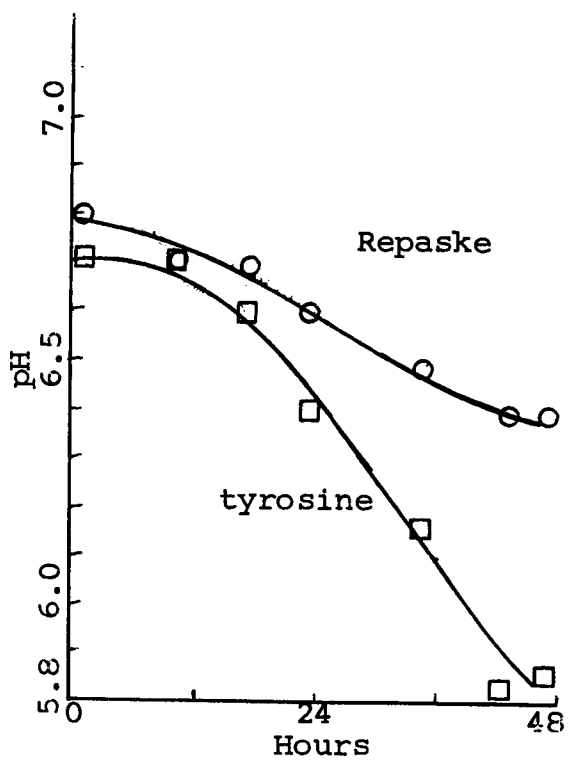
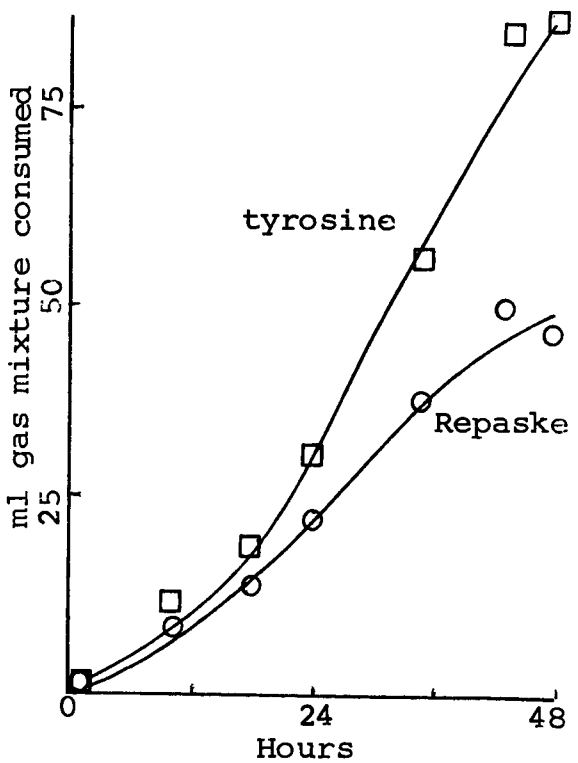


Figure 8. The effect of 1% tyrosine on the growth of Hydrogenomonas eutropha in Repaske's medium under stationary conditions with an atmosphere of hydrogen, oxygen and carbon dioxide. Incubation temperature was thirty degrees centigrade. Each point represents between ten and fifteen determinations.

1% tyrosine

Repaske control



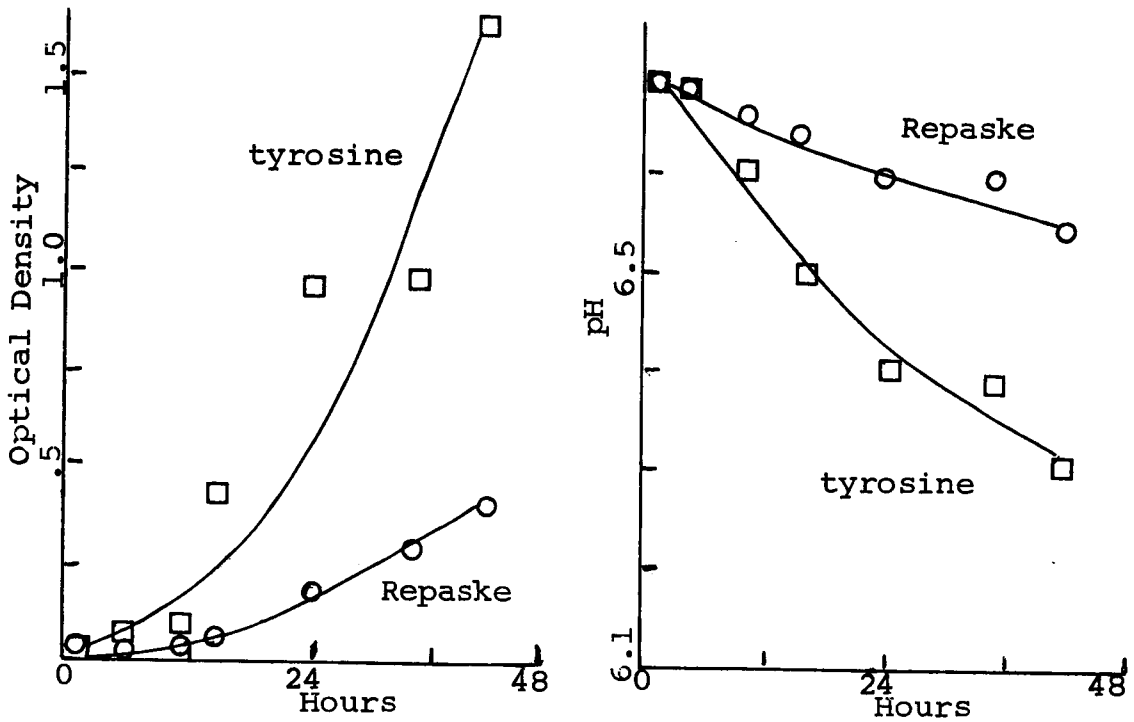
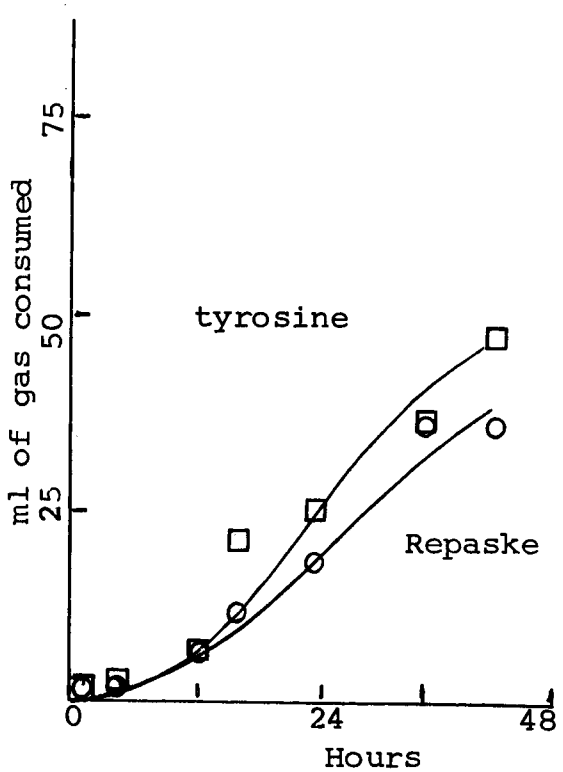


Figure 9. The effect of 1% tyrosine on the growth of Hydrogenomonas eutropha in Repaske's medium under stationary conditions with an atmosphere of hydrogen, oxygen, and carbon dioxide. Incubation temperature was thirty degrees centigrade. Each point represents between ten and fifteen determinations.

1.0% tyrosine

Repaske control



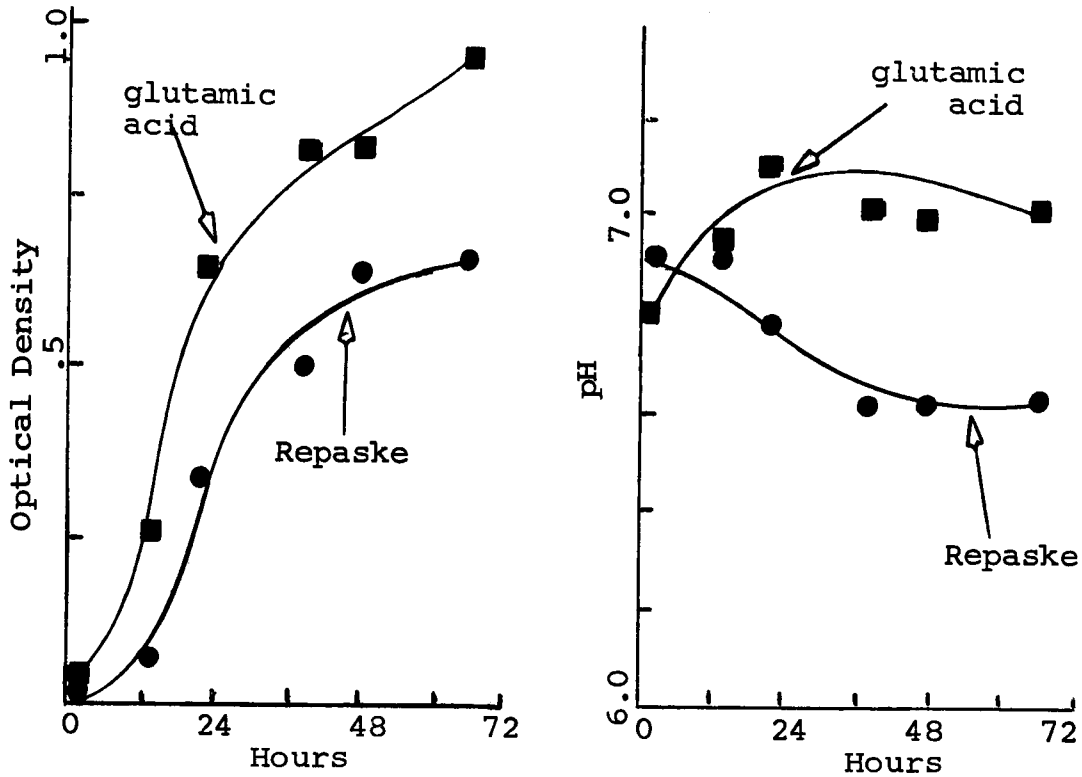
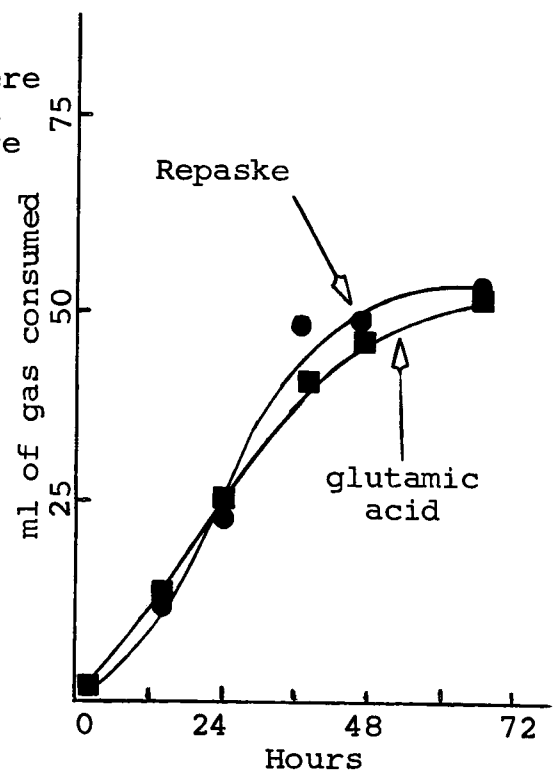
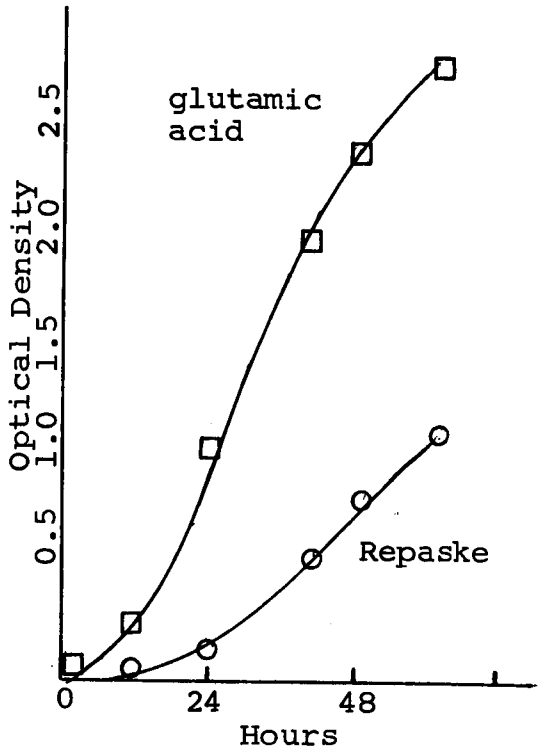


Figure 10. The effect of 0.1% glutamic acid on the growth of Hydrogenomonas eutropha in Repaske's medium under stationary conditions with an atmosphere of hydrogen, oxygen, and carbon dioxide. Incubation temperature was thirty degrees centigrade. Each point represents between ten and fifteen determinations.

0.1% glutamic acid

Repaske control





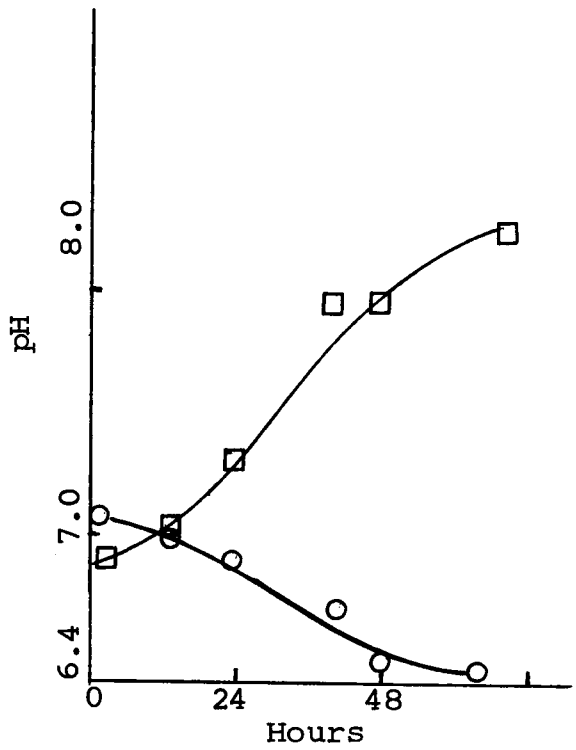
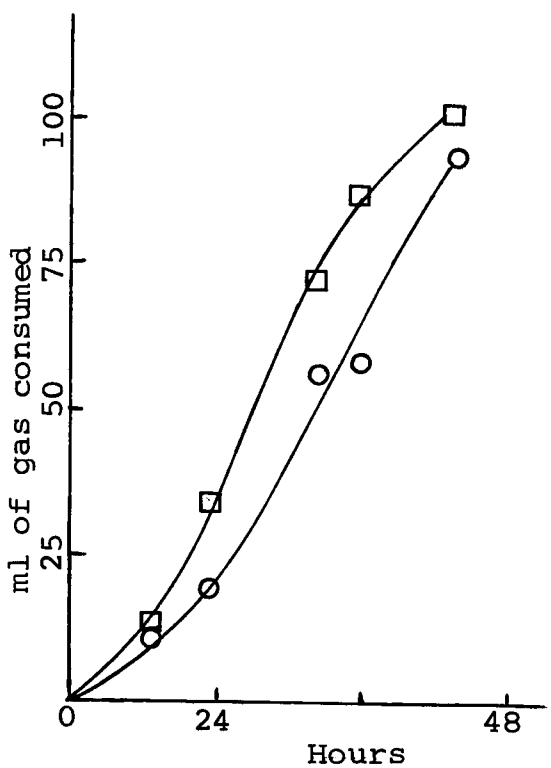


Figure 11. The effect of 1% glutamic acid on the growth of Hydrogenomonas eutropha in Repaske's medium under an atmosphere of hydrogen, oxygen, and carbon dioxide with stationary conditions. Incubation temperature was thirty degrees centigrade. Each point represents between ten and fifteen determinations.

1% glutamic acid

Repaske control



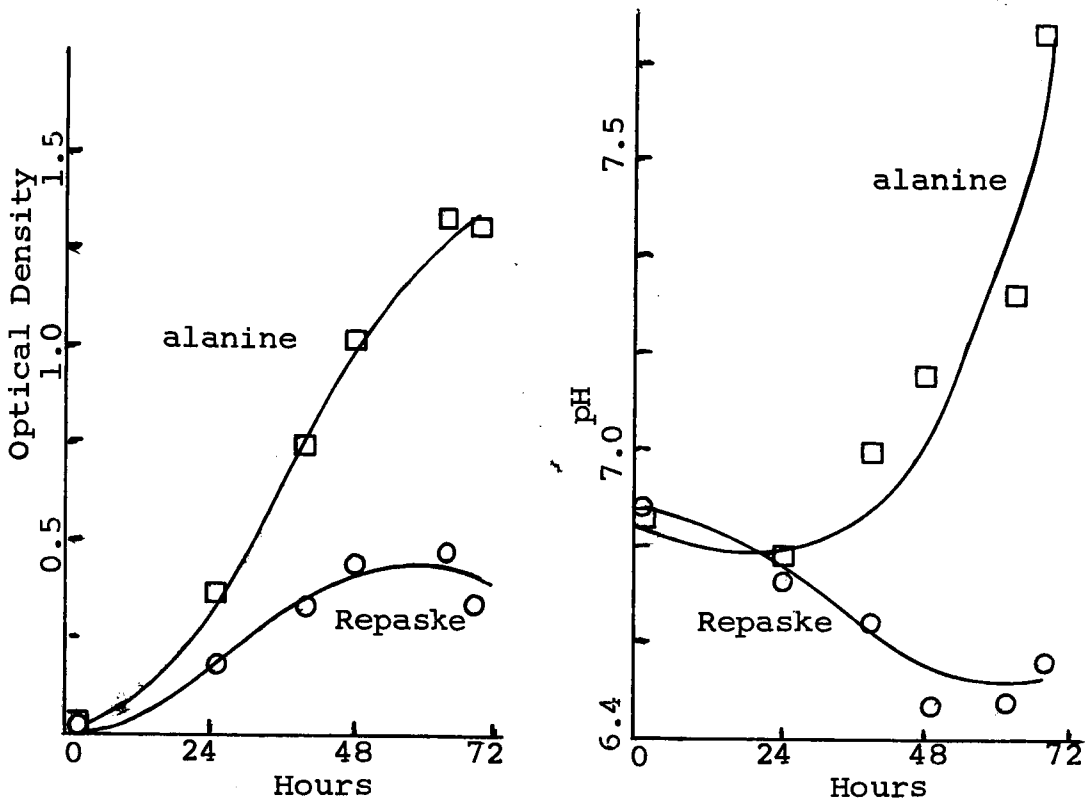
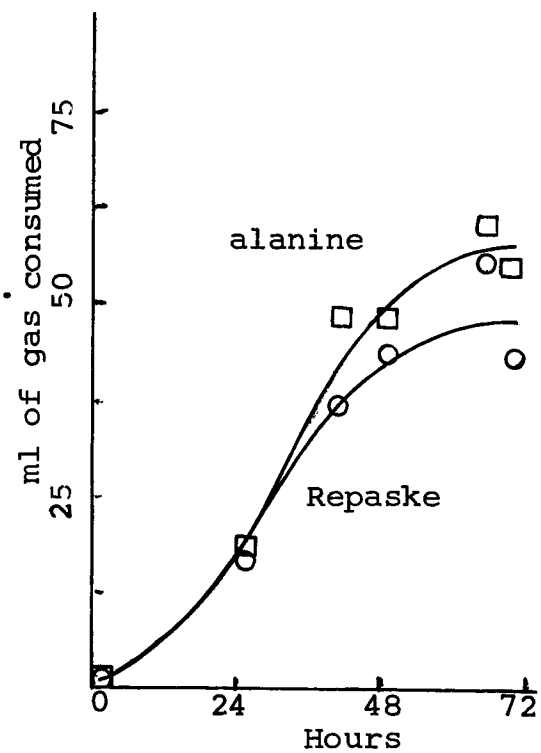


Figure 12. The effect of 0.1% alanine on the growth of Hydrogenomonas eutropha in Repaske's medium under stationary conditions with an atmosphere of hydrogen, oxygen, and carbon dioxide. Incubation temperature was thirty degrees centigrade. Each point represents between ten and fifteen determinations.

0.1% alanine

Repaske control



under shake conditions (Figure 3), it is found that there is a lengthening of the log phase of growth and the slope of the line during the log phase is less steep. These results suggest an increase in the generation time of the microorganism which can be attributed to a lack of gas exchange accompanying stationary conditions.

In the preliminary work those cultures growing in 0.1 percent glutamic acid, 0.1 percent alanine, or 0.1 percent tyrosine were allowed to enter the stationary phase of growth (Figure 3). In the experiments now to be discussed the cultures were in no instance allowed to enter the stationary phase.

The results obtained when employing tyrosine in Repaske's medium in concentrations of one percent (Figure 8) and 0.1 percent (Figure 9) were similar to those obtained in the preliminary experiments. The increase in turbidity, the fall in pH, and the total gas consumption show a pattern corresponding with the earlier work. The total gas consumption is low in both cases and indicated that the microorganism might have oxidized the organic substance in preference to the hydrogen. It should again be pointed out that these results are difficult to interpret due to the possible production of ammonia and carbon dioxide resulting from the presence of the amino acids.

In the concentrations of 0.1 percent glutamic acid and one percent glutamic acid the results (Figures 10 and 11)

appear to follow those obtained earlier (Figure 3, 4, and 5). The pH of the medium containing 0.1 percent glutamic acids remains close to seven suggesting the microorganism is growing on the amino acids and deamination is occurring.

In the concentration of 0.1 percent alanine the results (Figure 12) appear to follow those obtained earlier (Figures 3, 4, and 5) with the exception of the change in pH (Figures 12 and 3). In the present case the alanine in Repaske's medium was associated with a rise in pH approaching that shown by one percent glutamic acid in Repaske's medium (Figure 11).

The Effects of the Presence of the Three Amino Acids on the Growth of Hydrogenomonas eutropha in Repaske's Medium Under Stationary Conditions with an Atmosphere of Air.

With an atmosphere of air the one percent tyrosine medium supported an excellent response as indicated by turbidity measurements (Figure 13) and one percent glutamic acid a fair response (Figure 14), the media consisting of Repaske's basal medium plus 0.1 percent alanine (Figure 15), 0.1 percent tyrosine (Figure 16), or 0.1 percent glutamic acid (Figure 17) supported little growth while Repaske's medium appeared to support no growth (Figures 13, 14, 15, 16, and 17). Since these last four media under an atmosphere of the gas mixture supported growth, these results once more appear to indicate that these media do not provide a sufficient nutrient source. A lack of nutrients is also

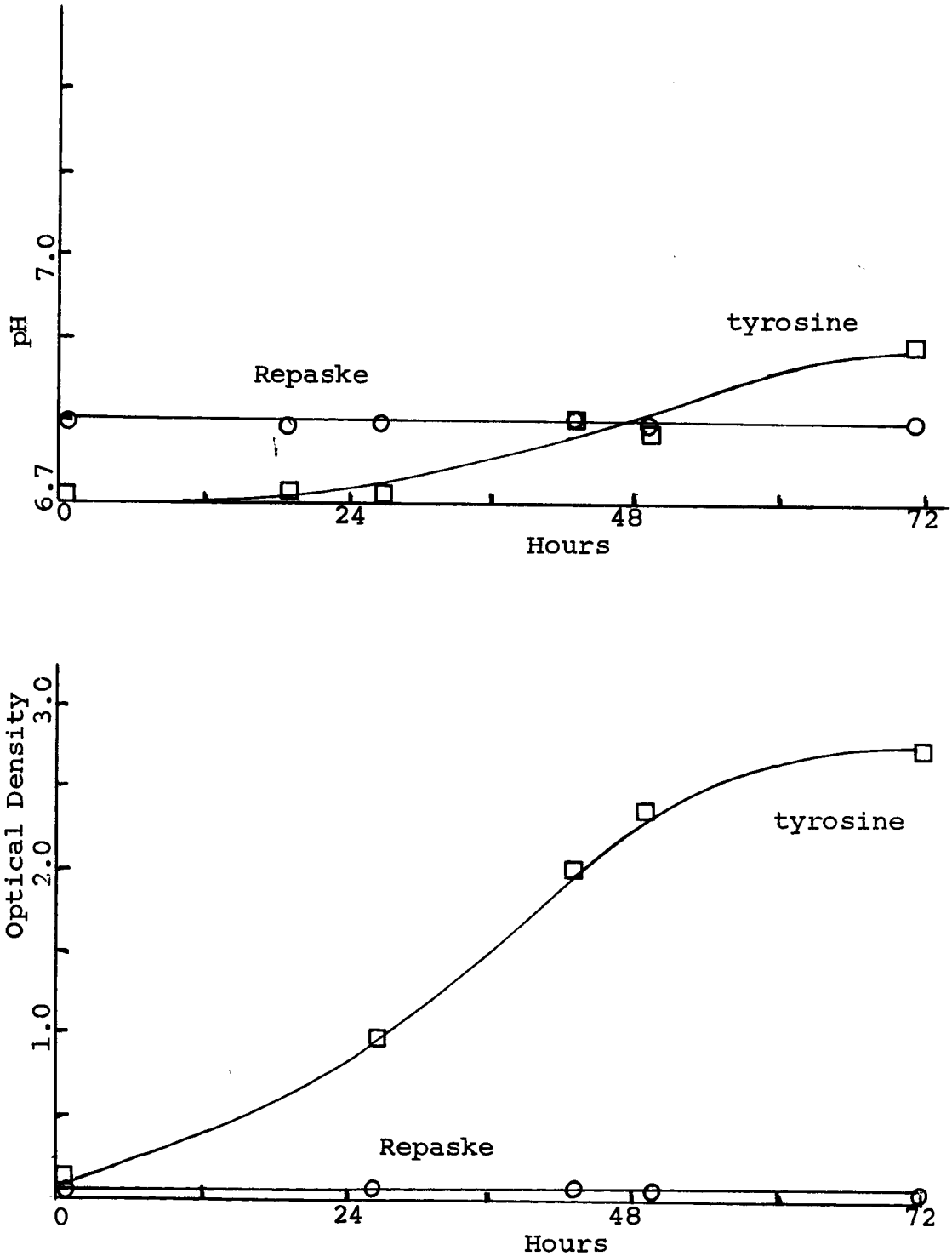


Figure 13. The effect of 1.0% tyrosine on the growth of Hydrogenomonas eutropha in Repaske's medium under stationary conditions with an atmosphere of air. Each point represents between five and nine determinations.

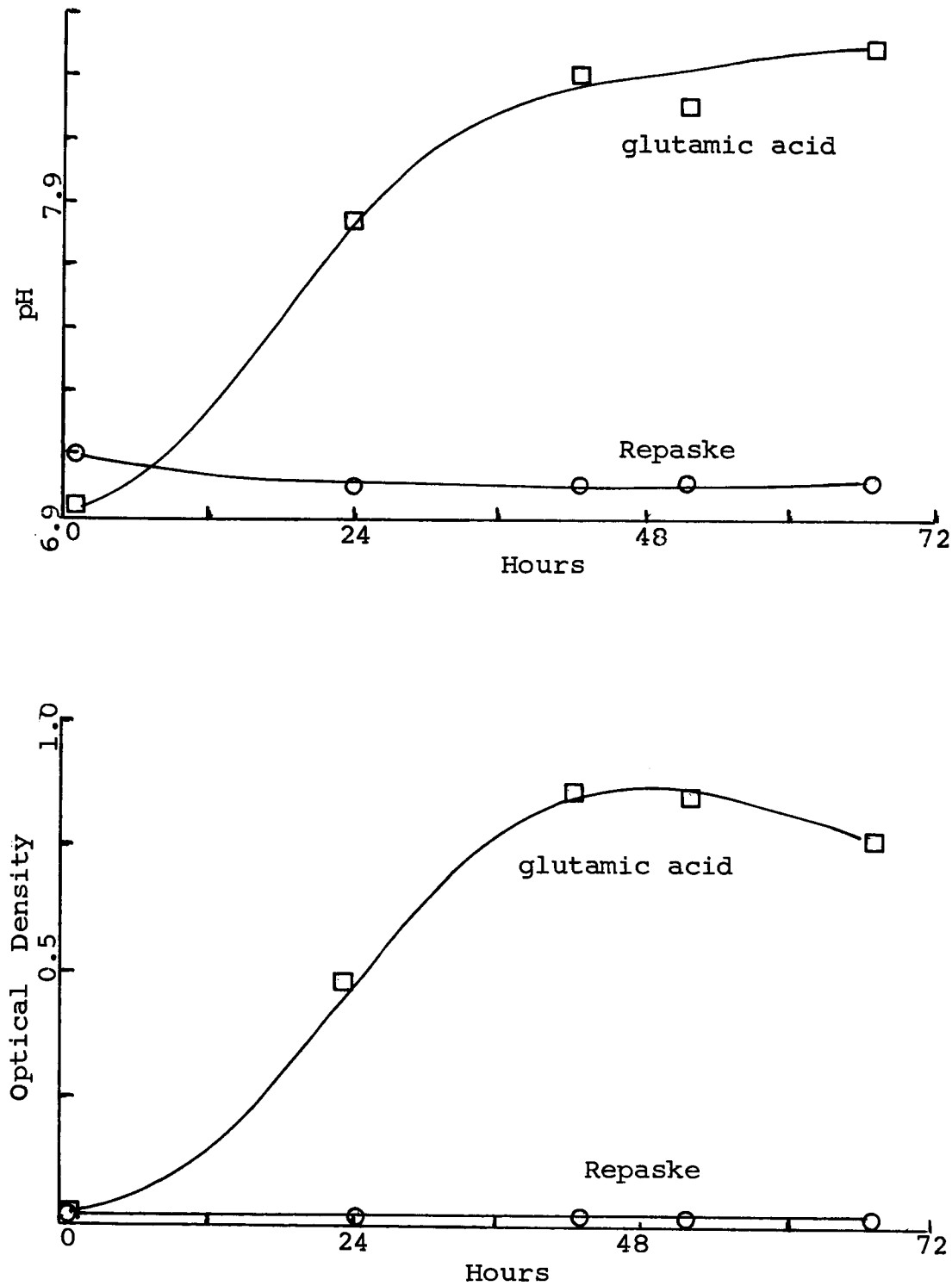
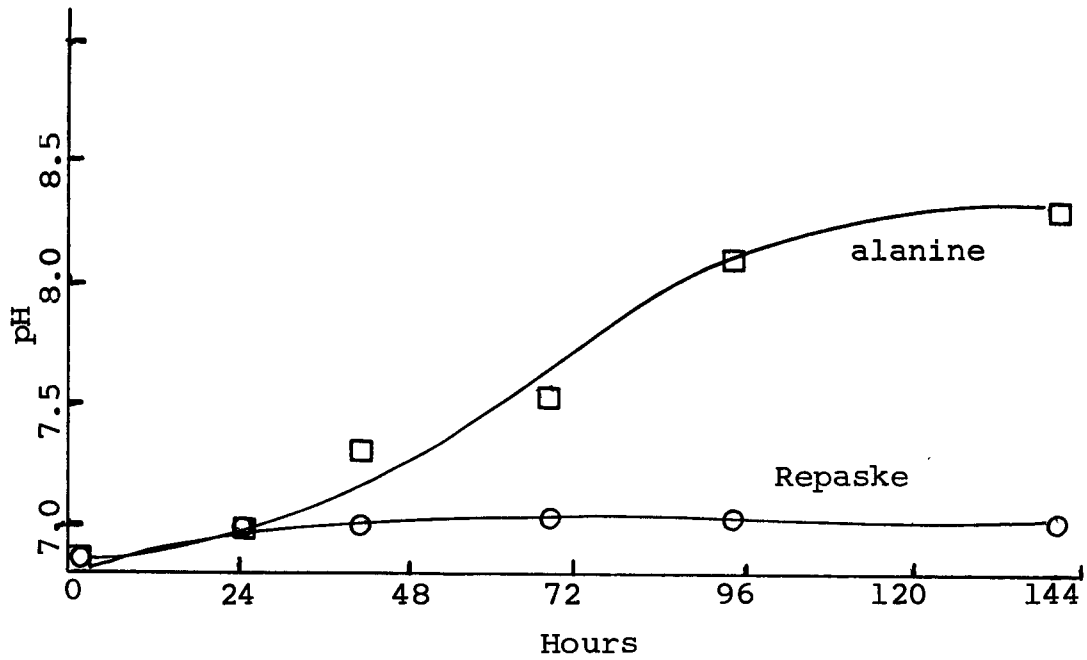


Figure 14. The effect of 1% glutamic acid on the growth of Hydrogenomonas eutropha in Repaske's medium under stationary conditions with an atmosphere of air. Each point represents between five and nine determinations.



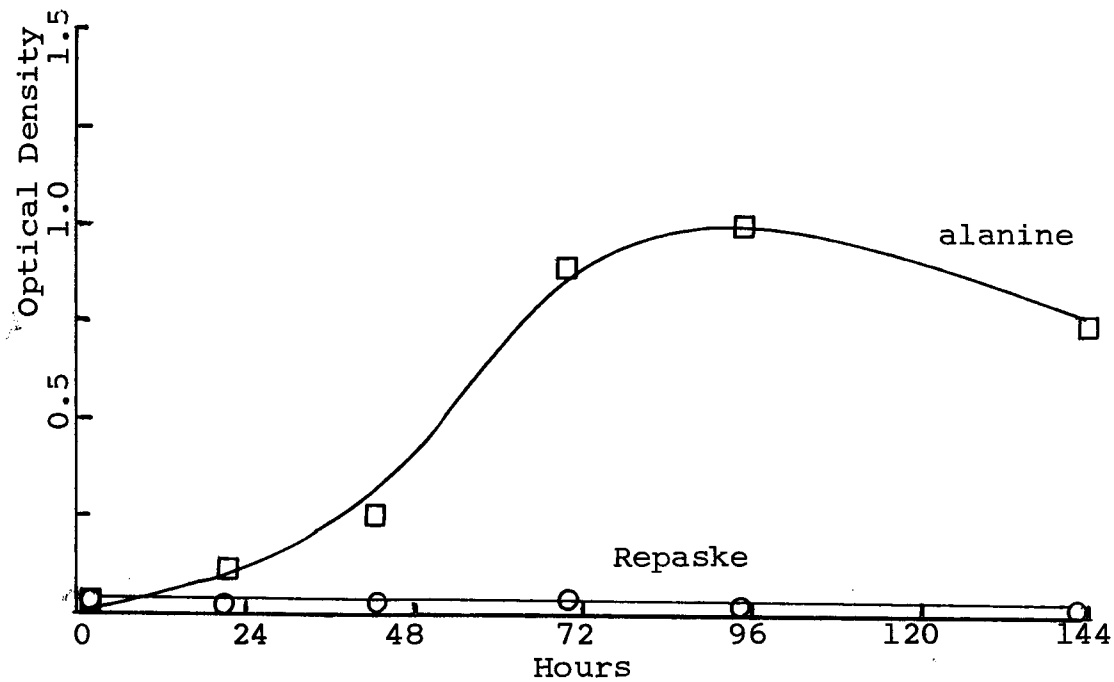


Figure 15. The effect of 0.1% alanine on the growth of Hydrogenomonas eutropha in Repaske's medium under stationary conditions with an atmosphere of air. Each point represents between five and nine determinations.

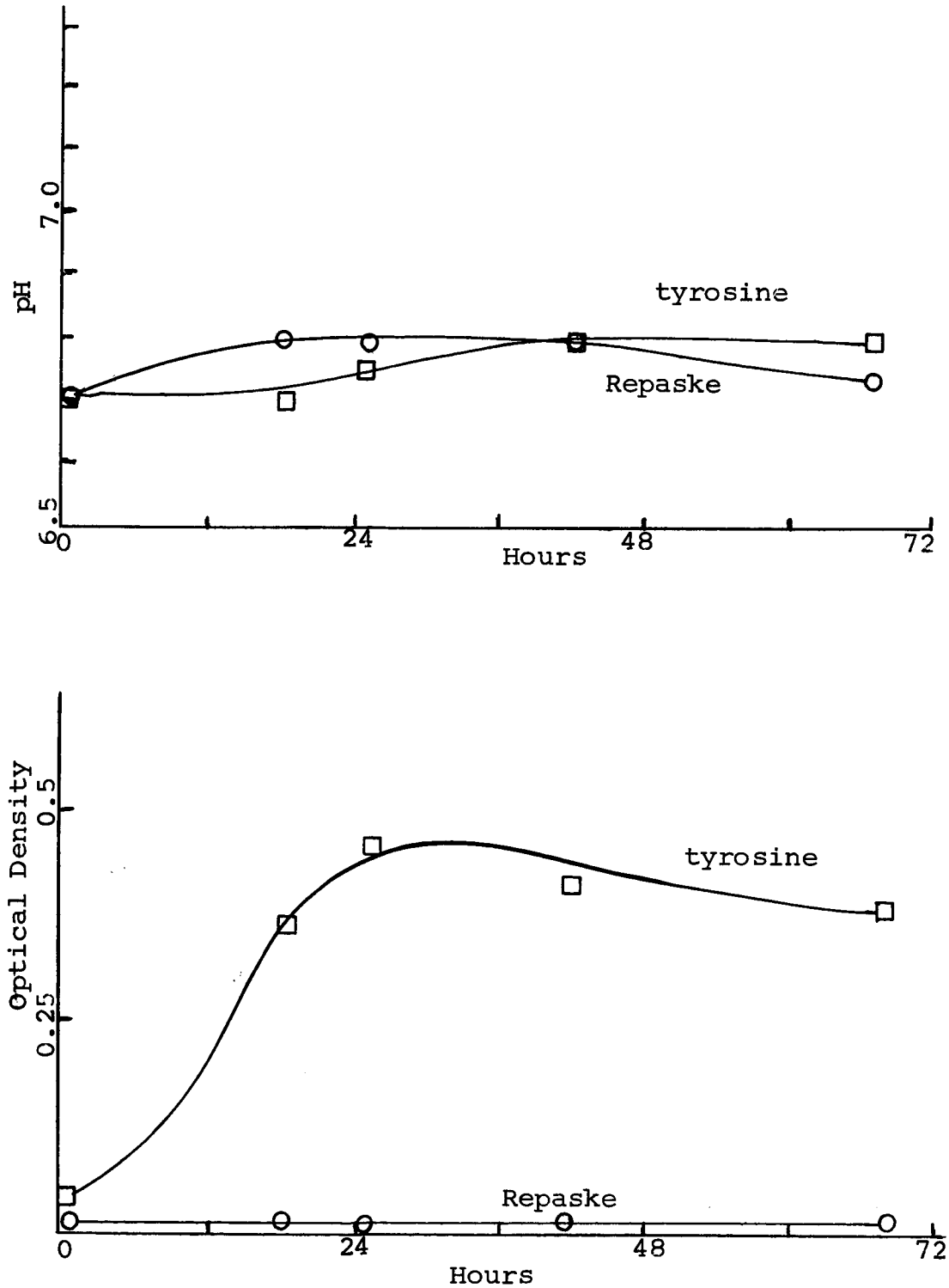


Figure 16. The effect of 0.1% tyrosine on the growth of Hydrogenomonas eutropha in Repaske's medium under stationary conditions with an atmosphere of air. Each point represents between five and nine determinations.

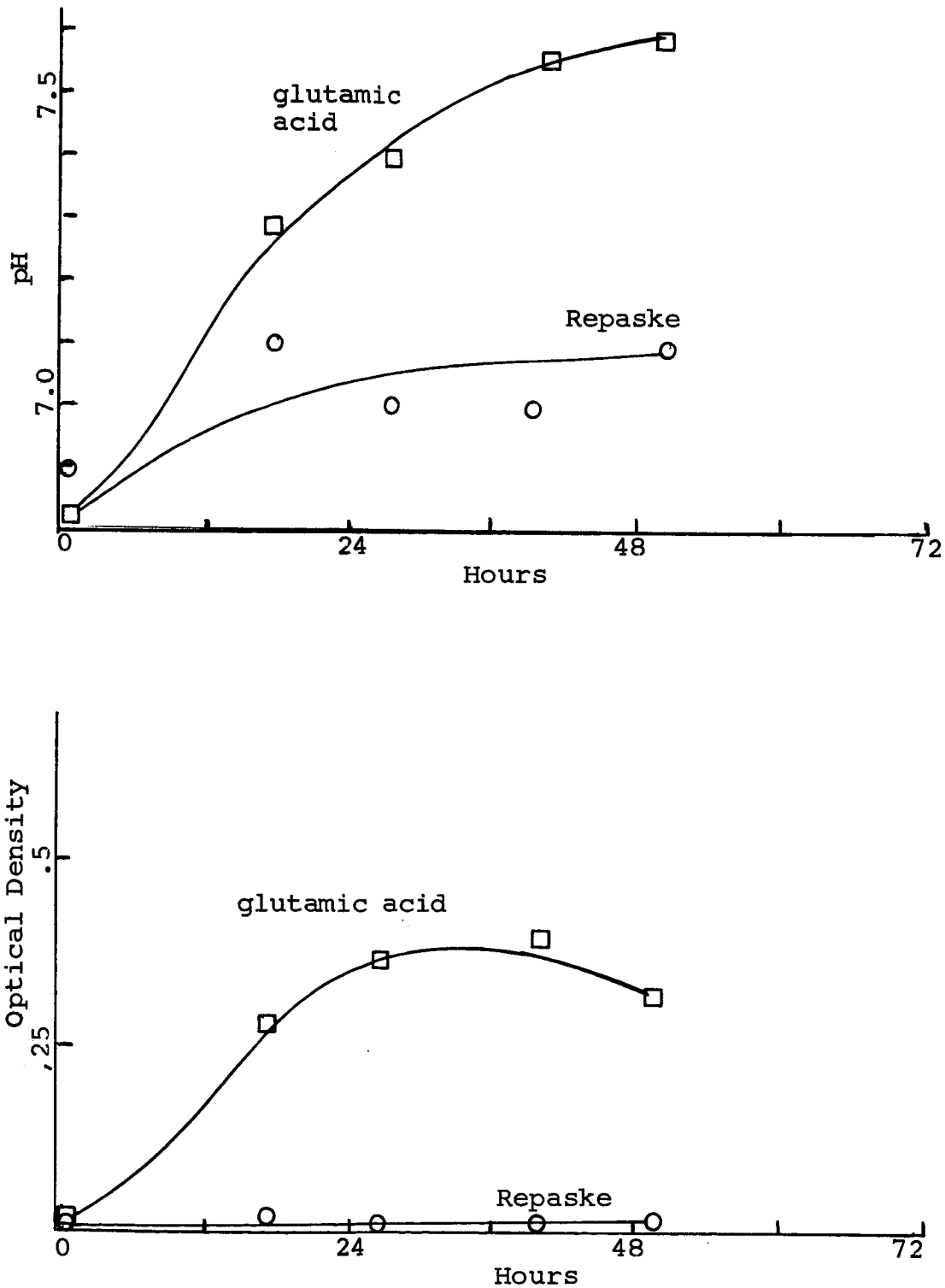


Figure 17. The effect of 0.1% glutamic acid on the growth of Hydrogenomonas eutropha in Repaske's medium under stationary conditions with an atmosphere of air. Each point represents between five and nine determinations.

suggested since Hydrogenomonas eutropha grew well under an atmosphere of air in Repaske's medium containing either one percent glutamic acid (Figure 14) or one percent tyrosine (Figure 13).

Results of the turbidity measurements shown in Figures 14 and 15 each reached a maximum and then began to fall. In both cases there was a sharp rise in pH preceding the decline in turbidity. As mentioned earlier, the optimum pH for growth of Hydrogenomonas eutropha is 6.4 to 6.9 (Repaske, 1962a).

The one percent tyrosine medium appeared to support growth (Figure 13) and the pH of the culture medium remained close to the optimum for growth. Since there is no rise in pH, the tyrosine is most likely not degraded by a pathway involving deamination. DeCicco and Umbreit (1964) had evidence that tyrosine was degraded by a pathway which involves breaking the ring structure of the molecule. The bacterium used by these workers was an induced auxotrophic mutant of Hydrogenomonas facilis.

Results of the Gas Chromatography Analyses of the Atmosphere Remaining in the Culture Units Following Incubation Under the Gas Mixture.

The results of gas chromatographic analyses of the atmosphere remaining in the culture units following incubation are given in Tables I and II. Since Table I gives the information necessary to obtain the milliliters of gas

Table I. Gases Remaining in the Culture Bottles as Effected by the Presence of the Three Amino Acids on the Growth of Hydrogenomonas eutropha in Repaske's Medium Under Stationary Conditions with an Atmosphere of Hydrogen, Oxygen, and Carbon Dioxide. These Values Represent an Average of Between Ten and Fifteen Determinations.

Culture Medium	Incubation time (Hours)	Gases Remaining in the Culture Units							
		Percentages				Milliliters			
		H_2	O_2	N_2	CO_2	H_2	O_2	N_2	CO_2
0.1% alanine	48	56.48	29.17	2.99	11.36	30.16	15.58	1.6	6.02
Repaske Control	48	55.94	29.47	4.1	10.48	32.45	17.09	2.38	6.08
initial		56.11	29.00	3.55	11.34				
1% glutamic acid	24	61.27	24.56	2.56	11.61	39.03	15.64	1.63	7.4
	48	60.65	23.21	3.69	12.45	6.55	2.51	.40	1.34
Repaske Control	24	61.24	25.35	2.7	10.71	49.3	20.41	2.17	8.62
	48	59.62	26.42	3.24	10.72	23.55	10.44	1.28	4.23
initial		56.11	29.0	3.55	11.34				
0.1% glutamic acid	24	63.7	22.76	2.2	11.34	47.39	16.93	1.64	8.44
Repaske Control	24	63.35	23.78	2.1	10.77	48.84	18.33	1.62	8.3
initial		62.65	23.97	2.29	11.09				

Table 1. (Continued)

Culture Medium	Incubation time (Hours)	Gases Remaining in the Culture Units							
		Percentages				Milliliters			
		H_2	O_2	N_2	CO_2	H_2	O_2	N_2	CO_2
1% tyrosine	24	63.18	21.77	4.59	10.46	44.8	15.43	3.25	7.42
	48	63.04	19.65	5.13	12.18	16.39	5.11	1.33	3.17
Repaske Control	24	64.89	22.68	2.41	10.02	49.64	17.35	1.84	7.67
	48	61.58	22.65	5.1	10.67	36.89	13.57	3.05	6.38
initial		60.81	22.98	6.54	9.67				
0.1% tyrosine	24	60.37	27.23	1.82	10.58	44.73	20.18	1.35	7.84
	48	57.5	28.12	4.4	9.98	30.30	14.82	2.32	5.26
Repaske Control	24	59.73	27.82	2.43	10.02	49.4	23.01	2.01	8.29
	40	57.66	29.17	4.14	9.03	31.09	15.72	2.33	4.87
initial		58.85	27.14	4.1	9.91				

Table II. Gas Change as Effected by the Presence of the Three Amino Acids on the Growth of Hydrogenomonas eutropha in Repaske's Medium Under Stationary Conditions with an Atmosphere of Hydrogen, Oxygen, and Carbon Dioxide. These Values Represent an Average of Between Ten and Fifteen Determinations.

Culture Medium	Incubation Time (Hours)	Gas Consumed (Milliliters)					Ratios		Optical Density	Dry Weight in 5 ml. (mg)
		Total	H_2	O_2	N_2	CO_2	H_2-O_2-N_2-CO_2	H_2/O_2		
0.1% alanine	48	46.6	29.95	13.42	1.95	5.27	5.7-2.5-.37-1	2.2	1.0685	30.625
Repaske control	48	42.0	23.66	11.91	1.17	5.26	4.5-2.2-.22-1	1.99	0.4454	13.125
1% glutamic acid	24	36.3	22.07	9.83	0.32	4.08	5.4-2.4-.07-1	2.25	.9515	27.5
	48	89.2	54.55	22.96	1.55	10.14	5.4-2.3-.15-1	2.38	2.2215	64.4
Repaske control	24	19.5	11.80	5.06	0	2.86	4.1-1.8-0-1	2.3	.2090	6.25
	48	60.5	37.55	15.03	0.67	7.25	5.2-2.1-.09-1	2.5	.835	25.0
0.1% glutamic acid	24	25.6	15.26	7.04	0.65	2.65	5.8-2.7-.25-1	2.17	.6825	20.65
Repaske control	24	22.9	13.81	5.64	0.67	2.79	4.9-2-.24-1	2.45	.3435	10.0

Table II. (Continued)

Culture Medium	Incubation Time (Hours)	Gas Consumed (Milliliters) Total	H_2	O_2	N_2	CO_2	Ratios H_2-O_2-N_2-CO_2	H_2/O_2	Optical Density	Dry Weight in 5 ml. (mg)
1% tyrosine	24	29.1	16.01	7.55	2.29	2.25	7.1-3.4-1-1	2.12	1.125	32.5
	48	74.0	44.42	17.87	5.21	6.5	6.8-2.7-.8-1	2.49	2.32	66.9
Repaske control	24	23.5	11.17	5.63	4.7	2.0	5.6-2.8-2.35-1	1.98	0.25	6.4
	48	40.1	23.92	9.41	3.49	3.28	7.3-2.9-1.06-1	2.54	0.56	16.25
0.1% tyrosine	24	25.9	14.12	6.96	2.75	2.07	6.8-3.4-1.3-1	2.03	.98	28.75
	40	47.3	28.55	12.32	1.78	4.65	6.1-2.6-.38-1	2.32	1.185	35
Repaske control	24	17.3	9.45	4.13	2.09	1.62	5.8-2.5-1.3-1	2.29	.21	6.25
	40	46.1	27.77	11.42	1.87	5.04	5.5-2.3-.37-1	2.43	.43	13.125

change discussion will be limited to Table II. These values represent an average of between ten and fifteen determinations.

When 0.1 percent alanine in Repaske's medium was the culture medium employed, the gas change was 46.6 milliliters but was only forty-two for the Repaske control. The values for cellular dry weights indicate that there was almost three times as much cell production in the culture medium containing alanine as in Repaske's medium. If deamination and decarboxylation had occurred, the 5.6125×10^{-5} mole of alanine in the five milliliters of the culture medium could yield about 1.4 milliliters each of these two gases. A system to detect ammonia in the gaseous atmosphere was not used. The possible presence of ammonia is suggested by the rise in pH. The pH for the culture medium containing alanine and for the Repaske's medium, following the forty-eight hour incubation period, was 7.16 and 6.47, respectively.

The presence of carbon dioxide in the gaseous atmosphere was detected by the silica gel column. The values for the carbon dioxide removed from both culture units were about the same. It must be borne in mind that the solubility of carbon dioxide in an aqueous solution increases with a rise in pH, which makes it difficult to evaluate these data. There is very little difference between the values of the hydrogen quotients (H_2/O_2), but the ratio of all the gases consumed

does show a difference between the amounts of gases consumed under the two conditions.

The volumes of gas removed from the atmosphere of the culture medium containing one percent glutamic acid were 36.3 and 89.2, respectively, for the twenty-four and forty-eight hour growth periods. The value for cellular dry weight was more than doubled from the termination of the first growth period to the end of the second growth period (from 27.5 to 64.4). In the control medium the cellular dry weight increased four times (from 6.25 to 25). During this length of time there was a three-fold increase in the volume of gas used, while the increase in volume of gas used in the medium containing glutamic acid was about 2.5. Although the cell mass in the culture containing the glutamic acid, at the end of forty-eight hours, was more than twice that in the Repaske's medium, the total volume of gas consumed was about 1.5 times that of the control medium.

Again there is very little difference between the values for the hydrogen quotient. The ratios of the gases consumed do show some variation after the first twenty-four hours. But the presence of glutamic acid in the growth medium caused a rise in pH to 7.31 in twenty-four hours and 7.94 in forty-eight hours. The values for the pH of the Repaske control for twenty-four and forty-eight hours were 6.9 and 6.45, respectively.

The low amount of gas consumed may be partly due to the

possibility that the 3.398×10^{-4} mole of glutamic acid contained in the five milliliters of the one percent glutamic acid medium could release as much as 8.5 milliliters of ammonia. Were both carboxyl groups to be decarboxylated, twice this amount of carbon dioxide would be released.

When 0.1 percent glutamic acid was present in the culture medium, the pH of the culture medium remained close to neutrality throughout the growth period (Figure 10). Since only 3.398 x 10-4 mole of glutamic acid was present in the five milliliters of the culture medium, the amount of ammonia and carbon dioxide that could be released due to deamination and decarboxylation would, at the most, be about 2.5 milliliters. For these reasons the data should be easier to interpret.

The value of cellular dry weight in the culture medium which contained glutamic acid was about twice that of the cellular dry weight from the Repaske medium. But the volume of gas consumed was 25.6 milliliters for the culture grown in the presence of 0.1 percent glutamic acid in Repaske's medium showed a gas change of 22.9 milliliters. The medium containing 0.1 percent glutamic acid in Repaske's medium showed a poor growth response under air. Since Hydrogenomonas eutropha grew well in this medium under an atmosphere of the gas mixture, it would seem that there was a deficiency of an energy source and/or nutrients.

It is of note that the volumes of gas consumed under

both conditions were about the same, although in the presence of glutamic acid better than two times the cell mass was formed in the culture medium. Since the values for carbon dioxide removed from the atmosphere are nearly the same for both treatments, it appears that when 0.1 percent glutamic acid is in the culture medium under an atmosphere of the gas mixture Hydrogenomonas eutropha uses the hydrogen for an energy source and the organic material augments the carbon source available to the microbe.

Reasons were given earlier for the suggestion that Hydrogenomonas eutropha possibly degrades tyrosine by a pathway that involves attacking the hydroxyphenyl group. The pH of the culture medium containing tyrosine dropped as growth progressed, and these values were close to those of the Repaske medium. For these reasons there was probably no ammonia in the atmosphere over the culture, and the solubility of carbon dioxide in the culture media was very likely close to the solubility in Repaske's medium. The gas analyses data concerning tyrosine in Repaske's medium can be interpreted more freely.

When tyrosine was employed in the culture medium, there was a considerable increase in cell mass as indicated by dry weight over that in the Repaske's medium. These increases in value ranged from 2.67 for the forty-eight hour culture of 0.1 percent tyrosine (13.125 and 35) to 5.078 (6.4 and 32.5) following a twenty-four hour incubation period for the

culture medium containing one percent tyrosine. There was no comparable increase in the total volume of gas consumed by the culture in the presence of tyrosine.

When the concentration of tyrosine in the medium was one percent, the value of the hydrogen quotient was close to that of the Repaske control. This quotient was altered by the presence of tyrosine in the concentration of 0.1 percent. The ratio of H_2-O_2-CO_2 consumed varied, in all cases, from the Repaske control. It appears that Hydrogenomonas eutropha continues to oxidize hydrogen in the presence of tyrosine, but the amount of carbon dioxide that is assimilated from the atmosphere is either decreased or rapidly replenished. The carbon source used by the microorganism when tyrosine is in a concentration of one percent would seem likely to come mainly from the tyrosine, since this concentration of tyrosine supports growth under an atmosphere of air.

In summing up, it is observed that the two ratios of the gases consumed differ, but these variations are slight. The most striking results are the increases in cell mass when the amino acids are present in the medium during incubation under the gas mixture. Since the media containing one percent glutamic acid and one percent tyrosine supported growth under an atmosphere of air, the microorganism appears to be able to use these compounds as sources of carbon and energy. When the culture is incubated

in these media under an atmosphere of the gas mixture, the culture continues to utilize the hydrogen. It then seems reasonable to conclude that the presence of these amino acids does not totally impair the bacterium's ability to oxidize hydrogen. Further study should be done to determine if Hydrogenomonas eutropha loses its ability to oxidize hydrogen after many transfers through media containing the amino acids.

When the three amino acids were in the concentration of 0.1 percent and incubated under an atmosphere of air, there was very little increase in cell mass. When the culture was incubated under an atmosphere of the gas mixture, there was, in all cases, a marked increase in cell mass. Again, these results seem to indicate that the oxidation of hydrogen appears to provide the necessary energy for utilization of these amino acids in this concentration for cell synthesis.

In the presence of the amino acids there was always an increase in the volume of the gas mixture consumed, but the volumes of these gases consumed per cell were less than under autotrophic conditions. This discrepancy may not be of importance since these experiments were conducted under stationary conditions. This system limited the amount of gas that was in contact with the cells. Further experiments using shake conditions would be necessary to determine if the presence of the amino acids limits the utilization of the hydrogen.

The theoretical value of 6-2-1 for the H_2-O_2-CO_2 consumed was never obtained. Marino and Clifton (1955) obtained a ratio close to the above value when using cells from a forty-eight hour culture grown under autotrophic conditions. These results were obtained from manometric studies. They found the values of hydrogen and oxygen consumed to the amount of carbon dioxide consumed increased with the age of the cells. Packer and Vishniac (1955a) also found that the amount of carbon dioxide assimilated and the efficiency of the over-all reaction decreases with the age of the cells. Their conclusions were also based on manometric studies. The work of Ruhland was based on growth experiments similar to those conducted in this work but lasted from one to four weeks (Ruhland 1922 and 1924). Ruhland (1922) stated that the hydrogen quotient is small in young cultures but will increase with older cultures, and only under the most favorable conditions will this value be two.

Manometric Studies with Hydrogenomonas eutropha under an Atmosphere of Air.

The results of manometric studies under an atmosphere of air using the three amino acids as substrate are given in Table III.

The variation in duplicate experiments was considerable, and further investigations would be needed to determine the

Table III. Results of Warburg studies under an atmosphere of air. The main compartment of the flasks contained phosphate buffer with a pH of 6.7. Potassium hydroxide was used to absorb carbon dioxide. The temperature was thirty degrees centigrade. The substrate is given in percentages and in micromoles per flask.

Ex.	Substrate (%)	μmoles per flask	oxygen μl/hr	dry wt. mg	nitrogen mg	QO_2	$QO_2(N)$	Growth Medium
1	.8% glutamic acid	1.0864	207	1.8	.14	115	1478	Repaske
2	1% glutamic acid	1.359	110	1.7	.14	65	786	Repaske
1	.8% glutamic acid	1.0864	116	1.5	.11	77	1055	Repaske, 1% glutamic acid
2	1% glutamic acid	1.359	163	1.4	.10	116	1630	Repaske, 1% glutamic acid
1	.5% alanine	1.122	70	1.8	.14	39	500	Repaske
2	1% alanine	2.246	74	1.7	.14	44	529	Repaske
1	.5% alanine	1.122	96	1.8	.14	53	686	Repaske, 1% alanine
1	1.1% tyrosine	1.214	361	4.1	.29	90	1245	Repaske
2	1% tyrosine	1.1038	259	1.7	.14	152	1850	Repaske

reasons for these differences. It is of note that the cell suspensions used in these studies cannot be considered "resting cells." The amino acids provided a nitrogen source, and, although the experiments lasted about two hours, there was the complication that the cells had all the conditions necessary for growth.

Considerable difficulty was encountered in showing oxygen consuming activity of the cells with tyrosine as a substrate. Although the exact nature of the difficulty was not uncovered, it may be that the enzyme system for the metabolism of these amino acids is labile. Meticulous attention must be paid to the preparation of the cells, and also to the preparation of the reagents involved. As a result of these difficulties only the results of those studies done on cells harvested the day of the experiment have been presented.

The results do show that Hydrogenomonas eutropha has a constitutive enzyme system for the metabolism of these amino acids since the cells which had been cultivated under autotrophic conditions consumed oxygen when any one of these amino acids was the substrate.

A cell suspension grown in the presence of tyrosine could not be obtained since the tyrosine could not be separated from the cell mass. Tyrosine is only slightly soluble in water and comes down in the pellet during centrifugation. An attempt to overcome this problem was made

by diluting the cell and tyrosine suspension in a sufficient quantity of saline solution to bring the tyrosine into solution. This suspension was then centrifuged in a Sharples Super Centrifuge. Unfortunately this procedure yielded a pellet of cells contaminated with debris and considered unfit for use in manometric studies, dry weight determinations, and nitrogen determinations. A further objection to using cells harvested in this particular centrifuge is that the centrifuge is not refrigerated.

Manometric Studies with Hydrogenomonas eutropha Under an Atmosphere of Hydrogen and Oxygen.

It was mentioned in the Review of the Literature that Kluyver and Manten (1942) had proven several basic facts about a species of Hydrogenomonas through manometric studies on resting cells which had been grown under autotrophic conditions. They showed that the gaseous uptake of these cells under an atmosphere of oxygen and hydrogen is increased by the presence of an organic substrate, in this case, lactate. When this same organic substrate was present in low concentrations, the increase in gas consumption was about equal to the respiration under an atmosphere of air, and this increased rate extended through the period of time necessary for the oxidation of the lactate under an atmosphere of air.

Kluyver and Manten also determined the hydrogen quotient of these same cells in the presence and in the absence

of the lactate. The quotient was decreased in the presence of the organic substrate which indicated that the increased gas consumption was due to increased oxygen consumption.

A similar experiment was performed by Wilson, Stout, Powelson, and Koffler (1953). There investigations showed that more of the hydrogen mixture is utilized in the presence of the lactate, and the quantity of lactate does decrease during the experiment. They concluded that greater amounts of oxygen in the mixture were used to oxidize the lactate.

Similar manometric experiments were conducted to determine if the three amino acids would produce the same results with cells grown under autotrophic conditions in Repaske's medium. The results of these experiments are given in Table IV.

Since the rate of gaseous uptake began to fall off during the second hour of the experiment when no organic substrate was added, it was decided to use the results based on the first hour. It was mentioned earlier that Bartha (1962) noted that the rate of oxidation of hydrogen is greater in the presence of carbon dioxide than in its absence. All of the flasks in these experiments contained potassium hydroxide to absorb the carbon dioxide.

We will refer to Tables III and IV to discuss the microliters of gaseous uptake per milligram of cellular dry weight per hour.

Table IV. Results of Warburg studies under an atmosphere of hydrogen and oxygen. The main compartment of the flask contained phosphate buffer with a pH of 6.7. The temperature was thirty degrees centigrade. The cells were grown under autotrophic conditions.

Ex.	Substrate	Hydrogen-Oxygen µl/mg/hr	Dry Weight mg.	Nitrogen mg.
1	none	192	4.1	.29
1	1.1% tyrosine	304	1.8	.14
1	.04% tyrosine	291	1.8	.14
1	.01% tyrosine	306	1.8	.14
1	.8% glutamic acid	381	1.8	.14
1	.03% glutamic acid	361	1.8	.14
1	.008% glutamic acid	346	1.8	.14
1	.5% alanine	236	4.1	.29
1	.02% alanine	211	4.1	.29
2	none	411	1.7	.14
2	1% tyrosine	465	1.7	.14
2	.004% tyrosine acid	430	1.7	.14
2	1% glutamic acid	392	2.0	.16
2	.004% glutamic acid	324	2.0	.16
2	none	352	2.0	.16

When 1.1 percent tyrosine was the organic substrate, the increase in consumption of hydrogen and oxygen was possibly due to the oxidation of the organic substrate. With 1.1 percent tyrosine as the organic substrate the uptake was 304 (Table IV) and 90 (Table III), respectively, for the mixture of hydrogen and oxygen and for oxygen alone. When no organic substrate was added, the cells (if one assumes that more than double the cellular dry weight in the main compartment of the Warburg flask would not introduce error) showed an uptake of the hydrogen and oxygen mixture of 192. The sum of this figure (192) plus the uptake of oxygen (90) is 282.

A duplicate experiment showed that when one percent tyrosine was the substrate, the gaseous uptake was 465 (Table IV) and 152 (Table III), respectively, for the mixture of hydrogen and oxygen and for the oxygen alone. When no organic substrate was present, the uptake of the hydrogen and oxygen was 411 (Table IV). The sum of this figure (411) plus the uptake of oxygen (152) is 563.

When 0.8 percent glutamic acid was the organic substrate, the uptake was 381 (Table IV) and 115 (Table III), respectively, for the mixture of hydrogen and oxygen and for the oxygen alone. When no organic substrate was present in the flask, the equivalent value was 192 (Table IV) for the mixture of hydrogen and oxygen. The increase in consumption of hydrogen and oxygen was possibly due to the

oxidation of the organic substrate, because the sum of this figure (192) plus that of the uptake of oxygen (115) is 307.

A duplicate experiment showed that when one percent glutamic acid was the substrate, the gaseous uptake was 392 (Table IV) and 65 (Table III), respectively, for the mixture of hydrogen and oxygen and for oxygen alone. When no organic substrate was present, the uptake of the hydrogen and oxygen mixture by the same cells was 352 (Table IV). The sum of this figure (352) plus the uptake of oxygen (65) is 417, and the increase in consumption of the hydrogen and oxygen was possibly due to the oxidation of the organic substrate.

When 0.5 percent alanine was the substrate the gaseous uptake was 236 (Table IV) and 39 (Table III), respectively, for the mixture of hydrogen and oxygen and for oxygen alone. When no organic substrate was present, the cells showed an uptake of the hydrogen and oxygen mixture of 192 (Table IV). The sum of this figure (192) plus the uptake for oxygen (39) is 231, and the increase in uptake of hydrogen and oxygen was possibly due to the oxidation of the organic substrate.

In the first series of experiments, cells in the presence of glutamic acid showed a greater uptake of the mixture of hydrogen and oxygen than with the other organic substrates. This uptake seemed to suggest that glutamic acid has a greater capacity to stimulate the uptake of the hydrogen mixture.

When 0.004 percent tyrosine was the organic substrate, the uptake of hydrogen and oxygen was 430, and when no organic substrate was added, the uptake of hydrogen and oxygen was 411 (Table IV).

When 0.004 percent glutamic acid was the organic substrate, the uptake of the hydrogen and oxygen mixture was 324, and the same cells showed an uptake of the gaseous mixture of 352 when no organic substrate was added (Table IV).

These results failed to prove that this low concentration of either glutamic acid or tyrosine would stimulate the uptake of hydrogen and oxygen and even seem to indicate a depression of the gaseous uptake. These differences are probably not conclusive. Concentrations of either organic substrate probably could be found since earlier work indicated that these values might be additive.

It was mentioned earlier the possible complication that could arise since these cells had all the necessary conditions for growth. A further complication with these experiments under discussion is that mercury was used as the manometer fluid. The readings on the manometer were changed by a factor of about 13.6, the density of mercury at thirty degrees.

In making an over-all consideration a point to ponder is: does a microorganism synthesize a compound that it can use as a carbon source?

The portion of the cells of Hydrogenomonas eutropha containing hydrogenase activity is soluble. For this reason the research concerning this microbe, with the exception of the work involving an atmosphere regenerating system, concerns studies on hydrogenase activity. We must go to another species for information concerning synthesis.

DeCicco and Umbreit (1964) used an induced mutant of Hydrogenomonas facilis in growth studies. Their work indicated that this microbe used tyrosine for synthetic purposes, but their mutant had lost its ability to synthesize tyrosine and would not grow unless tyrosine were added to the medium.

Studies on the path of carbon with Hydrogenomonas facilis have shown that alanine and glutamic acid are among the labeled compounds isolated after incorporation of radioactive carbon dioxide (Faust, 1958; McFadden, 1959). Intermediates of the Kreb's cycle have also been found (Bergmann, Towne, and Burris, 1958; Dewar, 1962).

Brown, Cook, and Tischer (1964) found these three amino acids in the cell-free medium after growth had been terminated during the late log phase. It was not determined if these compounds were due to excretion by the growing cells or if they were due to the autolysis of a small portion of the culture. The amount of the amino acids present was not determined.

An estimate can be made of the minimum quantities of these amino acids present. Among the techniques used to identify these amino acids was paper chromatography. The

minimum quantities of these amino acids detectable by paper chromatography are:[1]

amino acid	minimum quantity (micrograms)
alanine	0.2
glutamic acid	0.1
tyrosine	3.0.

The paper chromatogram was run on a 0.1 milliliter aliquot of the cell-free medium obtained when the cells had reached the late log phase. The aliquot was obtained from a one milliliter concentrate of the medium. The initial volume of the medium was one liter. A qualitative test for the presence of these amino acids was also made before concentrating the cell-free medium. It is to be assumed that the minimum and maximum quantities of the amino acids that would be present in the medium during the late log phase is approximately as follows:

amino acid	minimum quantity (grams per liter)	maximum quantity (grams per liter)
alanine	2×10^{-6}	2×10^{-3}
glutamic acid	1×10^{-6}	1×10^{-3}
tyrosine	3×10^{-5}	3×10^{-2}

These quantities are several-fold less than those added to the medium.

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[1] Lederer, Edgar, and Michael Lederer, Chromatography (Elsevier Publishing Company, Amsterdam, 1957), p. 310.

The results of all of these experiments give no evidence that these amino acids are inhibitory, but rather that they are used by the growing microorganism. The results of the gas analyses give evidence that these amino acids are used for cell synthesis. Since most biological reactions are theoretically reversible, it is then conceivable that Hydrogenomonas eutropha will metabolize compounds that it synthesizes. When these amino acids are added to the culture, less energy should be required for the manufacture of cellular material and this could account for the several-fold increase in cellular dry weight observed during these investigations when any of the amino acids were added to the medium and incubation took place under the gas mixture.

The next problem to be resolved is: why would material needed for cell synthesis be found in the medium when the culture was growing under the autotrophic conditions described by Cook, Brown, and Tischer (1964)?

Since these amino acids were found as the microbe was going into the late log phase there might be less demand for these materials needed for cell synthesis and as a consequence these materials might be excreted by the cell into the medium. There is also a likelihood that through some fault of the autotrophic nature of the microorganism, an excess of components needed for protein might be synthesized and these excesses would be excreted into the medium. Small polypeptides might have been formed and excreted by the

cells and these might later have been hydrolyzed during the preparatory treatment prior to performing the chromatograms.

However, a difficulty that presents itself in attempting to correlate the results obtained in these investigations with the results obtained by Cook, Grown, and Tischer (1964) is that, although the cultures were incubated under a gas mixture, these conditions were not autotrophic since the amino acids were added to the medium. The microorganism, therefore, had present during the entire growth period material that appears to be needed for cell synthesis and was able to adjust its synthesizing mechanisms to utilize this added source of cell material. As was mentioned above, with material that could be used for cell synthesis present, less energy would be needed for cell synthesis.

The attempts to answer these two questions posed are perhaps inadequate and of a speculative nature. It is hoped that this work will arouse the interest of others, and further work will bring the correct answers.

Most important of all in considering the use of this microorganism in an atmosphere regenerating system is that there is little likelihood that these amino acids would build up in the recycled medium during space travel, because the microbe can use them for a carbon source. It must be emphasized again that further work should be done to determine if Hydrogenomonas eutropha loses its ability to oxidize hydrogen after many transfers through medium containing the

amino acids.

A small inoculum was used to minimize the organic substances present in the growth medium. Work done by Mr. David W. Cook, in the Microbiology Department, Mississippi State University (Personal Communication) on autotrophic growth responses by Hydrogenomonas eutropha indicated that increasing the buffering capacity of the growth medium caused an increase in growth, as indicated by measurements of turbidity. A study of Figure 4 (3) indicates that during growth under autotrophic conditions, the pH of the medium drops below the pH optimum. Using a larger inoculum shortens the lag phase.

It would perhaps be of value to determine the growth response and volume of gas consumed if a larger inoculum were used in the presence of the amino acids in Repaske's medium with a greater buffering capacity. The studies should be done under shake conditions to allow the cells to come in complete contact with the gas mixture. The incubation time should allow the culture to enter the stationary phase.

SUMMARY

These investigations were undertaken to determine if the presence of organic material in the mineral salts medium would impair the growth and physiology of the hydrogen-oxidizing bacterium Hydrogenomonas eutropha. In a series of studies Repaske's mineral salts medium was supplemented with various concentrations of L-alanine, L-glutamic acid, or L-tyrosine and inoculated with cells grown under strict autotrophic conditions. Repaske's mineral salts medium was employed as a control. Growth under an atmosphere of six parts of hydrogen, two parts of oxygen, and one part of carbon dioxide was compared with growth under an atmosphere of air. The cultures were incubated under stationary conditions.

Preliminary experiments indicated that the presence of these amino acids in concentrations of 0.1 to one percent were not toxic.

Under an atmosphere of hydrogen, oxygen, and carbon dioxide all three of the amino acids caused a marked increase in turbidity of the culture medium as indicated by measurements of the optical density. This apparent increase in cell mass was always greater than that formed in the Repaske control medium. In the presence of one percent glutamic acid and 0.1 percent alanine there was a rise in the pH of the culture media, but in the presence of tyrosine the pH dropped as growth progressed. When glutamic acid in

the concentration of 0.1 percent was added to the medium, the pH remained close to seven.

Under an atmosphere of air all of the culture media, with the exception of Repaske's, were able to support some growth of the microorganism. An increase in turbidity was apparent within twenty-four hours when either glutamic acid or tyrosine was present in the medium. Neither 0.1 percent alanine, 0.1 percent glutamic acid, not 0.1 percent tyrosine gave a marked increase in turbidity. When alanine was present in the medium, there was little change in turbidity until after forty-eight hours. A rise in pH was observed where growth occurred for all media with the exception of tyrosine.

Although there was a marked increase in cell mass as indicated by optical density measurements when any of the amino acids were added to the culture medium and the culture was incubated under the gas mixture, the volume of gas consumed per unit of cell mass was low. These studies were conducted under stationary conditions, and there was less contact between the cells and the gas mixture.

Gas chromatographic analyses of the atmosphere remaining after termination of the growth period indicated that the presence of the amino acids caused an increase in cell mass without impairment to the bacterium's ability to oxidize hydrogen. The different conditions for growth did not cause a marked alteration of the ratios of gases consumed.

A consideration of the over-all results indicates that when any one of the three amino acids is added to the culture medium in a concentration of 0.1 percent Hydrogenomonas eutropha uses the amino acid as an added source of carbon and obtains energy from the oxidation of hydrogen.

Manometric studies were conducted with a Warburg respirometer on cells grown under strict autotrophic conditions. The results of these studies show that Hydrogenomonas eutropha has constitutive enzyme systems for the metabolism of these amino acids since these cells were able to consume oxygen in the presence of any of the three amino acids.

Autotrophically grown cells were tested in the Warburg respirometer for the ability to oxidize hydrogen in the presence of each of the three organic substrates. The flasks were flushed with a mixture consisting of ninety percent hydrogen and ten percent oxygen. Following equilibration, the substrate was tipped in. The data indicated that a larger amount of the hydrogen and oxygen mixture was consumed in the presence of the organic substrate, and this increase was apparently due to the oxidation of the organic substrate. This substantiates earlier conclusions that these organic substrates do not affect the ability of the microorganism to utilize hydrogen.

Difficulty was encountered in duplicating the results concerning oxidative assimilation. This may have been due

to the fact that these cells were not "resting cells" as all the conditions necessary for growth, including a nitrogen source, were present.

CONCLUSIONS

1. Preliminary experiments indicated that L-tyrosine, L-alanine, and L-glutamic acid are not toxic to Hydrogenomonas eutropha in concentrations of 0.1 to one percent.
2. These amino acids appear to be capable of supporting growth of this bacterium when no hydrogen is present.
3. The presence of these amino acids in the culture medium does not impair the bacterium's ability to oxidize hydrogen.
4. The microorganism appears to use the energy it obtains from the oxidation of hydrogen to utilize the amino acids for cellular synthesis when the amino acids are present in concentrations of 0.1 percent, and it appears that most of the amino acid goes into cellular material.
5. Cells grown under autotrophic conditions can metabolize the amino acids.

It appears that either one or all of these amino acids do not impair the ability of Hydrogenomonas eutropha to oxidize hydrogen, and since these amino acids are metabolized by this bacterium, their presence in the mineral salts medium should not constitute a problem in long-term continuous cultures.

ABSTRACT

Eva Elliott Blake, Master of Science, 1966

Major: Microbiology, Department of Microbiology

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ABSTRACT

Investigations were conducted to determine if the presence of L-tyrosine, L-alanine, and L-glutamic acid in the medium would impair the growth and physiology of Hydrogenomonas eutropha. These compounds had been found in the basic medium following growth under autotrophic conditions.

Preliminary studies indicated that these amino acids are not toxic.

In a series of growth studies using cells grown under autotrophic conditions, Hydrogenomonas eutropha was used to inoculate Repaske's medium and Repaske's medium containing the amino acids. Growth under an atmosphere of hydrogen, oxygen, and carbon dioxide in a ratio of 6-2-1 was compared with growth under air.

Under an atmosphere of the gas mixture all three of the amino acids caused a marked increase in growth as indicated

by turbidity measurements. The increase in cell mass was several-fold that in Repaske's medium. The least increase over that of the control medium was obtained when 0.1 percent glutamic acid was in the culture medium and was 2.06. The greatest increase occurred when one percent tyrosine was in the culture medium and was 4.12.

When any one of the three amino acids was present in a concentration of 0.1 percent and incubation was under air, there was little growth. Either one percent glutamic acid or tyrosine gave a marked improvement.

Gas chromatographic analyses of the atmosphere remaining after termination of the growth period indicated that that the amino acids did not impair the microbe's ability to oxidize hydrogen. Since there was little alteration of the ratios of the gases consumed, it was felt that most of the amino acids goes into cellular material.

The volume of gas consumed in the presence of the amino acids was low, but these results were expected since the experiments were conducted under stationary conditions.

Respirometric studies on the cells grown under autotrophic conditions indicated that Hydrogenomonas eutropha can oxidatively assimilate all three amino acids. Under an atmosphere of hydrogen and oxygen the results indicated that an increase in the gaseous uptake occurred which was probably due to the oxidation of the organic substrate.

It appears that either one or all of these amino acids

do not impair the ability of Hydrogenomonas eutropha to oxidize hydrogen, and since these amino acids are metabolized by this bacterium, their presence in the mineral salts medium should not constitute a problem in long-term continuous cultures.

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