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INFLUENCE OF METABOLIC ACCUMULATION OF PRODUCTS OF HYDROGENOMONAS CELLS ON THEIR CONTINUED GROWTH

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XXII. Wright, E.L. and R.G. Tischer. Effects of Spacecraft Level Vibrations and Gravities on Plectonema boryanum. Submitted to Aerospace Medicine, 1969. 26

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Hassan, Farook. Studies on Oxidation Reduction Potentials in Repaske's Medium.

Bullen, John. CO_2 Utilization by H. eutropha as Effected by Varying Magnesium Concentrations. (tentative title)

Al-Hindawi, Nassir. Assimilation of Methane and Carbon Dioxide by M. methanooxidans.

INTRODUCTION

Reports on theories, speculations, and research concerning closed or partially closed ecological systems, chemical systems, and combinations of these bear some early publication dates. The greatest emphasis, however, has been in the last two decades.

During this period the algal ecologies, plant ecologies, and several chemical bioregenerative systems were investigated. Most of these systems involved coprophagy, directly or indirectly and were not found to be satisfactory either esthetically or functionally.

The introduction of the electrolysis of water as a source of oxygen during manned space flight engendered consideration of the disposal or profitable utilization of the hydrogen produced during electrolysis.

Apart from the explosion hazard properly associated with the presence of hydrogen, no vital role was found for it in the proposed bioregenerative system. As a result it was suggested that the hydrogen be recovered in edible form by using it along with other inorganic nutrients and some of the oxygen produced by electrolysis for the growth of Hydrogenomonas eutropha cells. The astronauts could then eat these bacterial cells as a part of their diet, thereby increasing the efficiency of the system without any of the difficulties introduced by the use of algae or other plants or animals and without any involvement with the troublesome liquid and solid human wastes.

Among the problems introduced along with the use of electrolysis with hydrogen oxidizing organisms are:

1. The efficiency of the test organism in using hydrogen under various conditions of growth.
2. The influence of metabolic products of bacterial growth on continued growth.
3. The comparative value of H. eutropha and other hydrogen utilizing organisms especially thermophiles, which might be employed.

It has been the aim of this project to provide useful information about the foregoing problems which might lead to the formulation of an efficient, partially bioregenerative system for use by astronauts on long space flights.

I. Extracellular Products of Hydrogenomonas eutropha

The test organisms were routinely grown on Repaske's medium at 30°C with a gas mixture of the composition 70% H_2, 20% O_2 and 10% CO_2, which was later changed to 67% H_2, 22% O_2, and 11% CO_2 to take advantage of the small increase in growth afforded by this change.

Electrolytic and ion-exchange techniques provided proof that approximately 10% of the extracellular products of growth are neutral in character while 30% are acidic and 60% are amphoteric.

Extracellular products identified mainly by paper chromatography were ribose, glutamic acid, alanine and tyrosine. Subsequently arabinose, xylose and glucose were discovered.

These findings suggest that amino acids generally, and specifically alanine glutamic acid or tyrosine are not likely to appear as important growth inhibitors.

Arabinose, ribose, xylose and glucose were found to have no important influence on the growth of H. eutropha while fructose was metabolized under otherwise autotrophic conditions.

Although the foregoing statement and the appended publications indicate a considerable gain in information about the nature of H. eutropha metabolic products and their relationship to the growth of the organism, no large effects have been noted which might markedly influence the use of the test organisms in a regenerative system.

Spent medium was concentrated using a flash evaporator and distilled at one pH intervals from 4 through 9. Both the distillates and the residues were used in Warburg Respirometer tests on both growing and resting cells.

While some effects were associated with the extremes of pH in the distillates, the resulting influence on gas consumption was sufficiently small to invite its neglect in future planning.

Growth of H. eutropha for 150 hours in massive amounts of spent medium, centrifuge-concentrated cells, and new medium inoculated with new cells was measured using a Coulter counter and by spectrophotometry.

Use of the slopes of the resulting growth curves for comparison indicated that the presence of 60% spent medium produced a slope of .28. In massive doses of old cells the slope was 1.0 while new medium and new inoculum exhibited a slope of 1.1 under similar conditions.

These experiments indicate that spent medium inhibits growth proportional to its concentration. It is reasonable to subsume that the main effect is one of dilution of nutrients.

The presence of large amounts of old cells made no appreciable difference in the growth rate since their presence apparently did not dilute the supply of nutrients very much.

II. Studies on Hydrogenomonas thermophilus

The continued use of soil enrichment techniques produced a hydrogen utilizing organism which grows well at 50°C and has been characterized as H. thermophilus, the first thermophilic hydrogen-utilizing bacterium ever to be isolated.

Difficulties encountered in the culture of this organism led to the discovery that half-strength Repaske's medium and a gas mixture of 80% H_2, 10% O_2, and 10% CO_2 in conjunction with freshly prepared medium containing reduced iron allowed generous growth of this organism.

Colonization was only affected when specially prepared Repaske's medium was subjected to incubation (drying) at 65°C for several days prior to inoculation. The colonies were opaque, irregular in shape and size, slightly raised and faintly brown.

Curves relating the pH response of H. thermophilus at 55°C indicated a sharp maximum, in terms of optical density, at pH = 7.0 with growth falling off rather sharply within one pH unit in either direction.

Oxidation-reduction studies have shown marked response of the oxidation potential to the presence of cells. The addition of cells to the medium caused a shift in potential many times that shown by the gas mixture and for Fe^{++}. Apart from these three changes, assays of the influence of individual medium components were small and may be negligible.

Since the E_0' value employed in these investigations is of primary importance in determining the value of $C \ln \frac{(ox)}{(red)} = 0$, special efforts are being continued to arrive at a statistically valid estimation of this parameter of the Nernst equation.

The organism has been accepted by the ATCC as Culture no. 23388

Multiple temperature growth tests analyzed by means of Coulter counts or optical density show that organisms grown at 5°C intervals from 30°C to 55°C yield clear maxima between 42 and 48°C. These tests further indicate that the time-location of the growth maxima varies from one temperature to another, suggesting a very plausible dependence of time-to-maximum-growth on temperature of growth.

Attempts to evaluate the effects of oxidation state of the organism on its growth rate and ultimate utilization in a bioregenerative are being continued without support.

III. Future Research

Research has indicated that neither the algal ecologies nor the electrolytic-hydrogenomonas systems are acceptable for use in their present state.

The most encouraging aspects of this work, which it is expected will yield results applicable to bioregenerative systems of the future, are those associated with the discovery of the reaction mechanism characteristics of both of the subject organisms.

It is the hope of some scientists in this field that careful basic research will provide the basis for the closed-ecology shrinking method which was the original purpose of this effort.

IV. Project Spin Off

This project will have produced six Ph.D.'s and seven M.S. graduates during the period of its duration.

Associated research carried on without outside support included (1) investigation into the production of algal heteropolysaccharides for ultimate use as human food, (2) studies on the pathway of carbon dioxide assimilation by M. methanomonas and (3) studies on the oxidation-reduction potentials of H. thermophilus.

V. Cody, R.M., and R.G. Tischer, Microbial Synthesis of Animal Feeds From Human Wastes Substrates. Developments in Industrial Microbiology. 3:53-62. 1962.

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MICROBIAL SYNTHESIS OF ANIMAL FEEDS FROM HUMAN WASTE SUBSTRATES *

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Microbial synthesis by selected fungi grown on human feces for the purpose of producing animal feeds has been investigated.

Chromatographic techniques and iodometric titration procedures revealed that human feces were deficient in reducing sugars.

The mean value for the cellulose content of feces was 6.5%. The fecal cellulose was submitted to enzymic hydrolysis. The resulting glucose provided a readily available source of carbon, thus optimizing conditions for microbial synthesis.

Tests suggest that although the protein nitrogen of human feces supported good growth of molds, improved yields may be obtained by supplementing feces with nitrate nitrogen.

The component fractions of feces designated as carbohydrate, ether-soluble substance, protein nitrogen, and ash, added separately to an otherwise adequate medium, provided no practical advantage over normal feces in terms of increased mold growth.

Carbohydrate and fat served as a sole source of carbon to support mold growth. Although this investigation was initiated to evaluate microbial synthesis by molds in space feeding systems, it may be possible to extend these applications to the commercial feed industry.

Microbiologists have been pondering the idea of increasing the world's food reserve through microbial synthesis for several decades. One serious threat to the supply of food was experienced during World War II, particularly in the case of Germany. German scientists began to exploit the usefulness of yeast as food and fodder (Reusser *et al.*, 1958) at a time when the supply was becoming exhausted.

Even today, with the tremendous expansion of world population and the decrease in arable land, increasing demands on the food reserve again threaten depletion of stockpiles. Consequently, the interests of microbiologists are being renewed in microbial synthesis as an abundant source of food.

The need for producing food by microbial synthesis becomes even more essential because of the recent advances in rocketry and space technology. Thus, microbial synthesis may serve (1) as the primary source of food for the space man, and (2) for the disposal of his waste.

Several organisms have been investigated as a possible source of food when grown on agricultural waste. Roberts (1953) demonstrated the nutritive value of *Escherichia coli* for chicks. From this investigation, the conclusion was drawn that this protein can serve as an adequate feed supplement.

Yeasts have been found to contain a relatively large quantity of high-quality protein (Carter, 1944). Experiments employing yeast as the sole source of protein for swine revealed that these animals could be fattened to magnitudes similar to those observed with normal diets. Other observations, by Axelson (cited by Carter, 1944), indicated that yeast may supply 50% of the protein requirement of poultry. Attempts to use fungal protein as either the sole source of nutrients or as a feed supplement have not met with the same success as have experiments with bacterial and yeast proteins. Experiments (Skinner, 1934) have shown that *Penicillium flavoglaucum* supported better growth in young rats when fed at 18% of the regular diet than at 9%. However, best growth results were obtained when the

*This work was done in cooperation with the National Aeronautics and Space Administration, Washington, D. C.

mycelia were used with 9% casein or maize gluten, in which case normal rapid growth resulted in the rats.

Gorcica *et al.* (1953) were able to supply the vitamin B_1 (thiamin) requirement of chicks by supplementing an otherwise adequate ration with *Aspergillus sydowi*. Anderson *et al.* (1953) obtained improved growth in chicks when supplementing the diet with the mycelia of *Penicillium*.

Skinner and Muller (1940) found that the limiting growth factor in animals on a microbial protein-supplemented diet was due to cystine and methionine deficiency of the molds. These workers concluded that molds were deficient in sulfur amino acids.

The present investigation was initiated in an effort to discover more efficient means of utilizing human wastes as substrates for the synthesis of microbial products to be used as animal feeds or feed supplements and possibly as human foods, and to relieve the nuisance of waste accumulation during long space flights.

The objectives of this investigation have dealt, in general, with the conversion of human waste materials into fungal material to be used as feeds and foods. More specifically, the tests reported were designed (a) to measure the carbohydrate content of human feces, including cellulose; (b) to evaluate the products resulting from enzymatic hydrolysis of cellulose as a source of carbohydrate for the growth of mold; (c) to ascertain the nutritive value of carbohydrate, fat, protein, and inorganic mineral (ash) fractions of feces; (d) to establish the value of fecal protein nitrogen as the sole source of nitrogen for mold growth; and (e) to conduct mass cultivation trials of selected fungal species to be used in future animal feeding tests.

Determination of the Reducing-Sugar Content of Feces

In view of the fact that most fungi require carbohydrates as their usual carbon source (Cooke, 1957), it seemed plausible to investigate the carbohydrate content of human feces intended for use as mold substrates.

The quantity of reducing sugars in feces was determined by chromatography (Block *et al.*, 1958) and iodometric titration (Shaffer and Hartman, 1921).

Measurements by chromatographic techniques were employed primarily as a check against the titration procedure. In the chromatographic analysis, paper chromatograms were prepared from solutions containing 150 mg/ml of the monosaccharides, glucose, fructose, mannitol, and galactose, and the disaccharides, sucrose, maltose, and lactose, in both a distilled-water base and a 5% aqueous solution of feces. Fifty μl of these mixtures was spotted on filter paper and developed according to the method of Block *et al.*, (1958). By adding known amounts of carbohydrate materials to an aqueous feces solution, it was possible to ascertain the effect of feces on the Rf value of each individual carbohydrate.

A quantitative measurement of fecal reducing sugars was accomplished by the iodometric procedure of Shaffer and Hartman (1921). Because color constituents, protein materials, and other components of feces affect the titration by obscuring the end point, it was necessary to make comparative measurements on feces substrates freed of protein and most color constituents by lead acetate precipitation. In addition, the sensitivity of the iodometric titration for detecting fecal reducing sugars was evaluated quantitatively in terms of the known level of added glucose. To determine the sensitivity, glucose at levels of 0, 50, 100, and 200 mg/ml was added to both precipitated and nonprecipitated fecal sub-

strates, and to a control consisting only of distilled water. These trials were run in duplicate.

The reducing-sugar concentration, as measured by the iodometric procedure, was calculated according to Shaffer and Hartman (1921).

Results of single-phase paper chromatographic determinations for fecal sugars showed that sucrose, maltose, lactose, glucose, fructose, galactose, and xylose were detectable in feces solutions when added at a level of 150 mg/ml of substrate. The Rf values obtained for this concentration of sugar in two different substrates are shown in Table 1.

Table 1. Paper Chromatographic Determination of Fecal Sugars

Carbohydrate	Rf value	
	In water	In 5% feces solution
Sucrose	0.26	0.15
Maltose	0.19	0.16
Lacotose	0.19	0.16
Glucose	0.17	0.16
Fructose	0.32	0.29
Galactose	0.26	0.26
Xylose	0.33	0.33

Samples without added carbohydrate revealed no evidence of fecal carbohydrate.

It appeared from these chromatographic data that, in general, constituents of human feces produced a lower Rf value of glucose than did distilled water. Mean Rf values indicated that chromatography can be used successfully to detect fecal carbohydrates.

The data in Table 2 show that human feces was deficient in reducing sugars as measured by the iodometric titration procedure of Shaffer and Hartman (1921). The titration values determined for fecal solutions containing known concentrations of added glucose revealed that, in most instances, more than 80% of the added glucose was resolved by titration. Since we have no knowledge denying the

Table 2. Measurement of Fecal Reducing Sugar by Iodometric Titration

Level of glucose added, mg/ml	Untreated feces		Precipitated (PbAc) feces		Aqueous solution (control)	
	Glucose, mg	Glucose recovered, %	Glucose, mg	Glucose recovered, %	Glucose, mg	Glucose recovered, %
0	00.0	00.0	00.0	00.0	00.0	00.0
50	52.0	104.0	45.5	91.0	46.9	93.8
100	63.1	63.1	62.5	62.5	90.4	90.4
200	170.6	85.0	173.3	86.6	178.0	88.8

validity of these experimental results, it is our feeling that this recovery from feces represented acceptable efficiency. In the aqueous control, approximately 90% of the glucose was resolved. The assumptions made concerning the effect of colored and protein constituents on the results of the titration were found to be invalid since, in most instances, a higher percentage of glucose was resolved in the nonprecipitated fecal sample than in the precipitated one.

It was also observed that as the concentration of glucose was increased to a higher level, there also occurred a corresponding decrease in the percentage of sugar resolved by titration.

In all tests, the greatest percentage of glucose resolved was in the aqueous glucose control solutions. This occurrence was to be expected since distilled water contains no interfering substances.

These observed data indicated that the sensitivity of the Shaffer and Hartman method was satisfactory.

Cellulose Content of Feces

Gradwohl (1956) lists, in addition to the soluble and gaseous compounds, raw vegetables, fruit skins, nuts, berries, mucus, tissue remnants, epithelial cells, muscle fibers, connective tissues, fats, starch granules, and a variety of bacteria in the feces of normal healthy individuals. Thus, it would seem reasonable to expect that cellulose and other high-molecular-weight polymeric carbohydrates are present in human feces. Assuming that certain molds are capable of utilizing cellulose as a carbon source, a study was designed to measure the cellulose content of feces.

The purpose of quantitatively measuring the cellulose content of feces was to ascertain that amount present which might be made available to molds for growth in the form of monosaccharides. In the analysis, fresh samples of feces were taken randomly. Test samples were run in duplicate with a filter-paper control according to the method of Crampton and Maynard (1938). The control consisted of 1 g of macerated filter paper.

Enzymatic Hydrolysis of Cellulose

The amount of monosaccharide made available by enzymatic hydrolysis of fecal cellulose for microbial synthesis by molds was determined using a modification of Ramsey's (1959) procedure. Crude cellulase enzyme (Delta Chemical Works) was prepared in a 1% concentration in 0.05 M potassium acetate buffer. The enzyme preparation was sterilized by filtration. Fecal substrate was prepared by adding 100 g of fresh human feces to 100 ml of 0.05 M potassium acetate buffer and heat-sterilizing.

The enzyme preparation was added at a level of 16.25 ml per 100 g wet weight of feces. This level corresponded to 0.1 g/ml of enzyme to each calculated gram of fecal cellulose. This ratio was based on a mean value of 6.5 g cellulose per 100 g wet feces containing 25% total solids (Table 3).

The following controls were employed: (1) carboxymethyl cellulose, 1 g in 100 ml of potassium acetate buffer; (2) purified macerated filter paper, 1 g of filter paper in 100 ml potassium acetate buffer paper; (3) 100 ml of 0.05 M acetate buffer; (4) 100 g feces in 100 ml of potassium acetate buffer.

Controls 1, 2, and 3 were treated with 10 ml of enzyme preparation. The enzyme preparation was not included in the feces control.

Since all of the substrates were sterile and thus presented no difficulty due to contaminating bacteria, all enzyme mixtures were allowed to react for 48 hr.

Following the hydrolysis period, cellulase activity was measured in terms of the amount of reducing sugars produced, as measured by the method of Shaffer and Hartman (1921). In addition, cellulase activity and cellulose content of feces. were measured indirectly in terms of the amount of glucose produced.

The cellulose content on the dry-weight basis of human feces, as determined in this study, ranged from 4% to 9%. The mean value for the two trials was 6.5% (Table 3). Duplicate samples agreed quite closely in both trials. The variation between the trials was attributable to a difference in the cellulose content of different fecal samples employed in the analysis.

Table 3. Cellulose Content of Feces from Two Analyses

Crucible number*	Weight of crucible and sample, g	Weight after igniting, g	Weight difference, g	Cellulose, %
Trial 1				
1	8.2229	8.1355	0.0874	4.37
2	9.2431	9.1515	0.0916	4.58
3	10.7621	9.1419	1.6202	99.90
Trial 2				
1	8.1748	8.1095	0.6530	8.52
2	8.8185	8.7459	0.7270	8.90
3	9.3631	8.1086	1.2545	98.60

*No. 1 and 2 duplicate 2 g dried feces with no added cellulose (test samples). No. 3 contained only 1 g macerated filter paper (control).

Using the mean value of 6.5% cellulose and assuming that 25% of feces consist of solids, the actual cellulose content of the sample was 1.62 g per 100 g wet weight of feces.

The mean value for the cellulose content of the filter paper control was 99.25%.

Cellulase activity was demonstrated with all three of the cellulose substrates, as evidenced by the production of reducing sugars (Table 4).

Table 4. Reducing Sugar from Enzymatic Hydrolysis of the Cellulose Substrates Measured Iodometrically

Substrate	mg reducing sugar (glucose) per 50 ml of substrate
Feces (with added enzyme preparation)	121.75
Feces (without added enzyme preparation)	0.00
Cellulose gum	65.92
Filter paper	44.71
Acetate buffer (control)	0.00

The reducing sugars produced from 50 g of feces amounted to 121.75 mg. This value corresponds to a hydrolysis conversion efficiency of 14%. When this value is compared with only 9.0% with the filter paper control, it becomes obvious that

feces contain substances which have an enhancing effect on the cellulose enzyme.

The results of enzymatic hydrolysis of the cellulose component of feces clearly indicated that hydrolysis was a means by which simple carbohydrates can be made readily available for microbial synthesis.

Even though Holden *et al.* (1950) obtained only 50% hydrolysis of cellulose, it is hoped that further research will provide methods for increasing this value.

Nutritive Value of Four Components of Feces

Previous studies (Cody, 1961) have shown that some mold species grow less on a 5% aqueous feces solution than in the presence of a similar solution containing added carbohydrates. In view of this occurrence, a study was designed to determine whether various fecal components designated as carbohydrates, fats, proteins, and minerals, when added separately to an otherwise complete medium, can support growth of *Aspergillus niger* and *Rhizopus delemar*.

In order to measure the effect on mold growth of fecal carbohydrate components, it was necessary to hydrolyze the cellulose constituent of feces by means of a crude enzyme preparation. The carbohydrate component was prepared by reacting 175 mg of crude cellulose with 100 g of feces containing approximately 7.0% cellulose (dry weight), for a period of 48 hr. The protein was removed from the hydrolysis mixture by lead acetate precipitation. The resulting supernatant containing the glucose produced by cellulase hydrolysis was filtered and treated with potassium oxalate to remove excess lead ions.

The filtrate was then added to an equal volume of carbohydrate-free Czapek Dox medium (Difco) and sterilized. In this medium, the sole source of carbon was contained in the filtrate. The control employed consisted of 100 ml of Czapek Dox medium plus 2.0 g glucose.

The ability of fat to support microbial synthesis as the sole source of carbon was assayed in a manner similar to that described for the carbohydrate component. The fecal fat was prepared by extracting 100 g of fresh feces twice with 300 ml of petroleum ether. The extracts were combined, evaporated to dryness, resuspended in 25 ml of d[illegible] water, and homogenized in a Waring blender. Ten milliliters of the ether-soluble extract was added to 100 ml of the mineral medium.

The control consisted of 100 ml of mineral medium to which 1 g each of cholesterol, palmitic acid, stearic acid, and glycerol were added as sole sources of carbon. These additives were selected on the basis of data presented by Cochrane (1958).

The protein component was prepared by precipitating 200 ml of a 20% feces solution with 45 ml of saturated lead acetate, followed by 12 ml of saturated potassium oxalate. The precipitated protein was removed from solution by filtration. The protein residue then was suspended in 100 ml of glass-distilled water and mixed thoroughly by vigorous shaking. This suspension was filtered and washed with 100 ml of glass-distilled water. The wet weight of the residue was approximately 10 g. These solids were added to a mineral–glucose medium without a nitrogen source at the rate of 2 g per 100 ml of mineral medium. The control employed consisted of the same ingredients as those used with the carbohydrate system.

The mineral constituents of feces available for mold growth and metabolism were assayed by ashing 100 g of feces at approximately 600 C for 3 hr. Three grams of ash were treated with 10 ml of concentrated HCl and boiled for 30 min.

The HCl residue was made up to a total volume of 20 ml with glass-distilled water. Ten milliliters of this mineral solution was added to a basal medium consisting of 2 g of glucose and 0.2 g of urea. The urea was sterilized by filtration and added aseptically to the autoclaved glucose medium. All test substrates were prepared in duplicate. The test sample containing carbohydrate, ether solubles, protein and mineral substrates, and controls were inoculated with a spore suspension, one set with *A. niger* and the other with *R. delemar*. All cultures were incubated on a rotary-type shaker operated at 98 cycles/min for 5 days.

The ability of each of these fecal fractions to support the growth of *A. niger* and *R. delemar* was measured in terms of the dry weight of mycelia produced.

The results show that the ether-soluble fecal component designated as fat supported excellent growth of the fungi when compared with corresponding controls. Although no chemical or physical tests were made for the presence of fat, the posssibility existed that one or more ether-soluble compounds, other than fat, were responsible for supporting mold growth.

It was observed that the protein and mineral fractions supported less fungal growth than the carbohydrate and ether-soluble components. Since excellent fungal growth can be obtained from a medium composed of 5% feces in a basal solution containing 20 g of glucose per liter, two possible explanations suggest themselves. First, that by removing the protein from the feces, a growth factor was separated from the protein during the process; and second, it may be that forms of nitrogen in feces other than protein nitrogen serve as the nitrogen requirement to support the growth of mold on a complete feces-carbohydrate medium.

Observation suggested that some constituent normally present in feces was absent in the medium supplied with fecal ash. It can be noted from Table 4 that corresponding controls were also incapable of supporting fungal growth. This occurrence may be attributable to the breakdown of urea or the inability of the molds to utilize it as a source of nitrogen.

From these data, the most significant factor deduced was that the best growth and metabolism of fungi can be obtained on normal feces supplemented with carbohydrate.

The data in Table 5 show that the fecal component described as carbohydrate supported growth and metabolism to a somewhat greater magnitude than did the ether-soluble fat component or the protein and mineral components of feces. It was most important to note that the fecal carbohydrate supported growth as well or better than the control containing glucose. This seemed to indicate that growth factors, possibly vitamins and other water-soluble components, necessary for mold metabolism were being added along with the carbohydrate component. However, it did appear that the fecal carbohydrate component was being utilized as the sole source of carbon, based on the fact that this substrate provided better growth than the glucose in the control, indicating the presence of other carbon sources. Thus, the indication was that other carbon sources utilized by the molds were added with carbohydrate preparations.

Fecal Protein as a Source of Nitrogen for Fungal Growth

A study was designed to compare the nutritive value of lead-acetate precipitated fecal protein with the values of ammonium nitrate and amino acid nitrogen as the sole sources of nitrogen for the growth of *A. niger* and *R. delemar*.

Table 5. Value of Fecal Components for Growth and Metabolism of Two Molds

Fecal components assayed	Mycelial dry weight			
	A. niger, g	Control, g	*R. delemar*, g	Control, g
Carbohydrate	1.690	1.028	1.191	0.644
	1.598	0.974	1.118	0.759
Fat	0.527	0.027	0.805	0.042
	0.955	0.012	0.427	0.064
Protein	0.019	0.076	0.007	0.228
	0.023	0.050	0.027	0.064
Mineral (ash)	0.037	0.000	0.100	0.000
	0.036	0.000	0.195	0.000

The fecal-protein nitrogen described earlier was added to 200 ml of basal medium at three levels, 2, 5, and 10 g (wet weight), constituting 1, 2.5, and 5% solutions, respectively. The mineral medium employed was prepared according to Czapek's formula.

Two sets of controls were used, ammonium nitrate as the inorganic source and an amino acid mixture as the organic source. In the case of ammonium nitrate, 1, 2.5, and 5% levels were prepared. From the amino acid mixture containing 0.455 mg per ml of each amino acid, 1, 5, and 10 ml were added to basal medium and sterilized. The three levels of each substrate were prepared in duplicate. The inoculations were made with 1 ml of a spore suspension containing 30 spores per ml of *A. niger* and 70 spores per ml of *R. delemar*. Flasks were incubated on a rotary-type shaker operated at 98 cycles/min and at a temperature of approximately 24-26 C for a period of 5 days. Following this, the mold was removed by filtering onto weighed filter paper and dried in a 55 C oven overnight. After drying, samples of mold were reweighed. The ability of fecal protein to serve as the sole source of nitrogen as compared with an amino acid mixture and ammonium nitrate was measured in terms of the dry weight of mycelium produced.

The data in Table 6 show that fecal nitrogen was superior to the amino acid mixture but somewhat inferior to ammonium nitrate as the sole source of nitrogen for the growth of the test molds. It was also noted that, in the case of fecal nitrogen and ammonium nitrate, higher levels of the nitrogen source supported better growth of the mold in each case. The results show very little variation among duplicates.

These results, it would seem, may form a basis for explaining the termination of mold growth in fecal substrates adequate in carbohydrates. The protein nitrogen apparently becomes depleted and, since feces have been found deficient in nitrates (Air Force Report No. 7, 1961), growth terminates. This reasoning may be plausible considering the high growth yield with nitrate as sole sources of nitrogen. Fecal-protein nitrogen and inorganic nitrogen were not used in upper limits of concentration; thus, maximum growth was not observed in these trials because concentration was the limiting factor.

These trials definitely show that fecal nitrogen supported the growth of *A. niger* and *R. delemar* ; however, better growth yield may be obtained by supple-

Table 6. A Comparison of Fecal Nitrogen with Ammonium Nitrate and Amino Acid Mixture as the Sole Source of Nitrogen for Mold Growth

Type of nitrogen supplied	Organism	Mold growth, dry weight of mycelia, g		
		Level 1	Level 2	Level 3
Fecal-protein nitrogen	*Aspergillus*	0.051	0.049	0.171
		0.020	0.042	0.122
	Rhizopus	0.004	0.040	0.131
		0.003	0.055	0.124
NH_4NO_3	*Aspergillus*	0.636	1.317	1.482
		0.350	0.994	----*
	Rhizopus	0.773	1.244	1.359
		0.801	1.307	1.361
Amino acid mix	*Aspergillus*	0.008	0.048	0.062
		0.007	0.049	0.071
	Rhizopus	0.008	0.039	0.077
		0.007	0.046	0.109

*Lost sample.

menting the fecal protein nitrogen with ammonium nitrate nitrogen. Furthermore, it appeared that amino acids were not able to support mold growth. However, this occurrence may not agree entirely with results reported in the literature (Cochrane, 1958). At best, there is little agreement among other workers concerning the utilization of amino acids as sole sources of nitrogen by molds.

The results of these experiments have shown that the cellulose content of human waste may be made available, through enzymatic hydrolysis, to molds as sources of carbohydrates. It has been further established that one of the critical factors limiting the growth of selected fungi on fecal substrate was concerned with the availability and supply of suitable carbohydrates.

In addition to a deficiency of carbohydrate in feces, data showed that fecal-protein nitrogen was not as good a source of nitrogen for fungal growth and metabolism as ammonium nitrate nitrogen. It appeared that this occurrence may explain the termination of mold growth even in the presence of an adequate carbohydrate source. This suggested that improved yields of mold growth might be obtained by supplementing feces with ammonium nitrate nitrogen.

Although surprising, these results appeared to indicate that the mineral component of feces was unable to satisfy the mineral requirement of certain fungi when grown in what was believed otherwise to be an adequate medium.

The data indicated that feces *per se* supported better growth of molds than did carbohydrate, fat, protein, and mineral fractions used separately.

The research progress reported substantiates the opinion that feces are able to meet the several nutritional requirements of the molds employed in microbial synthesis, and demonstrates the practical potentiality for microbial synthesis employing selected molds.

The several treatments afforded feces in these tests indicated that the efficiency of the chosen molds for converting human feces into microbial protoplasm can be improved by supplementing feces with certain forms of nitrogen, carbon, and, possibly, vitamins.

Tests are currently in progress which are a prelude to chicken feeding trials in which the nutritive value of molds grown on human feces will be ascertained.

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Quantitative Measurement of Urinary Nitrogen and Total Solids Depletion in a Closed Ecological System During Microbial Synthesis[1]

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A series of experiments were run to investigate the depletion and metabolic fate of urinary nitrogen and total solids constituents of urine during microbial synthesis.

Tests were designed to ascertain the nitrogen contribution afforded by the different individual nitrogen constituents of urine during the fermentation process. Quantitative measurements for the allantoin, amino-acid, creatinine, hippuric-acid, urea, and uric-acid contents of urine were made periodically throughout the course of the fermentation. Having established the utilization of urinary nitrogen constituents by either mold or yeast species, their metabolic fate was determined by radioisotope-tracer analysis.

The efficiency of converting urinary nitrogen into microbial nitrogen was measured in terms of reduction in total nitrogen and other heretofore mentioned nitrogen constituents, economic coefficient and conversion coefficient.

Data will be presented to show that urea contributes the major source of nitrogen during fermentation of urine by selected molds and yeasts. The total solids of urine can be reduced by half in the fermentation process.

This study has indicated that human urine provides an excellent substrate for microbial synthesis of cellular materials. A process of "double and triple" semicyclic fermentation may reduce the constituents of urine to the extent that it becomes stabilized to the point suitable for drinking purposes.

A SUCCESSFUL closed ecological system will be able to provide man with a source of food, water, and oxygen and at the same time eliminate the problems of waste disposal.

Since human urine is known to be rich in several physiologically active end-products of metabolism, and because of the problem of its disposal in outer space, aerobic fermentation tests were made to ascertain the feasibility of employing urine as a growth substrate for selected microorganisms. These tests were designed to provide a measure of the stabilization of urine as related to the reduction of total nitrogen and total solids.

The present paper discusses the depletion and utilization of urinary nitrogen and solids by selected molds and yeasts in an aerobic fermentation process.

Preliminary tests revealed that urine contains very little, if any, carbohydrate material. In order to obtain maximum growth of molds and yeasts, it was necessary to add a usable source of carbohydrate.

The areobic fermentation process was conducted in either a large pyrex-glass column (9 liter capacity) measuring 9.6 × 120 cm or 2 liter Fernbach flasks. The columns

[1] This work was done in cooperation with the National Aeronautics and Space Administration, Washington, D. C.

were fitted with two air jets which provided aeration and liberal agitation. Flask cultures were incubated on a rotary shaker which had 64 cycles per minute. All cultures were incubated at room temperature (25° ± 3°C).

The substrate consisted of fresh, composited urine of human origin employed at its normal strength. The urine was either pasteurized by boiling 10 minutes or given no heat treatment prior to use.

The pH of the urine medium was adjusted, respectively, to 4.0 and 6.5 without and with pasteurization.

Cultures of *Aspergillus niger* and *Rhizopus delemar* were prepared in the form of a spore or pregerminated spore suspension in phosphate buffer solution.

Eighteen-hour cultures of washed *Torula utilis* cells were prepared for fermentation trials employing yeast.

Both the mold and yeast inocula were used at the 10% level of the entire culture medium.

Analyses made on unfermented and fermented urine in the course of investigation were: 1) glucose; 2) total nitrogen and solids; 3) conversion and utilization of amino acids, creatinine, urea, and uric acid; and 4) the production of carbon dioxide. The growth of mold and yeast species on urine was evaluated in terms of dry weight.

Methods used separately or in combination to evaluate the foregoing components made use of titration, colorimetric, or chromatographic techniques.

The potassium permanganate titration procedure (A.O.A.C., 1950) was used to determine glucose.

Total nitrogen was measured by the Kjeldahl method described by Hawk, Oser, and Summerson (1954). Total solids were determined by the method of Cody, Moore, and Tischer (1962). The amino acid content of urine was determined by paper chroma-

TABLE 1. *Urinary Nitrogen Reduction by* Rhizopus delemar

Incubation	Duplicate flasks	Total nitrogen (duplicate samples)	
		1	2
Hr	No.	g/l	g/l
0 (initial)	1	8.90	8.70
	2	10.10	10.22
24	1	8.70	8.10
	2	8.60	8.60
48	1	5.30	5.80
	2	6.40	6.60
72	1	5.10	6.00
	2	6.60	6.90
Total Reduction (%)		38.67	31.75

TABLE 2. *Urinary Nitrogen Reduction by* Torula utilis

Sample	Incubation	Urine volume	Total urinary nitrogen	Reduction in urinary nitrogen
	Hr	liter	g/l	%
A	0	2	6.58	0
	48	2	5.04	23.40
4 liters of 50%-strength fresh urine + 1 liter of A				
B	0	5	5.90	0
	48	5	4.60	23.03
4 liters of 50%-strength fresh urine + 2 liters of B				
C	0	6	4.96	0
	48	6	4.59	7.46

tography (Block, Durrum and Zweig, 1958). Creatinine levels were measured by Folin's colorimetric procedure (Hawk et al., 1954). The colorimetric procedure described by Benedict and Franks was used to measure uric acid (Hawk et al., 1954).

The utilization of total urinary nitrogenous constituents was determined by measuring the reduction in total nitrogen at various intervals during fermentation.

Fernbach flasks containing 1 liter of fresh composite urine were inoculated with a 1 ml heavy spore suspension of *Rhizopus delemar*.

Column culture vessels were employed to investigate urinary nitrogen reduction by yeast cells (*Torula utilis*). The urine medium was inoculated with 200 ml of a washed 18-hour culture of *Torula utilis*. After 30 minutes, a sample of culture was drawn for nitrogen determination. This sample represented the initial or 0-hour nitrogen reading. Incubation was continued for 48 hours with liberal areation at which time a second (48-hour reading) sample was taken. This set of samples was designated as Series A. Following this, 1 liter of the culture was drawn off, and 4 liters of fresh urine were added to the column. When this culture had mixed sufficiently, another sample was taken for nitrogen determination. Since new medium had been supplied to the column, it was necessary to establish a new initial nitrogen level. The incubation was continued for 48 hours before another sample was taken (Series B). A third set of samples was taken from the column which contained 4 liters of fresh composite urine added to 2 liters of the culture remaining from B (Series C). The treatments given C were similar to those for A and B.

Table 1 shows that total reduction in urinary nitrogen by *Rh. delemar* amounted to 38.67 and 31.75% in urine samples 1 and 2, respectively. The greatest amount of nitrogen was utilized between the 24th- and 48th-hour periods in both samples.

Table 2 shows that the total urinary nitrogen reduction by *Torula utilis* was 23.40, 22.03 and 7.46% during the three 48-hour incubation periods designated as A, B, and

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C, respectively. The largest amount of nitrogen reduction occurred in the first and second incubation periods. The lowest was observed in the third 48-hour period.

Rh. delemar was able to reduce the total nitrogen content of human urine by 41.03%. The variation in nitrogen reduction observed between flasks 1 and 2 was attributed to a positioning effect on the shaker.

Flask cultures were more efficient in reducing the total nitrogen content of urine than were column cultures.

Yeast cells were able either to convert or utilize about 22.0% of the total nitrogen of urine during the first 48 hours of fermentation. When incubation was continued through 96 hours, a marked decrease occurred in the total percent nitrogen reduction of the urine. *Rh. delemar* was more efficient in reducing the nitrogen of urine than was *Torula utilis* under the conditions described. This study indicated that nearly half of the total urinary nitrogen can be converted to microbial protein in these fermentation processes.

The nitrogen content of urine is composed of several organic constituents. Of these substances, urea makes up 80-90% of the total urinary nitrogen.

In tests to determine the utilization of urinary urea by *Rh. delemar*, 1600 ml of pasteurized half-strength urine containing 2.5% corn dextrose and having a pH of 6.5 were put into a glass-column culture vessel. After a sample of the medium was taken for urea determination, the column was inoculated with 15 ml of a 24-hour pregerminated spore suspension of *Rh. delemar*. After 24 hours incubation, and at each 24-hour interval for 96 hours, samples were again taken for urea determination. The centrifuged medium was used for the urea measurements. Other tests run in conjunction with urea determinations in a second trial were carbon dioxide production, reduction in glucose, total nitrogen, and total solids. Carbon dioxide was determined by titrating the KOH (1 N) used for absorption with 1 N H_2SO_4 to a bromphenol blue endpoint. It was believed that these tests would give some insight into the metabolism of *Rh. delemar* on a urine substrate.

Table 3 shows that *Rh. delemar* utilized or reduced the urea concentration of urine by a value of 38.75% over a 96-hour period. The most rapid reduction in urea nitrogen occurred in the first 24 hours of fermentation. The free ammonia increased only 0.138 g/l during the entire 96 hours of incubation.

Table 4 shows that the percent reduction in urea nitrogen, total nitrogen, and total solids amounted to 23.49, 40.47 and 25.00, respectively. A total quantity of 26.40 g/l of CO_2 was produced during the 96 hours fermentation.

TABLE 3. *Utilization of Urinary Urea by* Rhizopus delemar

Incubation	Urea nitrogen	Equivalent urea	Ammonia
Hr	g/l	g/l	g/l
0	5.434	11.654	0.132
24	3.792	8.132	0.135
48	3.772	8.089	0.137
72	3.428	7.352	0.202
96	3.328	7.137	0.270

Total urea utilized = 38.75%

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TABLE 4. *The Production of Carbon Dioxide, Reduction in Glucose, Total Nitrogen, Total Solids, and Urea of Urine by* Rh. delemar

Incubation	Urea Nitrogen	Total Nitrogen	Reducing sugar	CO_2 production	Total solids
Hr	g/l	g/l	mg/ml	mg/ml	%
0	3.49	4.2	19.78	0	3.2
24	3.25	4.2	17.00	6.16	3.0
48	2.91	3.8	17.88	15.16	3.0
72	2.63	3.7	12.04	24.31	2.6
96	2.67	2.5	11.52	26.40	2.4
Total reduction %	23.49	40.47	41.76	26.40	25.0

Total mold yield = 6.145 g.

The reducing sugar concentration was diminished by 26.40% (8.26 g/l). For the 96 hours of incubation, the total mold mycelia produced were 6.15 g/l.

In terms of percent reduction, the values of 23.49, 40.47, 41.76, and 25.00 for urea nitrogen, total nitrogen, reducing sugar, and total solids, respectively, were believed to constitute a reasonably efficient reduction in urinary constituents especially nitrogen. These results suggested that *Rh. delemar* was capable of efficiently metabolizing and converting urinary constituents to microbial substances.

Urea control tests show that urease enzyme hydrolysis was capable of measuring the urea content of urine with 95.62% efficiency. Thus, the utility of the enzyme procedure was demonstrated for the determination of urinary urea.

Quantitative tests were made to determine the depletion and utilization of the chief nitrogenous compounds present in normal human urine. The method of analysis were those cited herein. The tests were conducted with both cultures of *Aspergillus niger* and *Torula utilis*. The necessary determinations were made at 24-hour intervals for incubation period of 72 hours.

Table 5 shows that the following percent reductions occurred during the fermentation for cultures of *A. niger* and *T. utilis*, respectively: creatinine — 22.47, 30.35; urea — 25.45, 45.11; uric acid — 55.35, 49.25; total nitrogen — 10.49, 17.15; and glucose — 24.67, 165.15. The data show that there was a higher depletion value obtained with uric acid than with the other nitrogenous compounds by *A. niger*.

The results conclusively demonstrated the potential application of urinary nitrogen compounds for use in closed biological systems.

The amino acid content of urine was determined chromatographically (Block, Durrum, and Zweig, 1958) before and after fermentation by mold and yeast species. The desalting technique of Calmon and Kressman (1957) was used to remove interfering salts.

The criterion used to evaluate the utilization of amino acids was their disappearance from the culture substrate as compared with the initial density after a definite period of fermentation.

To establish that nitrogen was utilized in the course of fermentation, total nitrogen measurements were made in conjunction with the amino acid tests.

TABLE 5. *The Depletion of Urinary Nitrogenous Constituents by Mold and Yeast Species*

Culture	Incubation	Creatinine	Glucose	Ammonia & urea	Urea nitrogen	Ammonia nitrogen	Uric acid	Total nitrogen
Number*	hours	g/l	g/l	g/l	g/l	g/l	g/l	g/l
A(1)	0	1.40	53.10	7.44	7.20	0.24	0.66	7.23
A(2)		1.40	53.10	7.44	7.20	0.24	0.66	7.23
Y(1)	0	1.40	53.10	7.44	7.20	0.24	0.66	7.23
Y(2)		1.40	53.10	7.44	7.20	0.24	0.66	7.23
A(1)	24	1.20	47.40	6.71	6.34	0.37	0.30	7.00
A(2)		1.21	47.90	6.68	6.37	0.31	0.28	7.30
Y(1)	24	1.32	0.00†	5.39	4.80	0.59	0.37	6.65
Y(2)		1.31	0.00†	5.56	4.98	0.58	0.37	6.80
A(1)	48	1.19	40.50	6.12	5.52	0.59	0.27	6.44
A(2)		1.10	41.00	6.12	5.52	0.60	0.29	6.72
Y(1)	48	1.21	56.10	5.22	4.22	1.00	0.30	5.81
Y(2)		2.00	55.80	5.22	3.98	1.25	0.34	5.96
A(1)	72	1.09	40.00	6.10	5.41	0.70	0.29	6.30
A(2)		1.08	40.00	6.00	5.32	0.70	0.30	6.60
Y(1)	72	0.97	34.50	5.36	4.10	1.29	0.34	5.88
Y(2)		0.98	35.20	5.50	3.86	1.68	0.33	6.10
Totals %	A1	22.14	24.67	18.01	24.86	7.00	56.10	12.86
	A2	22.80	24.67	19.35	26.11	7.00	54.54	8.13
	Y1	30.71	82.50	28.00	43.10	12.90	48.50	18.67
	Y2	30.00	82.40	26.10	47.22	16.80	50.00	15.63

A = *Aspergillus niger*.
Y = *Torula utilis*.
* (1) and (2) represent duplicate cultures.
† 100 g/l glucose added.

The amino acids identified in urine were alanine aspartic acid, glycine, histidine, leucine, lysine, and serine.

Actively growing cultures of *Aspergillus niger* and *Rhizopus delemar* were not able to utilize any of the seven amino acids present in the substrate. *Torula utilis* was able to deplete the histidine complement of the urine substrate.

The total nitrogen reduction of the substrate was 21.76, 18.20 and 7.35% for *A. niger*, *Rh. delemar*, and *T. utilis*, respectively.

These results suggested that the test organisms employed preferred other nitrogenous constituents of urine over the amino acids.

A study was designed to measure the reduction or conversion of total solids of urine during aerobic fermentation by *Rhizopus delemar* and *Torula utilis*. Tests run to evaluate this occurrence were specific gravity measurements and the production of mold mycelia and yeast cells.

The relationship between specific gravity (density) and percent urinary solids was established by plotting the specific gravity readings against percent dry weight total solids. This represented a standard curve and was used to estimate the total solids content of urine.

One trial run was conducted in 2-liter Fernbach flasks for two 72-hour periods. To

enhance mold growth, 2.5% corn dextrose was added. The second run was made in a pyrex-glass column for three 48-hour periods. Since 10% glucose was added, it became necessary to determine the percent total solids by dry weight determinations.

The results of these tests (Table 8) showed that the total solids of urine was reduced by 20.0 and 36.0% during the first and second 72-hour incubation periods respectively. The growth of *Rhizopus delemar* was slightly higher in the first 72-hour period. In the second trial, the yeasts reduced the total solids about equally in each of the three incubation periods.

The reduction of total urinary solids occurred simultaneously with depletion in added carbohydrate. The confounding of these two factors prevented us from ascertaining the absolute reduction in total urinary solids. The metabolic relationship between reduction of glucose and solids was believed to be favorable enough to permit the determination of total solids utilized during aerobic fermentation by mold and yeast species.

The results of this study suggested that the total solids content of urine can be reduced appreciably by a process of aerobic fermentation. This suggested that urine can be appreciably stabilized in this process.

This investigation demonstrated that urinary components can be converted to microbial materials in an aerobic fermentation process by mold and yeast species. The results showed that it is possible to deplete 50% of the uric acid complement of urine on fermentation. Furthermore, it was feasible to reduce the concentration of most of the urinary nitrogenous constituents by 20 to 40% under the experimental conditions employed.

Future research efforts will be directed toward converting 80 to 90% of the nitrogen constituents of urine to microbial substance applicable for space travel.

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CHAPTER 23

Preliminary Studies on the Extracellular Products of *Hydrogenomonas eutropha*

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This paper describes the detection and identification of extracellular products formed during the autotrophic growth of *Hydrogenomonas eutropha* on Repaske's medium. Using carbon-14 tracer studies to determine the quantity of extracellular products formed, it was found that about 2% of the total activity appeared in the supernatant liquid during the log phase of growth. As the stationary phase of growth was reached, the activity increased to about 5% of the total. Paper chromatography in conjunction with radioautography was used for separation and identification of the products. Ribose, glutamic acid, alanine, and tyrosine are among the extracellular products which have been identified.

One of the most promising methods of supplying oxygen in a closed ecological system is the production of oxygen by the electrolysis of water. For this system to be applicable for long periods of time, the hydrogen produced during the electrolysis must be recycled by conversion into some reuseable form. At present, one of the most feasible methods of converting hydrogen into a non-explosive reusable form employs the use of organisms of the genus *Hydrogenomonas* (Lechtman, Goldner, and Canfield, 1964).

Hydrogenomonas eutropha is currently being studied by a number of workers to determine the feasibility of using this organism in continuous culture for the conversion of hydrogen to water and cellular material. This organism is capable of growing either autotrophically using hydrogen as the source of energy or heterotrophically with organic material as the energy source. If this organism secretes large quantities of extracellular organic compounds into the medium during autotrophic growth, and if this medium is recycled through the continuous culture system after removal of the cells and addition of inorganic nutrients, the organism may preferentially grow heterotrophically rather than autotrophically, thus affecting the rate of hydrogen utilization. Therefore, the purpose of this investigation was to determine the quantity of extracellular products formed by *Hydrogenomonas eutropha*, and, to identify some of the compounds formed.

The culture employed in this investigation was kindly supplied by Dr. L. Bongers of the Space Science Division of the Martin Marietta Company.

Carbon-14 tracer studies were performed to study the secretion of extracellular products during the growth of *H. eutropha*. These studies employed Repaske's medium (Repaske, 1962) (with the deletion of $NaHCO_3$) buffered at pH 7.0 and a gas mixture composed of 70% H_2, 20% O_2, and 10% CO_2. Carbon-14 labeled CO_2 was incorporated as part of the 10% CO_2.

One hundred milliliters of the medium were inoculated with 1 ml of a heavy suspension of autotrophically grown *H. eutropha* cells and placed in a sterile 250 ml culture flask. The flask was stoppered with a sterile rubber stopper assembly containing a cotton-filled glass bulb to filter the incoming gases. Following evacuation, the flask was attached to the gassing apparatus and filled with the C-14 labeled H_2-O_2-CO_2 gas mixture. The culture was incubated at 30 C and mixed with a magnetic stirrer. Samples

were removed periodically through the serum stoppered sampling port at the base of the flask with a sterile syringe for the determination of radioactivity and optical density.

The optical density was measured in a 1 to 5 dilution of the sample with distilled water on a Beckman DU spectrophotometer at 655 mμ.

The total radioactivity in the cells and medium was determined by placing an aliquot of the sample in a planchet, acidifying with HCl to remove dissolved $C^{14}O_2$, drying, and then counting. For determining the radioactivity in the extracellular portion, the sample was first filtered through an ultra-fine sintered glass filter to remove cells and an aliquot assayed by the above procedure. All counts were made on a gas flow counter and corrected for background.

The radioactivity of extracellular products in the cell free medium amounted to about 2% of the labeled carbon accumulated during the log phase of growth (Fig. 1).

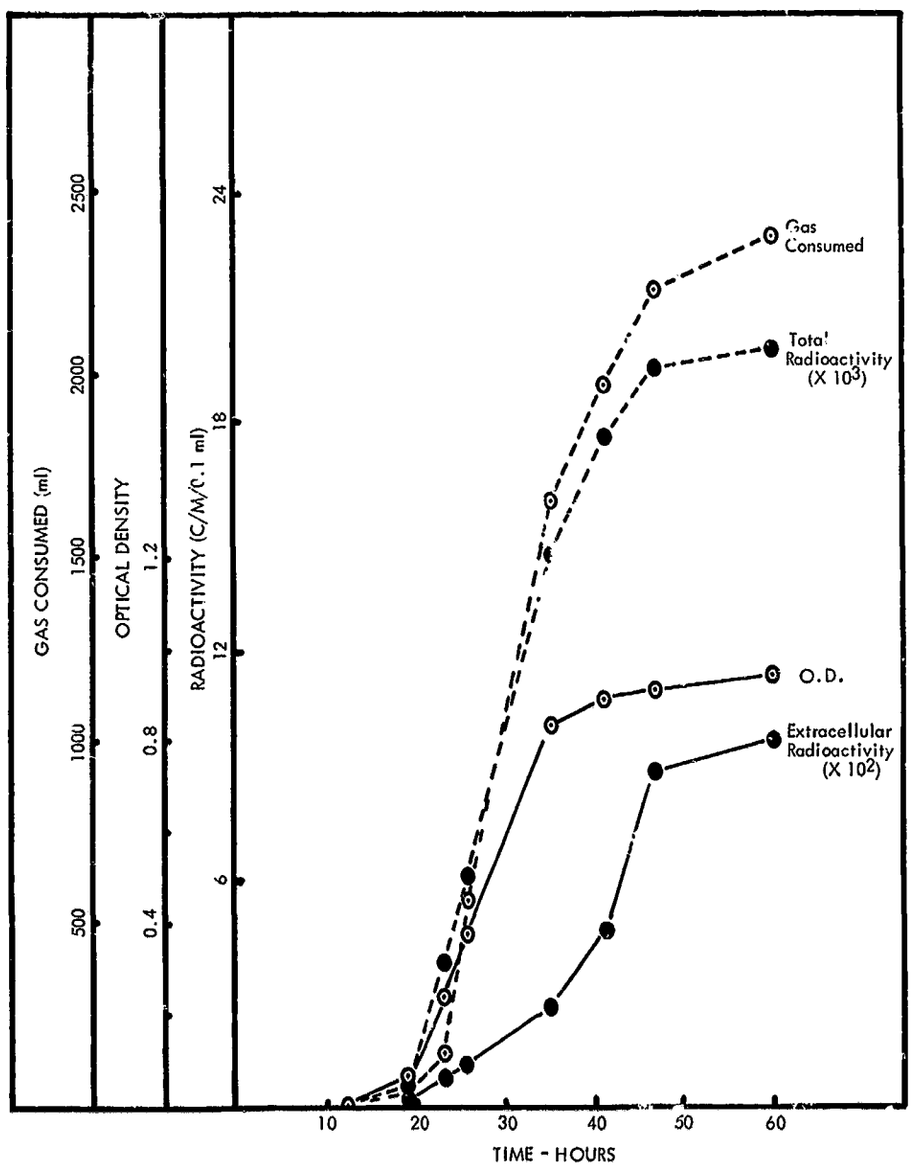

FIG. 1. Production of extracellular material during the growth of *H. eutropha*.

As growth entered the stationary phase as indicated by the optical density, the radioactivity of the extracellular products increased to about 5% of the total. However, the increase in radioactivity in the cell free medium subsequently leveled off.

In a continuous culture, the culture is normally held in the late log stage of growth where there are a maximum number of cells which are multiplying at the fastest rate. At this point in a continuous culture of *H. eutropha*, about 2% of the total carbon would be present as extracellular material. Therefore, in the following studies to determine the nature of the extracellular products produced by *H. eutropha*, growth was always terminated in the late log stage, and the cells immediately removed from the medium by centrifugation and filtration.

For isotopic studies the cultures were grown in 50 ml batches using the aforementioned apparatus and procedure. Most of the cells were removed by centrifugation at 4000 RPM for 30 minutes and then the remaining cells were removed by passing the medium through a Seitz filter.

In non-isotopic studies, the cultures were grown in liter amounts in Fernbach flasks on a reciprocal shaker at 30 C. Repaske's medium was always employed along with a gas mixture of 70% H_2, 20% O_2, and 10% CO_2. Most of the cells were removed from the medium with a Sharples centrifuge and then the medium was filtered through a Seitz filter. The medium was concentrated *in vacuo* on a rotary evaporator to about 1/20 of the original volume.

The cell free medium on which *H. eutropha* had grown was very light yellow in color. Upon concentration, the medium became a deep yellow color. The color could not be extracted from the medium with any of a wide range of organic solvents, both polar and nonpolar. However, the color was removed during desalting with a membrane type electrolytic desalter which employed a cation (Permaplet C-20) and an anion (Permaplet A-20) exchange resin membrane. The anion exchange membrane became discolored with the yellow material, thus indicating that the ion(s) or molecule(s) responsible for the color was negatively charged. However, the colored material was also retained on both Dowex 2 and Dowex 50W ion exchange columns and could be eluted from either with HCl.

Qualitative tests for carbohydrates, amino acids, and proteins were made on the medium both before and after concentration. All tests were negative on the medium before concentration. On the concentrated medium, the anthrone test for carbohydrates (Morris, 1948) was positive; Somogyi's test for free sugar (Nelson, 1944) was positive, and the ninhydrin test for amino acids (Feigl, 1960) using a citrate buffer to eliminate interference from the ammonium salts was positive. The Biuret test for protein (Mehl, 1944) was negative.

After desalting the concentrated cell free medium in the electrolytic desalter, the tests for carbohydrates and free sugars were positive, but the intensity of the color reactions was diminished considerably. The test for amino acids was negative.

Since these tests indicated a loss in extracellular material during desalting, studies were made to determine the extent of this loss. One and one-half milliliters of C^{14} labeled extracellular products containing 15470 C/M/ml were placed in the desalting chamber and desalted using a maximum voltage of 10 volts. After the current dropped to a minimum, the sample was removed and assayed for radioactivity. By desalting, the activity was decreased to 1200 C/M/ml. This was a loss of 92.2% of the radioactivity from the sample. There was no apparent loss in volume of the sample. Thus approximately 8% of the material was not charged and was not removed by the electrolytic desalting.

Ion exchange resins were used to determine the ionic nature of the materials in the extracellular products. Columns of ion exchange resins were prepared using Dowex 2 anion exchange resin in the hydroxyl form and Dowex 50W cation exchange resin in the hydrogen form. The mixed resin employed an equal mixture of Dowex 2 and Dowex 50W. The total capacity of each column was at least twice the value of the milliequivalents of salt in solution The samples were loaded on the columns and washed with 5 column volumes of distilled water. The effluent from the columns was then assayed for radioactivity (Table 1). Since the Dowex 2 column absorbed 90.0% of the total radioactivity and the Dowex 50W column absorbed 58.8% of the total radioactivity, it was speculated that much of the extracellular material was amphoteric in nature and would be taken up by either column. When the sample was passed through the mixed resin, 91.3% of the radioactivity was removed. This left 8.7% of the material which was neither anionic or cationic in nature. This figure agreed very well with the value obtained with the electrolytic desalter.

TABLE 1. *Absorption of C^{14}-labeled extracellular products with ion exchange resins*

Sample	Radioactivity not Absorbed by Resin	Radioactivity Absorbed by Resin
	C/M/ml	*Per Cent*
Original	1495	....
Dowex 50W	608	58.8
Dowex 2	150	90.0
Mixed Resin	130	91.3

The first group of compounds studied by chromatography was the free sugars. Since these compounds are nonionic, they were easily desalted with the electrolytic desalter.

On one dimensional chromatograms of the extracellular material, separated with n-butanol:acetic acid:water (4:1:5-vol) and sprayed with the silver nitrate reagent (Trevelyan et al., 1950) several spots developed, one of which was more intense than the others. By spraying a duplicate chromatogram with analine oxalate reagent (Horrocks, 1949), the intense spot was identified as an aldopentose by the color reaction. None of the other spots developed with this reagent, indicating that the other spots were either artifacts or that the sugars were not in sufficient quantities to be detected by the less sensitive analine oxalate reagent. Using two dimensional chromatography, the aldopentose was tentatively identified as ribose.

Labeled extracellular material, desalted with the electrolytic desalter, was chromatographed in two dimensions using n-propanol:NH_4OH:water (6:3:1-vol) in the first phase and n-butanol:formic acid:water (6:1:2-vol) in the second phase. X-ray films were then exposed to the chromatograms for 8 days.

After developing, seven spots were evident on the film (Fig. 2). One of the spots was identified as ribose by comparing Rf values with those obtained on the control chromatogram. Since the chromatographed fraction contained only the uncharged material, some of the other spots appearing on the film were also suspected of being sugars.

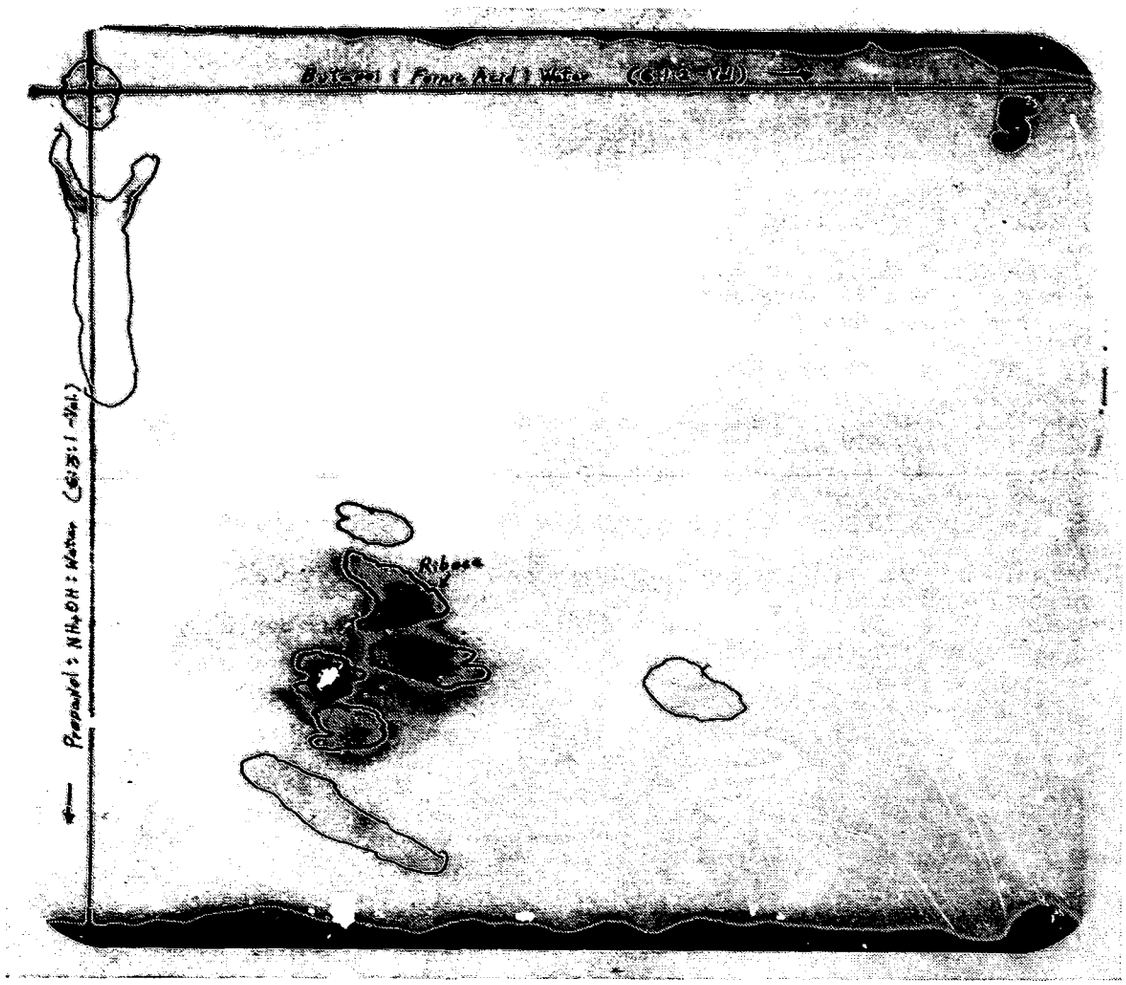

FIG. 2. Radioautogram of the nonionic fraction of the extracellular material formed by *H. eutropha.*

Ion exchange resins were used to desalt the extracellular material for the study of the amino acids (Roberts, 1963) by chromatography. The amino acids were separated with phenol:water (100:39-w:w) and n-butanol:acetic acid:water (2:1:1-vol) and detected by spraying with 0.2% ninhydrin in n-butanol followed by heating for 10 minutes at 75 C.

Of the eight spots which developed on the chromatograms, three were tentatively identified as glutamic acid, alanine, and tyrosine by comparison with control chromatogram.

In summary, it has been found that about 2% of the C-14 labeled carbon taken in by *Hydrogenomonas eutropha* during the log phase of growth appears extracellularly in the culture medium. Approximately 90% of this extracellular material is ionic in nature and is absorbed by ion exchange resins, while the remaining material is apparently non-ionic. Chemical tests indicated that amino acids and carbohydrates were present in the extracellular material. Ribose, glutamic acid, alanine, and tyrosine have been tentatively identified as being components of the extracellular material.

ACKNOWLEDGMENT

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Oxidative Metabolism of Citrate and Lactate by Pseudomonas aeruginosa and Serratia indica

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The oxidative metabolism of citrate and lactate has been investigated in *Pseudomonas aeruginosa* and *Serratia indica.* Respirometry and radiorespirometry studies have revealed that these organisms do not oxidize citrate readily. Tests indicated that lactate is oxidized appreciably, and provides the cell with a source of energy and carbon fragments for cellular synthesis. The inability of these organisms to oxidize citrate has been demonstrated to be related to permeability barriers.

Growth experiments demonstrated that excellent growth of the organisms can be obtained on a substrate containing either citrate or lactate as the sole carbon and energy source.

Studies in this laboratory have shown that human urine contains relatively low concentrations of oxidizable carbohydrate material and thus is not suitable as a growth substrate for many bacterial, mold, and yeast species. On the other hand, urine is known to contain appreciable quantities of citric and lactic acids. The occurrence of these acids in urine led to an investigation designed to determine their ability to replace carbohydrate as the sole carbon and energy source for *Pseudomonas aeruginosa* and *Serratia indica.*

The feasibility of citrate and lactate to replace carbohydrate as the sole carbon and energy source was evaluated by conducting respiration and growth experiments.

The ability of the test organisms to initiate growth on citrate, lactate, and various intermediates of the Krebs tricarboxylic acid cycle was determined by measuring the increase in cell density of cultures.

A washed stock culture suspension of *P. aeruginosa* and *S. indica* was inoculated into 200 ml of a basal medium described in *Difco Manual* (1953) and designated as Bacto-Yeast Nitrogen Base (for carbon Assimilation Tests). The carbon source was added to the basal medium in the form of a filter-sterilized solution to give a final concentration of 1%.

The cultures were incubated at 28 C with shaking. Immediately after inoculation, a 5 ml sample was taken for the zero-hour turbidity reading. The sampling procedure was continued at 24-hour intervals through 72 hours of incubation. The growth response was measured in terms of turbidity on a Coleman Jr. photometer at a wavelength of 430 mμ.

Respiration studies were made by the conventional manometric techniques described by Umbreit, Burris, and Stauffer, (1957). Resting cell suspensions were prepared from 24-hour cultures grown on Difco basal medium containing the desired carbon source.

* This investigation was supported in part by The National Aeronautics and Space Administration, Washington, D. C.

In order to maintain uniformity in respiration the cell density of the cell suspension used in the Warburg cups was adjusted to give 10% light transmittance in the photometer at a wavelength of 430 mμ.

Radiorespirometric experiments were similar to the conventional manometric procedures described except that C^{14}-labeled substrates were used to replace the unlabeled types.

Samples of respiratory $C^{14}O_2$, cells and cell-supernatants were collected for radioactivity measurements on completion of incubation. The usual counting techniques were used to evaluate the distribution of radioactivity. For this purpose, an end-window Geiger tube connected with a count-rate recorder was used. All counts were corrected for background and recorded as CPM (counts per minute).

TABLE 1. *Growth of* Pseudomonas *and* Serratia *on citrate and lactate*

Incubation (Hours)	Organism—Turbidity (430 mμ)			
	P. aeruginosa		*S. indica*	
	citrate	lactate	citrate	lactate
	%T	%T	%T	%T
0	96.5	95.6	95.0	95.0
24	60.0	52.8	33.0	63.0
48	33.0	47.3	27.0	40.1
72	33.5	29.6	29.0	35.6

The results of growth tests are presented in Table 1. The data show that *Pseudomonas aeruginosa* and *Serratia indica* were able to initiate significant growth on a medium containing either citrate or lactate as the sole carbon and energy source. Maximum growth of these cultures was obtained during the first 48 hours of incubation. *S. indica* grew well on all the TCA cycle intermediates when employed as the sole carbon source.

Respiration studies revealed that resting cell suspensions of *P. aeruginosa* and *S. indica* were not able to oxidize citrate appreciably. The data in Table 2 show that lactate oxidation was significantly greater in *S. indica* than *P. aeruginosa*. Furthermore, a substrate consisting of both citrate and lactate did not cause *P. aeruginosa* to consume larger quantities of O_2. However, when a resting cell suspension of a combination of both test organisms was employed, the O_2 uptake on citrate was considerably greater than that for either organism alone (Table 3). This occurrence was not observed with the lactate substrate. These results indicate that there existed an interrelationship of *P. aeruginosa* and *S. indica* relative to citrate oxidation.

The results of tests designed to trace the distribution of C^{14}-labeled lactate in growing cultures of *P. aeruginosa* showed that the distribution pattern was as follows: $C^{14}O_2$ – 46.6%, cells – 2.4%, residual in medium – 8.0%, and washings – 8.0%. Approximately 35.6% of the total activity could not be accounted for in these experiments. The results of duplicate cultures gave a similar distribution pattern to those reported.

TABLE 2. *Oxidation of citrate and lactate by resting cells of* P. aeruginosa *and* S. indica

Substrate*	Organism—Total O_2 uptake†	
	P. aeruginosa	*S. indica*
	μl	μl
Citrate	53	40
Lactate	108	392
Citrate + Lactate	115	385

* Concentration of carbon sources, 10 μM.
† The O_2 uptake was corrected for endogenous respiration and represents the total μl/2 hours.

These results show that a larger portion of the lactate metabolized by *P. aeruginosa* was used for energy and to a much lesser extent, cellular synthesis. Based upon these results, it was concluded that *P. aeruginosa* metabolizes lactate primarily as a source of energy.

Data thus far have revealed that *S. indica* is more active oxidatively on lactate than *P. aeruginosa.* For this reason, a more detailed study of lactate oxidation by this organism was made in an effort to demonstrate the pathway of lactate metabolism.

The data in Table 4 show that the μl of O_2 consumed by resting cells of *S. indica* on acetate and citrate were not significant. With all other substrates tested, however, appreciable O_2 uptake was evident. The largest amount of O_2 uptake was observed on lactate and succinate.

These results suggest that the test organism possessed enzymes of the Krebs tricarboxylic acid cycle. The fact that cells grew on citrate while resting cells were unable to oxidize it suggests that a cell permeability barrier was involved in relation to citrate oxidation.

Further tests using frozen cell preparations demonstrated that citrate was oxidized during respiration. This occurrence substantiated the contention that permeability barriers prevent the oxidation of citrate by whole cell preparations of *S. indica.*

TABLE 3. *Oxidation of citrate and lactate by a combination of a resting cell suspension of* Pseudomonas aeruginosa *and* Serratia indica

Substrate	Organism		
	P. aeruginosa	*S. indica*	*P. aeruginosa* + *S. indica*
	μl	μl	μl
Citrate	41	32	104
Lactate	137	446	311
Citrate + Lactate	145	415	415

TABLE 4. *Oxidation of tricarboxylic acid cycle intermediates by lactate-grown cells*

Substrate	Total O_2 uptake*
	μl/2 hours
Acetate	22
Citrate	14
Fumarate	66
Lactate	219
Malate	74
Pyruvate	90
Succinate	198

* All values corrected for endogenous respiration.

Tests designed to demonstrate citrate oxidation by cell-free extracts were unsuccessful. The inability of the cell-free extracts to oxidize citrate was attributed to the destruction of the specific enzyme during its preparation, or the absence of co-factors.

Dehydrogenase activity of whole-cell preparations was demonstrated on lactate, succinate, and malate. The activity on succinate and malate indicates that the enzymes necessary for the conversions of succinate to fumarate and of malate to oxaloacetate were present. Demonstration of these reactions is supporting evidence that the cells possessed a TCA cycle for terminal respiration.

Studies on inhibition of dehydrogenase activity of lactate-grown cells on lactate and pyruvate revealed that lactic acid dehydrogenase activity was inhibited by arsenite. This occurrence indicates that lactate is oxidized via pyruvate and excluded the possibility of a reaction via acetate.

The results of these tests provide adequate evidence that lactate is oxidized initially to pyruvate and subsequently via the TCA cycle.

Radioactivity measurements employing lactate labeled 1-C^{14} and 2-C^{14} suggest that the carboxyl (1-C^{14}) radical of lactate is oxidized to $C^{14}O_2$ whereas the 'active' acetate (2-C^{14}) fragment is distributed in the cells. These data indicated that in *S. indica* an approximately equivalent use is made of lactate for energy and cellular synthesis.

Since the TCA cycle is known to provide many organisms with a source of carbon fragments for cellular synthesis, it appears that lactate is metabolized via the TCA cycle.

The present investigation has presented data on the growth and oxidative metabolism of *P. aeruginosa* and *S. indica*. The results indicate that lactate, and indirectly citrate, is oxidized via the TCA cycle.

Also data are presented showing that the inability of resting cells to oxidize citrate can be attributed to cell permeability barriers.

The results of this investigation form a basis for evaluating the potential use of *P. aeruginosa* and *S. indica* in systems in which the sole carbon and energy source consists of either citrate or lactate.

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BIOSYNTHESIS OF EXTRACELLULAR POLYSACCHARIDES BY THE BLUE-GREEN ALGA ANABAENA FLOS-AQUAE

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Abstract

Extracellular polysaccharides were isolated from the blue-green alga *Anabaena flos-aquae* strain A-37. The polysaccharides are composed of glucuronic acid, glucose, xylose, and ribose in a molar ratio of 1:88:39:3. The extracellular polysaccharides comprise about 40% of the total carbohydrate produced by this alga.

Carbon utilization experiments revealed that only D-fructose could be substituted for carbon dioxide as a precursor of polysaccharides and cellular material.

The extracellular polysaccharides are derived from water-soluble intracellular polysaccharides of the same composition.

Fructose accumulates in stationary phase cells grown in CO_2 and the presence of the enzymes fructose diphosphate phosphatase and fructose diphosphate aldolase was demonstrated. Tracer studies showed the presence of phosphorylated compounds common to the photosynthetic carbon reduction cycle and the glycolytic pathway.

Introduction

The study of extracellular polysaccharides produced by freshwater algae has been concerned with the isolation of the polymers and identification of their constituents as shown by a number of workers (4, 11, 12, 14).

The uptake of C^{14} label from $NaHC^{14}O_3$, acetate-1-C^{14}, acetate-2-C^{14}, and formate-C^{14} into the intracellular polysaccharide of *Nostoc muscorum* was followed by Biswas (5). No previous communication could be found which described the kinetics of carbon dioxide fixation into extracellular polysaccharides or the intermediary pathway describing the synthesis of extracellular polysaccharides by the freshwater algae.

In a previous report (15) we showed that *A. flos-aquae* A-37 produces abundant polysaccharide material. The production of the extracellular polysaccharides by this alga follows cell growth (15), which is in contrast to the liberation of extracellular carbohydrates by some marine algae (7) and is similar to production patterns for *Palmella mucosa* (25) and *Chlamydomonas parvula* (12). The composition of the extracellular, capsular, and intracellular polysaccharides was shown to be glucuronic acid, glucose, xylose, and ribose (15).

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In this paper, we are reporting primarily on the nutrition relating to polysaccharide production.

Materials and Methods

Culture

The blue-green alga, *Anabaena flos-aquae* strain A-37, was isolated in pure unialgal culture in this laboratory. Bacterial contaminants were separated from the algal culture by a micropipette manipulation – enrichment technique (Tischer (26)).

The absence of contaminating bacteria in both the stock culture and cultures used throughout the project was checked by placing a 1-ml inoculum of algal culture in 10 ml of nutrient broth. These inoculated tubes were incubated under conditions identical with those for growing the algae for 48 hours. If, at the end of this incubation period, the nutrient broth tubes remained clear and contained no growth, the culture was termed "bacteria free."*

The removal of bacterial strains associated with the original culture did not hinder the propagation of the algal culture. This alga is different from most blue-green algae in that the optimum temperature for growth is 40 °C.

Growth Medium and Cultural Conditions

The alga was grown in modified Knop's mineral medium (19) at a pH of 7.0. Cultures were contained in Pyrex columns (1.5 liter capacity) and 200-ml prescription bottles. Other culture conditions have been previously described (15, 25). Inocula were always taken from mineral salts agar slants.

Carbon Source Studies

Carbon sources were added to the basal medium at a concentration to give 2 mg of carbon per milliliter of medium. The solutions were sterilized by Seitz filtration. Sodium salts of lactate, acetate, pyruvate, and related acids were used in all experiments. Experimental cultures were incubated for 5 days to evaluate the carbon sources.

In experiments involving the metabolism of C^{14}-labeled metabolites, these compounds were added at a concentration of 1 microcurie per milliliter of the basal medium or $M/15$ phosphate buffer. Unlabeled compounds were added at a level of 1 mg/ml.

Tracer experiments which involved C^{14}-labeled carbon dioxide were conducted in a gas-recycling apparatus designed with appropriate absorbers, generator tubes, and air pumps.

Radioisotopes and Chemicals

All radioisotopes and chemicals were the best commercial products available. $BaC^{14}O_3$ was converted to $Na_2C^{14}O_3$ by liberation of $C^{14}O_2$ with perchloric acid followed by the adsorption of the $C^{14}O_2$ in 1 N NaOH. Radioactivity measurements were made with a gas flow G-M detector.

Polysaccharide Determination, Isolation, and Hydrolysis

Extracellular polysaccharides in culture filtrates were determined by the anthrone method of Snell and Snell (24). Glucose was used as the reference.

*This alga was identified by Dr. G. W. Johnston, Head of the Botany Department at Mississippi State University, using the book Fresh-Water Algae of the United States by G. M. Smith. McGraw-Hill Book Company, New York. 1950.

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Extracellular polysaccharides in culture filtrates were isolated from growth media and phosphate buffer solutions by concentration of the cell-free medium or buffer to 1/10 volume under reduced pressure. The polysaccharides were then precipitated with 2 volumes of absolute ethyl alcohol. A fraction designated capsular polysaccharide was extracted as described by Lewin (12). Water-soluble intracellular polysaccharides were extracted by subjecting cell suspensions to sonic vibrations in a Raytheon 10 kc sonic oscillator for 10 minutes, centrifuging, and precipitating soluble protein, pigments, and nucleic acids with cold 10% trichloroacetic acid. The intracellular polysaccharides were then precipitated with 2 volumes of absolute ethyl alcohol. The ethanolic supernatant was concentrated to 0.1–5.0 ml under reduced pressure to obtain free carbohydrates. Repeated ethanolic precipitation and dialysis in cellulose tubing for 24 hours under tap water was used to purify polysaccharide samples for chromatographic analyses. The stringy polysaccharide precipitates were collected in alundum crucibles for weight determinations.

All polysaccharide and cell residue samples were hydrolyzed with 1 *N* H_2SO_4 (1 ml acid per 5 mg sample) in sealed glass ampoules for 6 hours at 100 °C. Hydrolysates were neutralized with $BaCO_3$.

Chromatographic Procedures

Paper chromatographic separation of monosaccharides and phosphorylated intermediates involved the use of the following solvent systems: phenol–water (100–39, w/w), *n*-butanol – propionic acid – water (4–1–5, v/v/v) and *n*-butanol – acetic acid – water (2–1–1, v/v/v). Monosaccharides and derivatives were detected on the chromatograms using aniline oxalate (10), orcinol (6), and ammonium molybdate (1). The elution of monosaccharides from chromatograms was performed by the method of Hawthorne (9).

The molar ratio of the extracellular and intracellular polysaccharide constituents was determined by elution of the polymer spots from chromatograms and colorimetric estimation using the modified Somogyi reagent (17).

Enzyme Assays

Phosphofructokinase was determined by the production of triose from fructose-6-phosphate and adenosine triphosphate (22). Fructose diphosphate phosphatase was measured by the liberation of inorganic phosphate from fructose diphosphate (20). Triose formation from fructose diphosphate was the indicator for aldolase (22). Phosphohexoisomerase was determined by the formation of fructose-6-phosphate from glucose-6-phosphate (23). *Anabaena flos-aquae* cells were grown in 200-ml prescription bottles containing the basal medium with appropriate carbon sources. The cells were harvested after 3 days and washed with phosphate buffer. One half of each set of cells was used for the whole cell assay procedure. The other half of the cell suspension was subjected to sonic vibrations in a Raytheon 10 kc oscillator for 2 minutes. Whole cells and sonic extracts were added to the incubation mixtures at the same concentration of protein per milliliter.

Photosynthetic Intermediates

Intermediary compounds formed during photosynthesis in $C^{14}O_2$ were separated by the method of Benson and Calvin (3).

Protein

Soluble cell protein in sonic extracts was estimated using the method of Lowry *et al.* (13).

Results

Polysaccharide Composition

Paper chromatographic analysis of the polysaccharide hydrolysates showed the constituents to be glucuronic acid, glucose, xylose, and ribose (15). Tests performed by the method described by Lowry *et al.* (13) for amino compounds and keto sugars were negative. Analyses of spot eluates showed the molar ratio of glucuronic acid:glucose:xylose:ribose to be 1:88:39:3. The capsular and intracellular polysaccharides were subjected to the same chromatographic separation. The carbohydrate composition was exactly the same.

Carbohydrate Distribution

The distribution of carbohydrates was determined with a 5-day, 1.5-liter column culture of the alga to ascertain the relationship of the extracellular polysaccharides to the other carbohydrates of the cells (Table I). Extracellular polysaccharides make up 39.7% of the total reducing sugars or 27.8% of the total organic cell matter produced. Cell residue carbohydrates are the only fraction higher than the extracellular polysaccharides.

TABLE I

Distribution of carbohydrates in different cell fractions (5-day incubation period)

Fraction*	Weight (mg)	Free reducing sugars† (mg)	Free reducing sugars, % of total	Free reducing sugars, % of total organic matter
Extracellular free reducing sugars	—	0.5	0.03	0.02
Extracellular polysaccharides	650.0	560.2	39.7	27.8
Capsular polysaccharides	12.3	10.8	0.8	0.5
Intracellular polysaccharides	239.1	224.8	15.9	11.0
Cellular free reducing sugars	—	13.1	0.9	0.6
Cell residue	722.7	603.0	42.7	30.9
TCA insoluble precipitate	700.0	—	—	29.9
Totals	2324.2	1412.2	100	100

*Cells extracted: 2264.00 mg.
†Free reducing sugars measured as glucose with the Somogyi reagent.

Carbon Dioxide Tracer Studies

The incorporation of $C^{14}O_2$ into polysaccharides (extracellular and intracellular), free intracellular monosaccharides, material insoluble in cold trichloroacetic acid, and non-extractable cell material were followed by exposing cells in $M/15$ phosphate buffer to $C^{14}O_2$ for intervals of 2.5 to 720 minutes (Table II). Intracellular and extracellular polysaccharides are labeled within 2.5 minutes. The increases of C^{14} into extracellular polysaccharides are dependent on the C^{14} incorporated into the intracellular polysaccharides.

Five sugar spots from the ethanolic cell extract were detected on paper chromatograms sprayed with aniline oxalate and orcinol. These included an unidentified spot having a low R_f value, glucose, sucrose, fructose, and xylose. The C^{14} of the above sugars for the period of 2.5–7.5 minutes exposure is

TABLE II

The time course of $C^{14}O_2$ fixation into various culture fractions. Results are expressed in 10^4 c.p.m. $C^{14}O_2$ added per exposure was 8.36×10^6 c.p.m./260 mg cells

Time, min	Fractions (in c.p.m. × 10^{-4})						
	ECE	EP	IP	TCA (insol.)	CR	Total	% fixed
2.5	3.1	0.2	0.4	1.2	0.3	5.2	0.6
5.0	4.7	0.3	0.4	1.0	0.2	6.6	0.8
7.5	6.6	0.3	1.4	2.3	0.3	10.9	1.3
10.0	11.1	0.2	2.7	2.6	1.0	17.6	2.1
20.0	7.9	0.3	2.3	3.0	0.9	14.5	1.7
30.0	25.6	0.9	6.5	10.9	1.4	45.3	5.4
40.0	33.3	1.1	8.9	14.5	3.4	61.2	7.3
50.0	111.5	3.5	24.1	31.5	5.2	175.9	21.0
60.0	86.9	4.9	19.4	69.1	5.5	185.8	22.2
120.0	115.2	6.9	33.3	59.0	7.3	221.7	26.4
360.0	92.2	19.7	32.3	62.3	27.5	264.0	31.6
720.0	154.9	21.5	67.6	64.1	24.4	332.5	39.7

NOTE: ECE = ethanolic cell extract; EP = extracellular polysaccharides; IP = intracellular polysaccharides (water-soluble); CR = cell residues; TCA = trichloroacetic acid.

TABLE III

Changes in $C^{14}O_2$ fixation with time in the ethanol-soluble monosaccharides of *Anabaena flos-aquae**

Time, min	Free monosaccharide C^{14}									
	Unknown		Sucrose		Glucose		Fructose		Xylose	
	c.p.m.	%	c.p.m.	%	c.p.m.	%	c.p.m.	%	c.p.m.	%
2.5	290	23.0	126	10.0	293	23.2	382	30.3	170	13.5
5.0	471	28.3	129	7.7	429	25.7	411	24.7	227	13.6
7.5	489	23.1	177	8.4	703	33.3	504	23.8	240	11.4

*Results are based on 50-μl aliquots from 2 ml of extract from 260 mg cells.

given in Table III. Fructose is soon heavily labeled and the unknown spot and glucose become more heavily labeled with time.

Carbon Source Experiments

Of 16 carbon sources tested the only one which would substitute for carbon dioxide in the synthesis of extracellular polysaccharides and cellular material was D-fructose. None of the common carbohydrates, phosphorylated derivatives, or tricarboxylic acids could replace carbon dioxide.

The incorporation of C^{14} from various labeled organic compounds in the presence of 5% carbon dioxide is given in Table IV. Fructose and sucrose were utilized very efficiently for the synthesis of extracellular polysaccharides, but sucrose was not incorporated into cell material to the degree of fructose. Approximately one-half as much glucose-C^{14} was incorporated into extracellular polysaccharides. Acetate utilization was equivalent to fructose in total cell C^{14}, but only one-fifth of that of fructose in the extracellular polysaccharides isolated from the cell-free growth medium. The high incorporation of citrate-C^{14} into extracellular polysaccharides is unexplained at this time.

Compounds associated with the Kreb's cycle were used primarily for cellular syntheses rather than for the formation of any large amount of extracellular polysaccharides.

TABLE IV

Fixation of C^{14} from labeled organic compounds by *Anabaena flos-aquae* in the presence of 5% CO_2*

C^{14} source	Total cell-C^{14}, % of c.p.m. added	Extracellular polysaccharide-C^{14}, % of c.p.m. added
Acetate-1-C^{14}	44.5	2.2
Acetate-2-C^{14}	40.8	1.6
Sucrose-C^{14} (μl)	11.9	11.8
Glucose-C^{14} (μl)	7.0	6.0
Fructose-C^{14} (μl)	40.1	11.1
Lactate-1-C^{14}	8.9	0.5
Pyruvate-1-C^{14}	26.3	2.9
Fumarate-1-C^{14}	0.5	0.6
Succinate-1-C^{14}	0.7	0.1
Citrate-1,5-C^{14}	0.2	6.5

*Results expressed as percentage of c.p.m. added. Total cell-C^{14} was determined by counting the C^{14} in an aliquot of washed cells. Extracellular polysaccharide-C^{14} was determined on the polysaccharides isolated from the cell-free growth media.

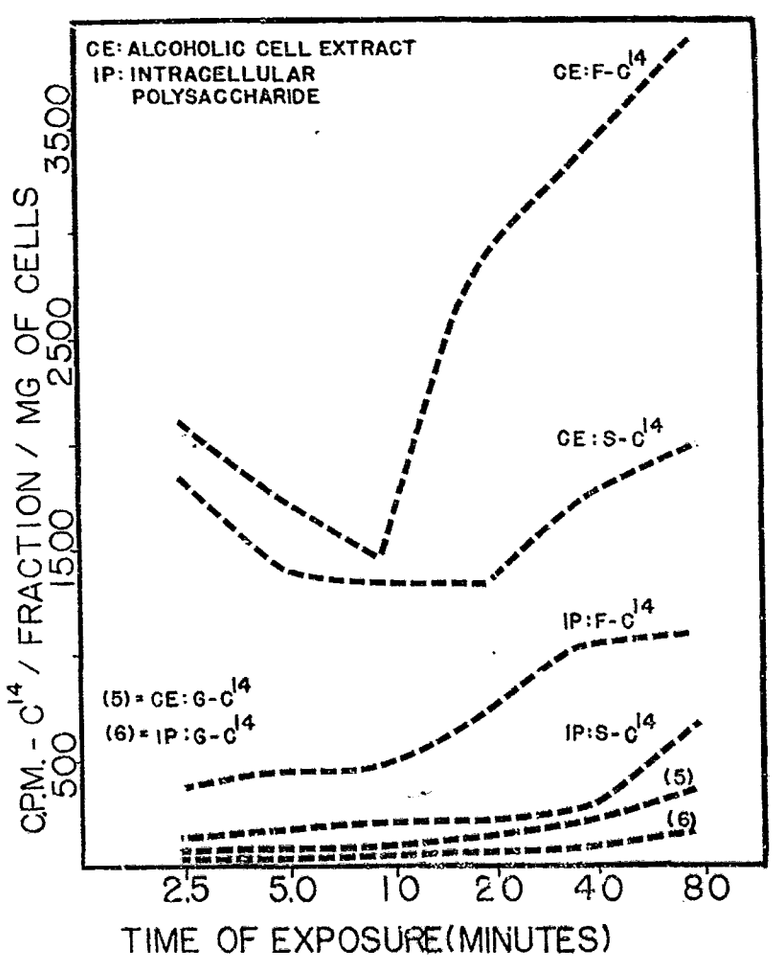

FIG. 1. Metabolism of glucose-C^{14}, sucrose-C^{14}, and fructose-C^{14} by *A. flos-aquae*. (Cells grown in CO_2 were suspended in $M/15$ phosphate buffer and aerated with CO_2-free air during the exposure periods.)

Cells of *A. flos-aquae* were allowed to metabolize unlabeled fructose, sucrose, or glucose and their respective C^{14}-labeled counterparts in the absence or carbon dioxide to determine the relationship of fructose to polysaccharide synthesis. The data in Fig. 1 show that fructose was readily incorporated into intracellular polysaccharides and ethanol-soluble compounds. Sucrose utilization was less rapid than that of fructose, presumably by incorporation of the fructose moiety from sucrose. Glucose utilization for polysaccharide synthesis was very slow by comparison. All of the constitutive monosaccharides of the intracellular polysaccharides were labeled in 2.5 minutes with fructose-C^{14}.

Although the transformation of glucose to polysaccharide material is adaptive and slow, labeling of the constitutive monosaccharides was attempted with glucose-1-C^{14} and glucose-6-C^{14} to determine the origin of the pentose skeleton for ribose and xylose. Studies with glucose-1-C^{14} showed that glucose was split at the number one carbon position to yield $C^{14}O_2$ and unlabeled pentoses. With glucose-6-C^{14}, little $C^{14}O_2$ was produced but the pentoses were radioactive. Metabolism studies with strawberry plants and wheat seedlings showed that carbon six of hexose was cleaved to form polysaccharide pentoses (16, 21).

Enzyme Assays

The carbon source and tracer studies had implicated fructose as a source of polysaccharide precursors. Aldolase activity, which is necessary for the condensation of triose units in the formation of the six carbon skeletons by a reversal of glycolysis, was determined to see if this system was operative under the conditions imposed.

The presence of aldolase activity in *A. flos-aquae* is clearly demonstrated in Table V. Sonic extracts of cells grown in carbon dioxide, fructose, or fructose diphosphate gave positive results for aldolase activity. The experiments were duplicated with appropriate controls each time.

In addition to the presence of aldolase, other enzyme assays showed the presence of phosphofructokinase and fructose diphosphate phosphatase, but the absence of phosphohexcisomerase.

Photosynthetic Intermediates

Some of the intermediates were detected by radioautography and identified by comparison of R_f's with pure compounds. These compounds identified by this method were ribulose-1,5-diphosphate, fructose diphosphate, fructose-6-phosphate, glucose-6-phosphate, 3-phosphoglyceric acid, and sedoheptulose. The free sugars were also present on the radioautograms. The spot which was earlier described as an unknown compound(s) was resolved into four spots when examined by radioautography. This area, on earlier chromatograms sprayed with aniline oxalate and orcinol, which could not be separated into more th[illegible] is explained by the fact that the spots are very small and bunched [illegible] revealed by the radioautograms. The R_f of these spots, whic[illegible] and 0.14 in butanol:propionic acid:water corresponds to p[illegible] the diphosphoglucose. The other compounds in this area [illegible] nucleotide complexes of the polysaccharide constituents

TABLE V

Assays for hexose diphosphate phosphatase, hexose diphosphate aldolase, phosphohexoisomerase, and phosphofructokinase in *A. flos-aquae* cells grown in the presence of 5% CO_2 in air, with and without 1 mg/ml fructose, 1 mg/ml fructose diphosphate, or 1 mg/ml glucose

Additions	Enzyme source	Product (μmoles/mg cell protein)
I. Hexose diphosphate phosphatase (product = P_i or F6P)		
None	Whole cells	3.3
None	Sonic extract	13.5
Fructose	Whole cells	2.9
Fructose	Sonic extract	24.3
Fructose diP	Sonic extract	16.3
II. Hexose diphosphate aldolase (product = triose)		
None	Whole cells	0.2
None	Sonic extract	4.0
Fructose	Whole cells	0.7
Fructose	Sonic extract	3.9
Fructose diP	Sonic extract	4.7
III. Phosphohexoisomerase (product = F6P)		
None	Whole cells	0.03
None	Sonic extract	0.06
Glucose	Whole cells	0.05
Glucose	Sonic extract	0.08
IV. Phosphofructokinase (product = triose)		
None	Whole cells	1.5
None	Sonic extract	4.7
Fructose	Whole cells	9.3
Fructose	Sonic extract	19.6

or their derivatives. It is known that sugar–nucleotide complexes are responsible for the transfer of simple monosaccharides into polysaccharides (8).

Discussion

The extracellular polysaccharides of *A. flos-aquae* are major end products of photosynthesis. These polysaccharides are composed largely of glucose and xylose with minor amounts of glucuronic acid and ribose. Water-soluble intracellular polysaccharides were isolated which had the same identity as the extracellular polysaccharides. The sequence of $C^{14}O_2$ incorporation into the extracellular and intracellular polysaccharides suggests that the polymer is synthesized intracellularly, diffuses through the cell wall, and subsequently sloughs off into the surrounding medium.

Experimental results were obtained which showed that D-fructose was an excellent substitute for carbon dioxide in the synthesis of extracellular and intracellular polysaccharides. The enzymes needed for the reversal of 3-phosphoglyceric acid to fructose phosphates were present. The photosynthetic intermediates that were isolated suggest that *A. flos-aquae* fixes carbon dioxide by the cycle described by Bassham and Calvin (2) for green algae and Norris *et al.* (18) for blue-green algae. Tentative results also show compounds of unknown identity which may be associated with the conversion of fructose to the polysaccharide constituents.

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Extracellular Polysaccharides of Algae: Effects on Life-Support Systems

Abstract. *The amount of extracellular polysaccharide produced by eight species of green and blue-green algae ranges from 174 milligrams per liter to 557 milligrams per liter. Most of the polymers are composed of four monosaccharides: a hexose, a pentose, a methyl pentose, and uronic acid. The production of excessive amounts of these photosynthetic end products will undoubtedly influence the effective recycling time of growth media in life-support systems.*

In "life-support systems" where media will be recycled for the continuous growth of algae, algal end products will undoubtedly influence the efficiency of the recycling process. Few microorganisms have been shown to be capable of breaking down their respective polysaccharides for carbon and energy (*1*). Therefore, in an efficient medium recycling process, not only will it be necessary to replace depleted minerals but some provision will have to be made to remove the accumulated extracellular by-products. Accordingly, the selection of an alga to be used in a life-support system will depend not only on its efficiency in utilizing CO_2 and producing O_2 but also on the quantity and nature of its extracellular by-products under the cultural conditions employed.

Since the early 1950's, studies have been carried out to explore the possible use of algae as a source of food for overpopulated regions of the world (*2*). In more recent years, the use of algae in life-support systems designed for long space flights has been studied (*3*). These efforts were initially designed to study mass culture, efficiency of oxygen production, suitable substrates, and so forth. One of the basic problems now concerns the production of extracellular end products of algal metabolism and their influence on the growth-medium recycling processes which would be used in life-support systems.

At least three classes of organic compounds are known to be liberated by some species of freshwater algae: organic acids (*4, 5*), nitrogenous material such as polypeptides and free amino acids (*6*), and carbohydrate polymers (*5, 7, 8*). Lewin (*7*) has presented quantitative studies on the production of extracellular polysaccharides by 18 species of green algae isolated from soil samples. The yields of the extracellular polymer ranged from 3 to 113 milligrams per liter.

In this report we describe the production and composition of extracellular polysaccharides by eight species of mucoid green and blue-green algae used for studies on life-support systems. Quantitative data are also presented for capsular and water-soluble intracellular polysaccharide production.

Table 1. Yields of polysaccharide and dry weight of cells from eight species of freshwater algae. The cultures were bacteria-free, unialgal cultures isolated from samples of fresh water and oxidation-pond water.

Algal culture	Incubation temperature (°C)	EP* (mg/liter)	CP (mg/liter)	IP (mg/liter)	TP (mg/liter)	Cells (mg/liter)
Anabaena f[illegible]quae	40	557	13	126	696	1379
Nostoc sp.	40	415	15	23	453	1315
Palmella m[illegible]cosa	25	271	36	196	503	2133
Chlorella vulgaris	25	235	34	75	344	3203
C. ellipsoidea	25	234	15	26	275	1959
Chlamydomonas sp.	25	224	19	62	305	1391
Oocystis sp.	25	197	22	48	267	2206
Chlorella sp.	25	174	15	26	215	1959

*EP, extracellular polysaccharide; CP, capsular polysaccharide; IP, water-soluble intracellular polysaccharide; TP, total polysaccharide.

Table 2. Increase in dry weight of cells and production of extracellular polysaccharide by *Anabaena flos-aquae*.

Time (days)	Extracellular polysaccharide (mg/liter, as glucose)	Cells (mg/liter)
2	25	268
4	44	323
6	50	1128
8	77	1203
10	138	1748
12	205	2068

Bacteria-free, unialgal cultures were isolated from samples of fresh water and oxidation-pond water gathered in the vicinity of this laboratory. The algae were cultured for 12 days in modified Knop's mineral medium (*9*), *pH* 7.0, contained in sterilized 2-liter Pyrex columns (48 mm in diameter). The cultures were maintained either at 25°C or 40°C in the presence of 13.4 kilolux of continuous light intensity supplied from a bank of white, 40-watt fluorescent lamps, and were aerated with a mixture containing 5 percent of CO_2 in air.

At 2-day intervals, samples were removed for the determination of polysaccharide in the cell-free medium and for determination of the dry weight. An appropriate amount of the cell-free medium was treated with 2 volumes of absolute ethyl alcohol, mixed, and centrifuged; the precipitate dissolved in 1 ml of distilled water, and the polysaccharide was determined as the glucose equivalent by the anthrone procedure (*10*). Dry weight was determined by drying overnight at 100°C.

At the end of the growth period, the cells were removed by centrifugation. The cell-free supernatants were concentrated to one-tenth volume with a rotary evaporator at 60°C, deionized with weak ion-exchange resins, and the extracellular polysaccharides were precipitated with two volumes of absolute ethyl alcohol. The stringy precipitates were collected in tared alundum crucibles and weighed after drying. The ash content of all extracellular polysaccharides, determined by combustion at 600°C, was subtracted to give yields of organic matter. The harvested cells were killed with 2 ml of a 2:1:1 mixture (by volume) of chlorobenzene,

dichloroethane, and chlorobutane, the capsular polysaccharides were extracted with distilled water at 10°C for 24 hours, precipitated with 2 volumes of absolute ethyl alcohol, and weighed. Intracellular polysaccharides (water-soluble) were extracted by subjecting the cell suspensions to sonic vibrations (frequency = 10 kcy/sec) for 15 minutes; the soluble protein and soluble pigments were removed with cold 10 percent trichloroacetic acid, and the polymer was precipitated with 2 volumes of absolute ethyl alcohol. For the determination of monosaccharide constituents of the extracellular polysaccharides, 50-mg samples were hydrolyzed with 4 ml of 1*N* H_2SO_4 in sealed ampules at 100°C for 6 hours. Barium carbonate was added to neutralize the H_2SO_4 to congo red and the $BaSO_4$ was removed by centrifugation. The hydrolyzates were then concentrated to 0.5 ml for chromatographic analysis. Portions of the hydrolyzates and standards were placed in spots on Whatman No. 1 filter paper and developed with a mixture of phenol and water (100:39 by weight), and a mixture of butanol, acetic acid, and water (2:1:1, by volume). Sugar spots were located and differentiated on the chromatograms with the aniline-oxalate mixture described by Horrocks and Manning (*11*). The tentative identification of the monomeric constituents of the polysaccharide was made by comparison of the unknowns to standard reference sugars.

The yields of polysaccharide in the various fractions and cells are given in Table 1. These strains produced much more extracellular polymer than those reported by Lewin (*7*). The range of extracellular polysaccharide production was from 174 mg per liter to 557 mg per liter. The yield of extracellular polymer was greatest (557 mg/liter), with the high-temperature blue-green alga, *Anabaena flos-aquae* (*12*). Another high-temperature blue-green alga, *Nostoc* sp., produced the next highest amount of extracellular polymer (415 mg/liter). Among the lower-temperature (25°C) green algae, the mucoid *Palmella mucosa* produced the most extracellular polymer (271 mg/liter). Yields with the other room temperature green algae (25°C) were slightly lower. The yield of capsular polymer did not deviate widely among the eight cultures tested, that is, 13 to 36 mg per liter. The yield of intracellular polymer was highest with *Palmella mucosa* (197 mg/liter), and next highest with *Anabaena flos-aquae* (126 mg/liter). The yields with all other cultures were somewhat lower (23 to 75 mg/liter). Synthesis of extracellular polysaccharide occurs more extensively than the accumulation of capsular or intracellular polysaccharides. Very little capsular polymer was expected to be produced because of the vigorous aeration of the cultures during growth.

Table 3. Monosaccharides detected in the hydrolyzates of the extracellular polysaccharides investigated. G, glucose; Ga, galactose; A, arabinose; X, xylose; R, ribose; Gl, glucuronic acid; F, fucose; Rh, rhamnose.

Algal culture	G	GA	A	X	R	Gl	F	Rh
Chlamydomonas sp.	X*			X			X	
Nostoc sp.	X		X			X	X	
Chlorella ellipsoidea	X		X			X	X	
C. vulgaris	X			X		X		X
Palmella mucosa	X		X			X	X	
Oocystis sp.		X	X			X	X	
Chlorella sp.	X		X			X		X
Anabaena flos-aquae	X			X	X	X		

* X indicates the presence of a particular tentatively identified monosaccharide.

With all of the cultures, the production of the extracellular polymer was dependent upon the age of the culture, the most significant increase being during the late log phase of the growth cycle. These results are in contrast to those reported for some species of marine algae in which the extracellular carbohydrate accumulated only after the cultures reached the stationary phase of the growth cycle (*13*). Polysaccharide production continues slightly after the cells reach the stationary phase of the growth cycle. The data for *Anabaena flos-aquae* are given in Table 2.

The large values for extracellular polysaccharide may have been the result of the vigorous aeration afforded in column culture. Conversely, Guillard and Wangersky (*13*), and Lewin (*7*) obtained much smaller yields from gently aerated flask cultures.

The monosaccharide components of the extracellular polysaccharides were hexose, uronic acid, pentose, and methyl pentose (Table 3). None of the polymers contained more than four components. The most common were glucose, arabinose, fucose, and glucuronic acid. Organisms which did not show this general pattern were *Chlamydomonas* sp., *Chlorella vulgaris*, and *Anabaena flos-aquae*. In the extracellular polysaccharides of the two green algae, (*Chlamydomonas* sp and *Chlorella vulgaris*), no uronic acid moiety was found. In the extracellular polysaccharide of the blue-green alga, *Anabaena flos-aquae*, no methyl pentose was found, and the polymer contained two pentose components, ribose and xylose. Hydrolyzates of capsular and water-soluble intracellular polysaccharides from *Palmella mucosa* and *Anabaena flos-aquae* yielded the same constituents as found in the extracellular polysaccharide of the respective alga.

The results of the extracellular polysaccharide screening tests designed to measure the amount of soluble extracellular carbohydrate polymers produced by certain algae used in studies of life-support systems show that these metabolic end products accumulate in the growth medium. The accumulation of these end products will undoubtedly decrease the effective use of growth media.

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Palmellococcus Species as a Carbon-Energy Source for the Growth of Torula utilis*

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Factors affecting the availability of photosynthetic products for other organisms have been investigated in this laboratory.

It has been found that by subjecting *Palmellococcus* species to sonic vibrations, that water soluble carbohydrate increases by a factor of ten and the dry weight of the algal cells decreases by factors up to 86-96%.

The feasibility of employing sonic extracts of *Palmellococcus* species to supply carbon and energy for the growth of *Torula utilis* has been investigated. Respiration and growth studies have shown a favorable response of *Torula utilis* to sonic extracts of *Palmellococcus* species.

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The roles of algae in extended space flights are fourfold; to furnish a source of food, to replenish the available oxygen supply, to utilize respiratory carbon dioxide, and to utilize the waste products of the manned craft.

The last three roles (oxygen production, CO_2 utilization, and waste degradation) can be demonstrated easily with most of the common green and blue-green algae.

The food concept has been studied extensively, but no favorable results have been obtained with human subjects. The protein, carbohydrate, and fat content of algae is comparable with yeast and other microorganisms.

Protein has been studied the most extensively. Fischer and Burlew (1953) reported that algal proteins did not stimulate growth in the rat as efficiently as other proteins of high biological value owing to a deficiency in the sulfur amino acids. A report of Prosky's work (Leveille, Sauberlich, and Shockley, 1962) stated that the addition of algae alone did not show as good a response as with the casein supplemented diet. Combs (1952) stated that chicks gained in weight and had higher feed conversions when 10% *Chlorella* was substituted for soybean meal, but the increased growth was later shown to be caused by the carotene content of the algae which supplied vitamin precursors to the chick. Leveille et al. (1962) reported that chicks and weanling rats fed several species of algae as a protein source did not compare with the control group on standard protein sources. The algae were deficient in methionine, histidine, and glycine for the chick and rats. Lubitz (1963) showed that *Chlorella* 71105 has a PER which is slightly under the PER for casein and about three-fourths the PER of whole egg. The protein was shown to be 86% digestible, the fat was 93% digestible, and total carbohydrate was 72% digestible. Dyer and Gafford (1961) reported that white mice showed a net gain in weight after being fed *Chlorella pyrenoidosa* TX 71105 for 112 days. An

* This work was done in cooperation with the National Aeronautics and Space Administration, Washington, D. C.

initial weight loss occurred before the gain was obtained. These authors attributed this to the low fat content and high protein content of the algae.

Powell, Nevels, and McDowell (1961) have presented data on the utilization of algae by humans as a sole food source. First, the algae were not acceptable by the subjects in taste and odor even when supplemented with other foods. Second, when large amounts of the algae were fed to human subjects, several undesirable symptoms developed (nausea, cramping pains, vomiting, etc.). These workers concluded that the algae might be used if supplemented with other foods but that improvements in digestibility and acceptability of the algae are needed before the algae can be used as a sole food source. Cook and Lau (1961) reported almost the same conclusions in that the rat and human could use the algae supplemented with other foods, but the algae alone did not meet the nutritional requirement of the rat and human. This same approach was used by Morimara and Tamiya (1954) in formulating algae containing diets.

Since yeast cells can serve as a food or supplement for the human subject, this investigation was initiated to determine if algal extracts could supply carbon and energy for the cultivation of yeasts acceptable as a diet for human consumption.

The objectives in this primary investigation were (a) to obtain a method for efficiently preparing algal extracts, (b) to test the algal extracts for energy sources in the form of carbohydrates, (c) to test the growth response of a selected species of yeast on the algal extract, and (d) to determine the oxidation of algal extracts by a species of yeast.

The source of algae for these experiments was *Palmellococcus* species grown under bacteria-free conditions in Knop's mineral medium at 23 C and 1200 foot-candles light intensity. This alga was selected because of its high cell carbohydrate content and its ability to produce an extracellular polysaccharide composed chiefly of glucose in high yields (Moore, 1963). The cells were harvested from the growth medium by centrifugation in the cold and resuspended in known concentrations in a yeast-basal medium deficient in carbon (Difco Labs., 1953).

Effects of Soneration on Palmellococcus

The method selected for preparing algal extracts was soneration. A Raytheon 10KC sonic oscillator was used to disrupt the cells. The effect of soneration on *Palmellococcus* in terms of dry weight, decrease in packed cell volume, and release of soluble carbohydrate is given in Table 1. Dry weight was determined by difference measurements using tared gooch crucibles with asbestos mats at 90 C. Packed cell volume was determined in calibrated centrifuge tubes. Soluble carbohydrate was determined by the Anthrone procedure of Matton et al. 1955.

A quick and efficient method for the rupture of algal cells (*Palmellococcus* in particular) is soneration as represented by the data in Table 1. Packed cell volume trials indicate that within 15 minutes the greatest amount of solubilization is approached. The release of soluble carbohydate into the medium (expressed as glucose by anthrone analysis) shows the same trend. Within 15 minutes, the soluble carbohydrate in cell fragment-free extracts increases by a factor of about 8 over the nonsonerated samples. The carbohydrate detected in the nonsonerated samples is the result of the excretion of cellular and extracellular polysaccharides into the medium. The amount of solubilization achieved with the algae varies with cell concentration as would be expected. With more dilute concentrations (less than 1 mg/ml), over 95% solubilization can be achieved in 15 minutes exposure. More concentrated solutions give solubilization figures lower than this. The trend with concentrated cell solution is to get more solubilization with

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TABLE 1. *Effects of Soneration on* Palmellococcus

Exposure (minutes)	Packed Cell Volume		Increase in Soluble Carbohydrate		Decrease in Dry Weight			
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1		Trial 2	
	ml/5 ml	ml/5 ml	μg as glucose	μg as glucose	mg/ml	%	mg/ml	%
0	0.15	0.13	49.0	65.0	6.34	——	0.828	——
15	0.03	0.05	395.0	595.0	3.94	37.85	0.040	95.17
30	0.01	0.02	394.0	590.0	3.92	38.17	0.029	96.49
45	0.01	0.01	395.0	595.0	3.42	46.05	0.028	96.62

long exposure in contrast to dilute solutions where almost total solubilization is achieved in less than 15 minutes.

Carbohydrates in the Algal Extract

To determine the relative amounts and forms of carbohydrates in the algal extract, the following analyses were performed. Fifty ml of a *Palmellococcus* cell•suspension (6.93 mg/ml) was sonerated 45 minutes. Ten ml samples of the cell-free 0- and 45-minute samples were concentrated to 0.1 ml for chromatography to determine free-reducing sugars.

Water-soluble polysaccharides were determined by precipitating 10-ml samples of the cell-free 0- and 45-minute samples with 20 ml of ethanol. The alcoholic precipitates were hydrolyzed with 4 ml of 1N HCl for 6 hours at 100 C in sealed ampoules. The hydrolysates were evaporated to dryness at 60 C in vacuo and resuspended in 0.1 ml of water for chromatography.

The solvents used for developing the chromatograms were phenol; water (100: 39 — w : w) and n-butanol: acetic acid: water (2 : 1 : 1 — 1 : 1 : 1). Reducing sugars and acidic monosaccharides were detected on the chromatograms by spraying chromatograms with aniline hydrogen oxalate and heating to 75 C for 15 minutes. The results of the chromatographic analyses are given in Table 2.

Soneration of the algal cells releases two classes of carbohydrates as exhibited by the data in Table 2. Exposure of the algal cells to sonic vibrations for 45 minutes releases three free monosaccharides from the cells. These were glucose, arabinose, and fucose. The relative amounts showed that glucose was the sugar present in the highest concentration. The qualitative analysis showed that an array of monosaccharides are released from the algal cells in the form of polysaccharides. The types of the bound monosaccharides of *Palmellococcus* were glucose, mannose, arabinose, ribose, fucose, and glucurone. Arabinose and glucose were the main sugars present by estimation of spot intensity on the paper chromatograms. The bound sugars obtained without soneration were attributed to the cellular sloughing off of polysaccharide material. The carbohydrate analysis shows that several different sugars and different forms are made available when the algal cell is ruptured. The presence of mannose is attributed to soluble cell-wall [illegible] Glucose, arabinose, fucose, and glucurone are the monosaccharides found in

TABLE 2. *Carbohydrates in algal extracts*

Sample	Phenol Rf*	Butanol Rf*	Mono-saccharide	Relative Amount
Free Reducing Sugars—0 Minutes	.....	.....	.....	.....
Free Reducing Sugars—45 Minutes	0.3573	0.1871	Glucose	3+
	0.4730	0.2387	Arabinose	+
	0.5552	0.2967	Fucose	+
Polysaccharides				
(1) 0 Minutes	0.3525	0.1742	Glucose	+
	0.5150	0.2413	Arabinose	+
	0.6300	0.3163	Fucose	+
	0.7600	0.3550	Glucurone	trace
(2) 45 Minutes	0.3735	0.1957	Glucose	4+
	0.4231	0.2198	Mannose	+
	0.5192	0.2359	Arabinose	5+
	0.6177	0.2841	Ribose	trace
	0.6322	0.3271	Fucose	2+
	0.7596	0.3538	Glucurone	+
Standard Sugars	0.3590	0.1983	Glucose	.....
	0.5102	0.2459	Arabinose	.....
	0.6128	0.3217	Fucose	.....
	0.082	0.1740	Glucuronic Acid	.....
	0.6100	0.2900	Ribose	.....
	0.3936	0.2149	Mannose	.....
	0.7200	0.3500	Glucurone	.....

* Owing to cellular polysaccharide of cells. All Rf values are averages.

the cellular and extracellular polysaccharides of this alga. Ribose was detected only in trace amounts.

Palmellococcus *Extract as a Carbon Source for the Growth of* Torula utilis

Torula utilis strain NRRL Y-900 was grown in the yeast-basal medium with carbon supplied in the form of the algal extract. The inoculum of *T. utilis* was prepared as follows: *T. utilis* was streaked on a sterile agar slant containing 25 ml of nutrient broth, 1% glucose, and 25 ml of a sonerated algae suspension. The yeast cells were washed off the agar slant, centrifuged, and suspended in 50 ml of sterile phosphate buffer, pH 7.0. Cell concentration was determined to be 9.5×10^6/ml with a counting chamber.

Palmellococcus cells (1.169 g) were suspended in 400 ml of yeast-basal medium giving a cell concentration of 2.93 mg/ml. Fifty ml amounts were sonerated for 15 minutes. Dry weight after soneration was 0.42 mg/ml. The percent solubilization being 85.66 and for each 50 ml of sonerated algal suspension, there were added 125.5 mg of solubilized, algal cell contents.

One ml of the yeast inoculum was inoculated into 50 ml of the algal extract and into yeast-basal controls. Samples were taken at 24, 48, and 72 hours to determine the yeast cell concentration with a counting chamber. The results are given and illustrated

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The algal extract as a growth substrate for *T. utilis* gave encouraging results. The number of yeast cells in the algal extract increased by a factor of about 4 in 72 hours and most of the gain was from 0 to 24 hours. Growth in the control was negative as evidenced by the decrease in the number of countable cells. The response to the extract shows that this species of yeast can utilize some of the solubilized algal cell contents as a source of carbon for cellular synthesis.

Oxidation of Algal Extracts by T. utilis

To determine the oxidation of algal extracts, manometric techniques were used to measure the oxygen uptake on extracts and modified extracts by *T. utilis*. Standard Warburg techniques were used as described by Umbreit, Burris, and Stauffer, 1959. For extracts, the flasks contained 2.8 ml of the test sample with 0.2 ml of 10% KOH in the center well and 0.2 ml of the yeast cells in the side arm. Oxygen uptake was determined at 30 C.

a. Uptake of Oxygen by *T. utilis* on Algal Extracts vs. Time of Soneration. Samples of *Palmellococcus* cells in yeast-basal medium (6.93 mg/ml) were sonerated for 0, 15, 30, and 45 minutes. Each sample was centrifuged after soneration to remove intact cells and debris. The samples were heated at 100 C for 5 minutes to retard enzymatic activity and bacterial growth. Samples were then tested for oxygen uptake using *T. utilis*. Each side arm contained 5 mg of *T. utilis* cells. The results are given in Table 3. The extracts were compared to a heat-killed suspension of *Palmellococcus* cells and to a sugar solution containing 800 μg of glucose.

The oxidation of the algal extracts gave results which indicate *T. utilis* can utilize the algal extracts as energy sources.

Oxygen uptake vs. time of soneration (Table 4) shows that the maximum response is gained when the algae are sonerated for 45 minutes, however, this difference is not large enough to merit exposing the algal cells to sonification for over 15 minutes. Oxygen uptake on extracts was about 3.5 times as much as on the intact cells. The response

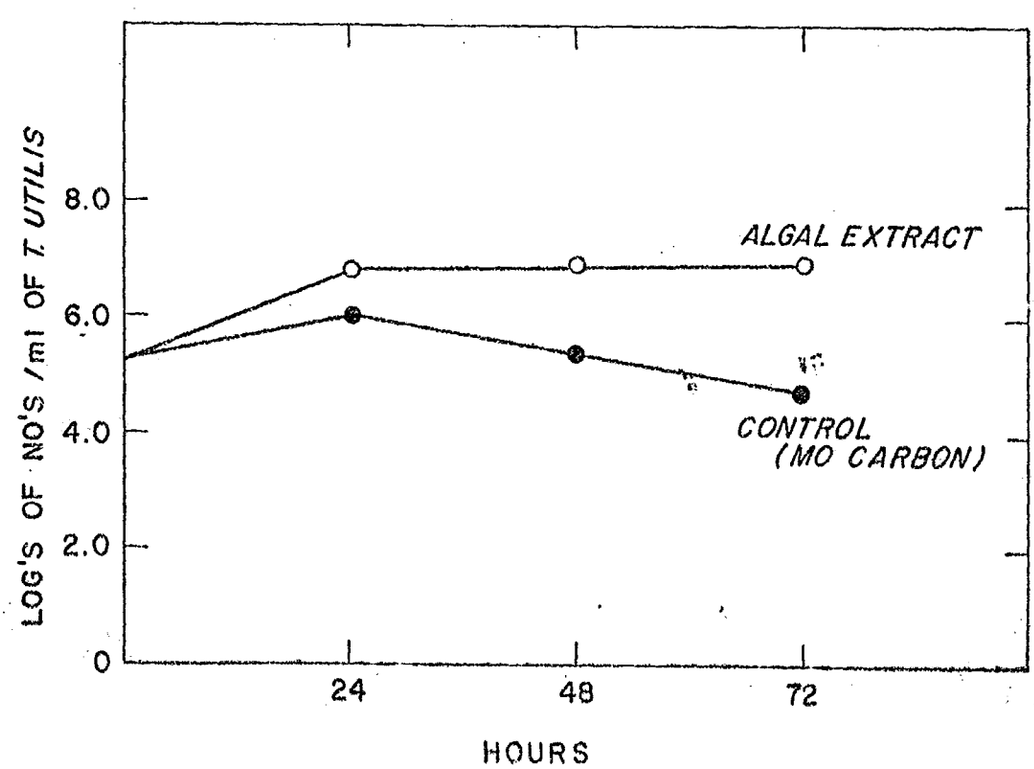

FIG. 1. Growth of *T. utilis* on algal extracts.

TABLE 3. *Oxidation of algal extracts by* T. utilis *vs. time of soneration*

Time (minutes)	O_2 uptake						
	End	AE–0	AE–15	AE–30	AE–45	HKC	SS
	μl	μl	μl	μl	μl	μl	μl
0	—	—	—	—	—	—	—
15	—	19	31	20	22	13	11
30	3	24	55	39	41	20	23
45	1	17	69	52	70	42	57
60	—	24	121	108	120	66	85
75	6	35	161	137	157	69	94
90	—	20	179	155	179	71	103
105	1	21	216	211	234	83	122
120	1	21	261	254	280	81	108

End: Endogenous.
AE–0, 15, 30, 45: Algal extracts over time.
HKC: Heat killed cells.
SS: Sugar solution.

on extracts was also about 2.5 times greater than the response to 800 μg of glucose in a control flask.

b. Oxidation of Algal Extracts by *T. utilis* With and Without Supplementation. Because of the oxidative response of the yeast to the algal extracts, a plan was adapted to determine if this response could be removed by supplementation of an energy source and a protein source. In one series, protein and chlorophyll were removed with lead acetate to determine what effect this might have on the oxidative response.

A 12-day column culture of *Palmellococcus* was centrifuged and the cells suspended in yeast-basal medium to a dry weight cell concentration of 7.1 mg/ml. After soneration for 15 minutes, the dry weight was 2.42 mg/ml corresponding to a solubilization percentage of 65.91. Test samples were prepared in 25 ml amounts. Samples containing the extract had the equivalent of 177.5 mg of solubilized algal cell contents.

Sample Protocol:

Series	Contents (25 ml)
A	Yeast-Basal Medium
B	Algal Extract
C	Algal Extract + 117.5 mg Glucose
D	Algal Extract + 117.5 mg Peptone
E	Algal Extract + 117.5 mg Glucose + 117.5 mg Peptone
F	Algal Extract – (Protein and Chlorophyll)*
G	Heat-killed cells
H	Yeast-Basal Medium + 117.5 mg Glucose

* Protein and chlorophyll were removed by adding a saturated solution of lead acetate to the extract and then neutralizing the excess lead with a saturated barium oxalate solution.

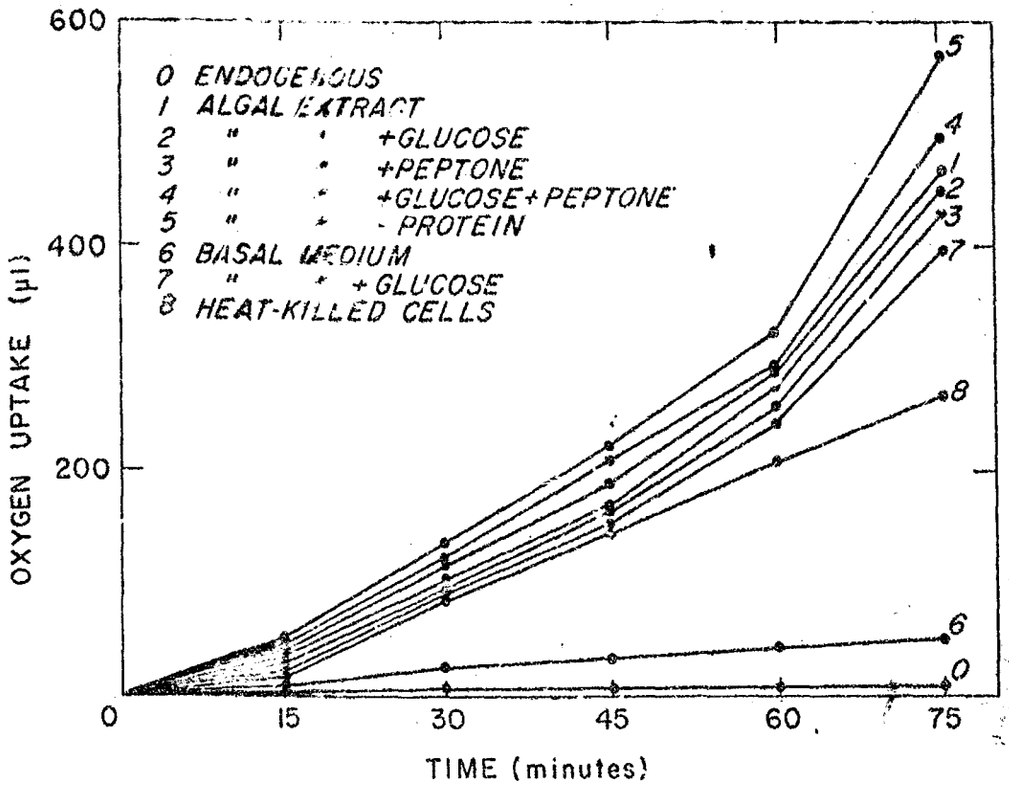

FIG. 2. Oxidation of algal extracts with and without supplementation by *T. utilis*.

The flasks were sterilized for 5 minutes at 121 C and 15 psi. Flasks containing the extract had a green precipitate after autoclaving. The series with protein and chlorophyll removed contained no precipitate and was perfectly clear. Aliquots of 2.8 ml were tested for oxygen uptake using 1.5 mg of *T. utilis* cells per Warburg flask. The response is given in Fig. 2.

Supplementing the algal extracts with glucose and peptone increased the oxidative response of the yeast but not to a large extent (Table 4). When glucose and peptone were added singly, the response to the algal extract was higher than the supplemented responses. The algal extract also gave a response much higher than a flask containing the same amount of glucose added to the basal medium as the extract contained solubilized cell contents. The response to heat-killed cells was about one-half that of the extract. The significant results of the experiment show that when the algal protein and chlorophyll are removed, a response is achieved just as great or greater than the response on the extract, with and without supplementation. This represents a method of converting algae grown on waste substrates, etc. to a microbiological medium for cultivating other microorganisms. The medium was almost perfectly clear after autoclaving and did not have any of the characteristic odors of algae (fresh or cooked). The odor problem was encountered when human subjects were fed whole algal cells and according to the subjects, this odor was very disagreeable.

c. Effects of Removing Algal Protein and Chlorophyll Upon Oxidative Response of *T. utilis* to Algal Extracts. It was shown in the previous experiment that by removing the protein and chlorophyll a response was obtained which was a little higher than for the agal extract with and without supplementation with glucose and peptone. The significant point is that the removal of protein and chlorophyll gave a medium which was

almost totally devoid of any color and algal odor. This same series was repeated along with series without protein and without chlorophyll.

Fifty ml of a *Palmellococcus* cell suspension (12.86 mg/ml) was sonerated for 15 minutes. Dry weight of the extract was 3.89 mg/ml corresponding to a solubilization percentage of 69.75.

Protein and chlorophyll were removed by the lead acetate: barium oxalate procedure.

For removing only protein, the extract was precipitated with $ZnSO_4$ (6 g/100 ml), precipitate removed by centrifugation, residue extracted with 5 ml of absolute ethanol and centrifuged, chlorophyll extract added back to original supernatant, and the ethanol driven off by heat.

For removing the chlorophyll only, the algal extract was precipitated with $ZnSO_4$, the

TABLE 4. *Effects of removing protein and chlorophyll from algal extracts on oxidative response of* T. utilis

Time (minutes)	O_2 uptake on autoclaved substrates					
	End	AE	AE−(P+C)	AE−P	AE−C	Y-B
	μl	μl	μl	μl	μl	μl
0	—	—	—	—	—	—
15	14	216	217	226	229	21
30	24	471	479	491	444	32
45	28	745	720	748	657	40
60	33	1054	982	1041	925	46
75	37	1270	1163	1392	1112	54
90	40	1427	1273	1531	1250	57
105	44	1560	1430	1713	1425	62
120	47	1766	1574	1868	1582	66
	O_2 uptake on heat-shocked substrates					
0	—	—	—	—	—	—
15	14	237	216	237	193	21
30	24	520	492	488	345	32
45	28	803	753	739	476	40
60	33	1096	1037	1015	612	46
75	37	1304	1232	1213	707	54
90	40	1436	1387	1339	766	57
105	44	1614	1551	1508	849	62
120	47	1773	1706	1660	923	66

End: Endogenous.
AE: Algal Extract.
AE−(P+C): Algal Extract minus Protein and Chlorophyll.
AE−P: Algal Extract minus Protein.
AE−C: Algal Extract minus Chlorophyll.
Y-B: Yeast-Basal Medium.

residue extracted with 5 ml of absolute ethanol, the extract discarded, and the residue was added back to the original supernatant.

One series of the samples was autoclaved for 15 minutes at 121 C and 15 psi. Another series of the samples was heated for 5 minutes at 100 C.

Aliquots of 2.8 ml were tested for oxygen uptake with 5 mg of cells (dry weight) of *T. utilis*. The results are given in Table 4 and the results of the autoclaved substrates are graphed in Fig. 3.

The effects of removing the protein and chlorophyll separately did not seem to alter the oxidative response of the yeast (Table 4). Since the oxidative response is not effected by the removal of protein and chlorophyll separately or in combination, the soluble algal carbohydrate, protein, etc. serves as an excellent energy source for *T. utilis* as evidenced by the oxidative responses. The heat-shocked substrates gave variable results each time this was duplicated.

The use of algae in this role would seem to be more of energy supply than a source of carbon in a closed ecology. Evidently the protein of the algae cannot be used efficiently as a source of carbon for other organisms as evidenced by this study. This would be of utmost value because it has been found in this laboratory that various molds, yeast, and bacteria can be grown in large quantities on waste substrates if supplemented with an energy source. By using algae for this energy source, a microbial food for human consumption could be realized. This would furnish the ecological cycle an end-point in furnishing food, waste utilization, and gas recycling. The efficiency of this system will depend on just how much energy has to be supplied in terms of light energy to cultivate the algae in mass quantities. This approach would seem to be much better than the use of whole algal cells as carbon-energy sources which has not yielded any astounding results so far in terms of supplementing the human diet.

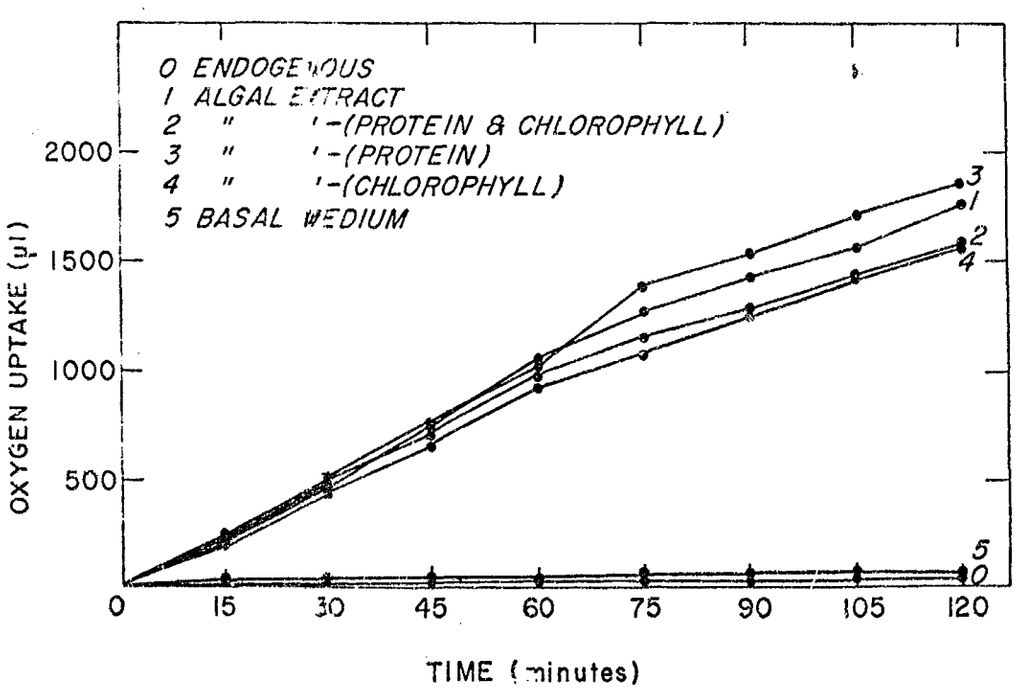

FIG. 3. Oxidative response by *T. utilis* to algal extracts with protein and chlorophyll removed.

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An Extracellular Polysaccharide Produced by Palmella mucosa Kütz.

By

R. G. TISCHER and B. G. MOORE

With 2 Figures in the Text

(Received May 23, 1964)

Some species of green and blue-green fresh-water algae liberate varying amounts of organic material into their surrounding environment. One such class of compounds is extracellular polysaccharides. These polymers may range from simple two and three to eight component polymers. LEWIN (1956) reported on seven algal polysaccharides from *Chlamydomonas* species which were composed of only two units, arabinose and galactose. Also LEWIN isolated a polysaccharide from *Chlamydomonas sphagmophila* which contained seven monosaccharide units. Extracellular polysaccharides from *Nostoc commune*, *Nostoc muscorum*, and *Anabaena cylindrica* contained seven, eight and six units respectively (JONES et al. 1952; BISWAS 1957; BISHOP et al. 1954). Almost all of the common monosaccharides can be found in these polymers. Hexoses, pentoses, uronic acids and methyl-pentoses are the usual constituents in varying combinations. The amount of extracellular polysaccharide produced varies widely from culture to culture with some variation probably due to differences in culture chambers and polysaccharide analyses. Quantitative data are available from the works of LEWIN (1956) and MOORE and TISCHER (1963). The species of algae examined by LEWIN (1956) produced the polymers in concentration from 3—113 mg per liter of growth medium. Mucilaginous species examined by MOORE and TISCHER (1963, 1964) produced from 174—557 mg per liter of growth medium. Thus, the amounts produced depend upon the strain employed.

Palmella mucosa Kütz. produces extracellular, capsular, and water-soluble intracellular polysaccharides (MOORE and TISCHER 1963). This report is concerned with the extracellular polysaccharide: its composition; the effects of different carbon and nitrogen sources on its synthesis; the rate of its formation; and its biosynthetic route.

Materials and Methods

Extracellular polysaccharides were extracted by a ten-fold concentration of the cell-free medium followed by the addition of 2 volumes of absolute ethanol. Capsular polysaccharides were extracted from harvested cells with distilled H_2O

at 5° C for 24 hours and precipitated by the addition of 2 volumes of absolute ethanol. Watersoluble intracellular polysaccharides were extracted by rupturing the cells in a Raytheon 10 KC sonic oscillator for 10—30 minutes. The supernatant liquid was treated with cold 10% trichloroacetic acid and the polysaccharide extracted with ethanol from the deproteinized solution.

Palmella mucosa Kütz. was cultured in the modified Knop's mineral medium (pH 7.0) (PHILLIPS and MYERS 1954) at 25° C and were exposed to a continuous light intensity of 1250 foot-candles. All cultures were supplied with a 5 % CO_2-air mixture except where designated.

Extracellular polysaccharide production as a function of time was measured by determining the amount of the polymer liberated as estimated by the anthrone procedure of SNELL and SNELL (1958). The dry weight of the cells was determined by drying at 100° C for 24 hours.

Polysaccharide samples were hydrolyzed with 1 N H_2SO_4 in sealed ampoules by heating in a boiling water bath for 6 hours. Residual H_2SO_4 was then removed by neutralization with $BaCO_3$ to congo red paper. $BaSO_4$ was removed by centrifugation and the sample volume reduced to 0.1 to 0.5 ml *in vacuo* at 60° C.

Chromatographic separations of the constituent monosaccharides were performed using phenol : water (100 : 39 — W : W) and butanol : acetic acid : water (2 : 1 : 1 — V : V : V). Sugar spots were located on the dried chromatograms using the procedure and reagent of HORROCKS and MANNING (1949). Reference sugars were run with all analyses.

To determine the molar proportion of the constitutive monosaccharides, the procedure of HAWTHORNE (1947) was followed. A sample of the polysaccharide hydrolysate was separated chromatographically on paper. The individual spots, located by spraying duplicate chromatograms, were cut out and eluted with distilled water. The amount of each monosaccharide was separately determined and the molar ratios calculated.

To determine the effects of carbon sources on extracellular polysaccharide production, the alga was grown on various carbon sources supplied at a level of 1% and the response was determined in terms of dry weight of cells and as extracellular polysaccharide produced (expressed as glucose by the anthrone procedure). Carbon dioxide was omitted from the aerating gas for carbon sources other than 5 % CO_2. Nitrogen effects were determined in a similar fashion with the nitrogen sources being added at the same level of nitrogen as the usual source, 2.52 g KNO_3/liter.

The preliminary biosynthetic studies were performed using $NaHC^{14}O_3$, $HC^{14}OOH$ and uniformly-labeled glucose-C^{14}. Cells were exposed to the C^{14} sources in appropriate chambers and the polysaccharide fractions extracted and assayed for radioactivity. Radioactivity was measured with a thin-window G-M tube.

Results

The extracellular, capsular and water-soluble intracellular polysaccharides all contained glucose, glucuronic acid, arabinose and fucose in molar ratios shown in Table 1. The average of the analyses gives a molar ratio of 11 : 6 : 3 : 1 for glucose : fucose : arabinose : glucuronic acid.

The production of extracellular polysaccharide and cell dry weight as a function of time is presented in Fig. 1. The data represent the production of polymer expressed as anthrone glucose equivalents.

Table 1. *Molar ratio of monosaccharides in the extracellular polysaccharide of Palmella mucosa Kütz.*

Hydrolysate	Spot	Concentration µg	Concentration µm	Molar ratio units	Average, closest whole number*
A	Glucose	89.09	0.4945	13.36	11
	Arabinose	19.31	0.1290	3.50	3
	Fucose	32.90	0.2010	5.45	6
	Glucuronate	7.15	0.0370	1.00	1
B	Glucose	87.91	0.4871	10.50	—
	Arabinose	23.53	0.1567	3.37	—
	Fucose	38.39	0.2339	5.03	—
	Glucuronate	9.02	0.0460	1.00	—
C	Glucose	76.92	0.4269	9.61	—
	Arabinose	16.67	0.1100	2.48	—
	Fucose	46.71	0.2845	6.34	—
	Glucuronate	8.72	0.0449	1.00	—

* Average of hydrolysates A, B and C.

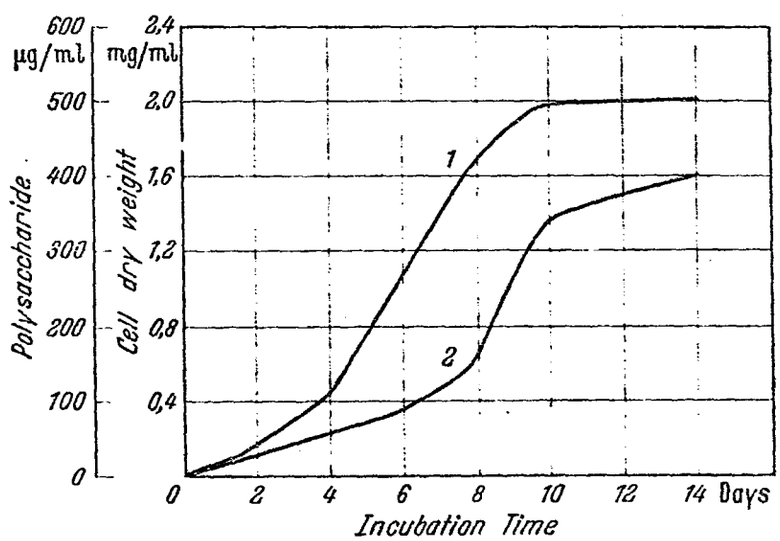

Fig. 1. Extracellular polysaccharide production and increase in cell dry weight as a function of time. *1* **Cell dry weight;** *2* **Extracellular polysaccharide**

The utilization of various carbon sources for cell and extracellular polysaccharide formation was measured after incubation for 9 days. The data are presented in Table 2. After incubation, the extracellular polysaccharides were examined for any variation in constituents. Ten mg's of the extracellular polysaccharide fractions were hydrolyzed and 50 µl spotted and developed chromatographically. The results are given in Table 3.

The effects of several nitrogenous compounds on extracellular polysaccharide production are described by the data in Table 4.

Table 2. *Cell dry weights and extracellular polysaccharide production (as glucose) of Palmella mucosa Kütz. grown on various carbon sources*

Carbon source	Mean dry weight mg/ml	Mean extracellular polysaccharide µg/5 ml as glucose	pH Change of medium Δ pH units
CO_2	2.849	438.00	0.0
Glucose	6.050	700.00	+ 2.7
Acetate	0.398	140.00	+ 2,7
Lactate	1.118	250.00	+ 2.1
Pyruvate	1.225	280.00	+ 3.7
Glycerol-phosphate	1.387	215.00	+ 1.3
Formate	1.193	171.00	+ 1.8
Control	0.209	58.50	+ 2.0

Table 3. *Extracellular polysaccharide composition of Palmella mucosa Kütz. formed in the presence of different carbon sources*

Carbon source	Spot on chromatogram	Monosaccharide	Carbon source	Spot on chromatogram	Monosaccharide
CO_2	1	Glucose	Lactate	1	Glucose
	2	Arabinose		2	Arabinose
	3	Fucose		3	Fucose
	4	Glucuronate		4	Glucuronate
Glucose	1	Glucose		5	2-Deoxyribose
	2	Arabinose	Pyruvate	1	Glucose
	3	Xylose		2	Arabinose
	4	Fucose		3	Fucose
	5	Glucuronate	Glycerol Phosphate		
	6	2-Deoxyribose		1	Glucose
	7	unknown		2	Arabinose
Acetate	1	Glucose		3	Fucose
	2	Arabinose	Formate	1	Glucose
	3	Fucose		2	Arabinose
				3	Fucose
				4	Glucuronate

Table 4. *Dry weight and extracellular polysaccharide production by Palmella mucosa Kütz. grown with various nitrogen sources*[1]

Nitrogen source	Dry weight[2] mg/ml	Extracellular polysaccharide[2] µg/5 ml as glucose	pH Change of medium Δ pH units
KNO_3	1.18	170.00	+ 0.2
$Ca(NO_3)_2 \cdot 4H_2O$	1.62	181.00	+ 0.4
NH_4NO_3	0.40	62.50	− 4.2
$NaNO_3$	0.89	95.75	− 0.1
No nitrogen[3]	0.30	51.30	− 1.0

[1] Incubation time: 8 days.
[2] Mean value of duplicate cultures.
[3] Atmospheric ammonia was not trapped from the control.

The preliminary biosynthetic studies were carried out with: (a) cells suspended in Knop's medium without a nitrogen source and (b) with cells growing in normal Knop's medium.

The incorporation of C^{14} from $NaHC^{14}O_3$ into the extracellular polysaccharide and cells during different time exposures is plotted in Fig. 2.

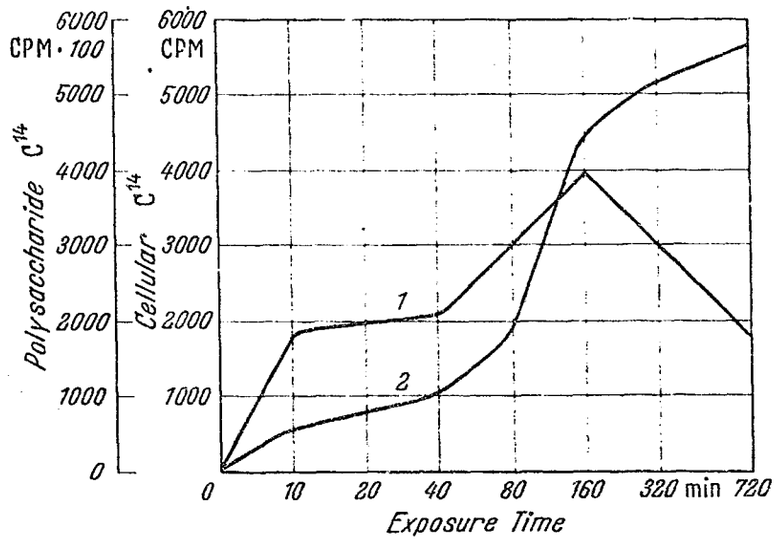

Fig. 2. The incorporation of $NaHC^{14}O_3$ into the extracellular polysaccharide and cellular material of *Palmella mucosa* Kütz. *1* Cellular C^{14}; *2* Polysaccharide C^{14}

Table 5. *Incorporation of radioactivity from $NaHC^{14}O_3$ and uniformly labeled glucose-C^{14} by resting cells into polysaccharide fractions in 12 and 24 hours*

C^{14} Source and fraction	Time hours	Total CPM in fraction cpm*	% of added radioactivity %
EP[1] : $HC^{14}O_3^-$	12	298	0.06
CP[2] : $HC^{14}O_3^-$	12	49	0.01
ICP[3] : $HC^{14}O_3^-$	12	1,000	0.21
EP : $HC^{14}O_3^-$	24	3,225	0.67
CP : $HC^{14}O_3^-$	24	12,680	2.64
ICP : $HC^{14}O_3^-$	24	24,000	4.99
EP : glucose-C^{14}	12	20,727	4.48
CP : glucose-C^{14}	12	12,000	2.59
ICP : glucose-C^{14}	12	8,920	1.93
EP : glucose-C^{14}	24	29,093	6.28
CP : glucose-C^{14}	24	6,920	1.50
ICP : glucose-C^{14}	24	9,800	2.20

[1] EP: Extracellular polysaccharide.
[2] CP: Capsular polysaccharide.
[3] ICP: Intracellular polysaccharide (water-soluble).
* cpm added per exposure: $NaHC^{14}O_3$ 480,200; glucose-C^{14} 462,700.

The results show that polymer production is directly related to the incorporation of CO_2 by the cells and the subsequent transfer to extracellular polymer. After 80 minutes exposure, the radioactivity of the cells starts to decrease, while the radioactivity of the polymer increases rapidly and continues to increase after the decline in cell C^{14} count.

The labeling of extracellular, capsular and water-soluble intracellular polysaccharides formed by resting cells exposed to $NaHC^{14}O_3$ and uniformly-labeled glucose-C^{14} for 12 and 24 hours is given in Table 5.

Table 6. *Incorporation of radioactivity from $NaHC^{14}O_3$ and uniformly labeled glucose-C^{14} by growing cells into polysaccharide fractions*

Polysaccharide fraction	Total CPM in fraction CPM*	% of added radioactivity %
EP[1]: $NaHC^{14}O_3$	—	—
CP[2]: $NaHC^{14}O_3$	—	—
ICP[3]: $NaHC^{14}O_3$	795	0.34
Residual cell C^{14}	12,150	5.26
EP: glucose-C^{14}	8,794	1.01
CP: glucose-C^{14}	4,140	0.48
ICP: glucose-C^{14}	37,680	4.33
Residual cell C^{14}	458,150	52.66

[1] EP: Extracellular polysaccharide.
[2] CP: Capsular polysaccharide.
[3] ICP: Intracellular polysaccharide (water-soluble).
* CPM added: $NaHC^{14}O_3$ 231,000; glucose-C^{14} 870,000.

The labeling of extracellular, capsular and water-soluble intracellular polysaccharides formed by growing cells from $NaHC^{14}O_3$ and glucose-C^{14} during 12 hours is shown in Table 6. Since extracellular polysaccharide production followed the curve for cell dry weight, the data show that the synthesis of other cell constituents with either of the two C^{14} sources takes precedence over that of polysaccharide.

The long-time labeling pattern of the polysaccharide fractions formed by cells exposed to $NaHC^{14}O_3$, glucose-C^{14} and $HC^{14}OOH$ is displayed in Table 7. These experiments were performed to determine if the radioactivity would be concentrated in the polysaccharide after exposure to the C^{14} sources during the complete growth cycle. From Table 7 it is apparent that approximately one-half of the total amount of C^{14} fixed is found in the polysaccharide fractions and is highest in the extracellular polymer.

Discussion and Conclusion

The extracellular polysaccharide produced by *Palmella mucosa* Kütz. is relatively simple in composition. The presence of the uronic acid moiety suggests that it may act as the core to which the other sugars of the polysaccharide are linked. The major components are glucose and fucose as shown by the molar ratio.

Table 7. *Incorporation of radioactivity into polysaccharide of Palmella mucosa Kütz. after 8 days exposure to radioactive carbon source*

Polysaccharide fraction and C^{14} source	Total CPM in fraction CPM*
1. $NaHC^{14}O_3$	
EP[1]	4,878
CP[2]	380
ICP[3]	3,052
Residual Cells	8,950
2. $HC^{14}OOH$	
EP	8,623
CP	640
ICP	3,600
Residual Cells	11,600
3. Glucose-C^{14}	
EP	154,151
CP	5,700
ICP	34,885
Residual Cells	251,550

[1] EP: Extracellular polysaccharide.

[2] CP: Capsular polysaccharide.

[3] ICP: Intracellular polysaccharide (water-soluble).

* CPM added: $NaHC^{14}O_3$ 576,240 cpm/100 ml; $HC^{14}OOH$ 904,080 cpm/100 ml; glucose-C^{14} 1,044,000 cpm/100 ml.

The production of extracellular polysaccharide is sharply dependent upon the age and metabolic activity of the algal cells. Polysaccharide production by growing cells closely parallels the curve for increase in cell dry weight with two exceptions: (1) a four day longer lag phase (4—8 days incubation) is evident with polysaccharide liberation and (2) the liberation of the polysaccharide continues slightly after the cells reach the stationary phase of the growth cycle.

Palmella mucosa Kütz. can grow at the expense of a variety of organic carbon sources. Glucose serves as a better carbon source for both cell and polysaccharide production than CO_2. The other carbon sources tested (acetate, lactate, pyruvate, glycerol-phosphate and formate) did not efficiently substitute for CO_2. As noted from Table 2, the oxidation of the organic acids (sodium salts) to provide energy and carbon for growth resulted in substantial increases in the pH of the medium. This trend towards increased alkalinity seems to be a limiting factor in cell growth by *Palmella mucosa* Kütz. However, the increase in pH was also evident with glucose as the carbon source but growth is not limited. Detrimental effects were observed with glucose as the sole carbon source. If the culture was successively carried in glucose solutions or in mineral salts agar containing glucose, almost all cells would be inactive on either CO_2 or glucose after about five transfers.

On examination of the constituents of the extracellular polysaccharides from cells grown in media with different carbon sources, it was found initially and confirmed on re-examination that variation in detectable monosaccharides exists with different carbon sources. With glucose as the sole carbon source, three additional spots were present of which two corresponded to R_f values for xylose and 2-deoxyribose while one R_f value could not be attributed to any of the common sugars or sugar derivatives. The 2-deoxyribose spot was again detected with lactate as the carbon source. The uronic acid moiety could not be demonstrated when acetate, pyruvate and glycerol-phosphate were substituted for CO_2 as the sole carbon source. The explanation for this variation cannot be given at this time.

Under the conditions used, calcium nitrate serves as the best source for cell and polysaccharide syntheses. Potassium nitrate produces a response slightly lower than with calcium nitrate. The increase of alkalinity of the medium with KNO_3 and $Ca(NO_3)_2$ was approximately the same. Sodium nitrate was not utilized as efficiently as the above nitrate salts and a slight decrease in pH towards acidity was recorded. Ammonium nitrate is undesirable as a nitrogen source because cell and polysaccharide production is barely above the response for the control. A drastic pH change towards acidity was recorded indicating the medium used could not counteract the excess hydrogen ions produced by the assimilation of ammonium nitrate. This phenomenon has been shown by other workers (Pratt and Fong 1940) using ammonium nitrate as the nitrogen source in media with low buffering capacities.

Bicarbonate-C^{14} is not incorporated into extracellular polysaccharide except after prolonged exposure. Later results showed that the intracellular polysaccharide is labeled first, suggesting that it is subsequently transferred to capsular polysaccharide and eventually sloughed off into the medium as extracellular polysaccharide. The 12 and 24 hour labeling patterns of the three fractions showed the stepwise transfer with the $NaHC^{14}O_3$ and no initial build-up of C^{14} was evident in the intracellular polysaccharide.

When growing cells are exposed to $NaHC^{14}O_3$, most of the label is found in the non-polysaccharide fraction. However, when glucose-C^{14} is supplied to growing cells, a large amount of C^{14} is still found in the non-polysaccharide fraction but the polysaccharide is also significantly labeled.

The exposure of *Palmella mucosa* Kütz. to $NaHC^{14}O_3$, $HC^{14}OOH$ or glucose-C^{14} during prolonged incubation results in a large uptake of C^{14} into the extracellular and intracellular polysaccharides.

Summary

An extracellular polysaccharide composed of glucose, fucose, arabinose and glucuronic acid in a molar proportion of 11 : 6 : 3 : 1 is a major end-product of photosynthesis by *Palmella mucosa* Kütz.

The liberation of polysaccharide is related to the age of the culture. Glucose can substitute efficiently for CO_2 as the source of carbon for polysaccharide synthesis. Nitrate-nitrogen from sodium, potassium and calcium salts can be used in the mineral salts medium with little differences in carbon metabolism of the alga. Ammonium nitrate produces an acidic medium which limits polysaccharide production.

The incorporation of C^{14} into the polysaccharide from $NaHC^{14}O_3$ shows initially a trend toward intracellular synthesis. The C^{14} appears in the extracellular polysaccharide after prolonged exposure. Glucose-C^{14} is actively transformed to polysaccharide material which is an indication that glucose may play an important role in the synthesis of polysaccharide by *Palmella mucosa* Kütz.

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Pure Culture of *Anabaena flos-aquae* A-37

SEVERAL methods have been proposed for the isolation of bacteria-free cultures of algae[1-6]. Methods reported to provide some measure of success, using antibiotics, ultra-violet light, chlorine compounds, etc., are fairly drastic and, in some cases, mutagenic. The problem of separating algae from bacteria may be related to a binomial distribution function in which the probability of choosing a bacteria-free algal colony in any trial varies in an unknown fashion from one trial to another. Successful, practical use of the proposed method may be expected only when the chance of choosing a bacteria-free algal colony exceeds about one in twenty.

Since the skill of the operator doubtless contributes to success, the method succeeds in proportion to the amount of operator bias employed in choosing uncontaminated colonies. This method has as its aim the separation of algae from the contaminating bacteria by the simple techniques of 'positive operator bias' coupled with numerous replication.

Accordingly, the method consists of (*a*) creating artificial predominance of the chosen alga by accumulation with a micropipette, (*b*) culture of the accumulated algal cells in liquid HGZ medium, a *tris*-buffered modification of the medium employed by Hughes, Gorham and Zehnder[7], (*c*) culture on HGZ agar dilution plates, (*d*) repetition of (*b*) and (*c*) alternately to obtain uni-algal cultures and to show which is the highest dilution in which algal colonies grow well separated and in small numbers, (*e*) preparation of 20 replicate plates of the plate dilution which affords good colony separation, (*f*) daily microscopic observation of plates from which approximately 20 sub-cultures are made into liquid HGZ, (*g*) after incubation and growth, inoculation of 1 ml. of the HGZ liquid cultures into nutrient broth. Observation at 48 h indicates the presence or absence of bacteria.

All the algae employed have been isolated from either pond water or sewage, using micropipettes to collect several units of a species for culture. Isolates were cultured alternately in agar dilution plates and in liquid medium to produce uni-algal cultures. Cultures were incubated in the light at 40° C on HGZ. Serial dilutions of isolates in HGZ agar were observed at least once each 24 h. A low-power, wide-field microscope was employed, using magnifications of the order of 90×.

It was soon found that several replatings reduced the apparent number of types of bacterial contaminants which would grow on HGZ medium from as many as 10 to one or two types. These plating trials also indicated the limits of dilution for any given culture, beyond which

growth of the alga could not be expected with useful frequency. It also suggested that, contrary to the opinions of some research workers, the bacteria often outnumber the algae by more than ten to one in what might appear to be a fairly 'clean' culture of algae grown in mineral salts medium.

Having determined the limiting dilution for the growth of a culture of algae, 20 poured plates were prepared at the limiting dilution less one. Growth was meticulously observed daily, using the wide-field microscope, to find the algal colonies growing at maximal distances from each other and from bacterial colonies. All the colonies which appeared to afford good separation from the surrounding bacteria were cut out of the agar with an inoculating needle or a micro-scalpel and placed in liquid HGZ medium for incubation. If possible, 20 cultures were made. As soon as growth was observed, sub-cultures were made in nutrient broth. Absence of growth in nutrient broth after 48 h of incubation was accepted as proof of the absence of bacteria.

When this procedure was employed, *Anabaena flos-aquae A*-37, which was isolated in this laboratory from an oxidation pond located in Mississippi, grew through the 10^7 dilution. Of 16 sub-cultures on liquid HGZ, 11, or almost 70 per cent, were free of bacteria as judged by sub-culture on nutrient broth. Frequent sub-culture of these bacteria-free isolates continues to prove the absence of bacteria which will grow either on HGZ medium or in nutrient broth.

While it is admittedly impossible to provide complete proof of the purity of any algal culture from all bacteria, the 'positive operator bias' technique appears to increase the chances of success by at least one order of magnitude without engendering serious metabolic disturbance. It has been employed successfully in this laboratory, to provide bacteria-free cultures of two *Anabaena*, two *Polycystis*, two brown *Oscillatoria* and four green *Oscillatoria*. It is being used routinely in this laboratory for the bacteria-free isolation of other types of algae which may be of interest. This work was done in co-operation with the National Aeronautics and Space Administration.

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Nitrogen Fixation by the Blue-green Alga *Anabaena flos-aquae* A-37

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Abstract

Nitrogen fixation has been demonstrated in a bacteria-free culture of a semi-thermophilic blue-green alga, *Anabaena flos aquae* A-37, employing the criterion of an increase in cellular nitrogen as a basis for determining nitrogen fixation. This is apparently the first time that nitrogen fixation has been demonstrated in a pure culture of a blue-green alga which grows at 40 C and which produces abundant quantities of an extracellular heteropolysaccharide.

Introduction

Algal nitrogen fixation has been demonstrated only in members of the Myxophyceae, the blue-green algae. For many years it was believed that nitrogen-fixing blue-green algae belonged only to the family *Nostocaceae* (Fogg 1956, 1962). However, nitrogen fixation has now been demonstrated in *Mastigocladus laminosus*, family *Stigonemataceae* (Fogg 1951), *Tolypothrix tenuis*, family *Scytonemataceae*, *Calothrix brevissima*, family *Rivulariaceae* (Watanabe *et al.* 1951), and *Oscillatoria subbrevis*, family *Oscillatoriaceae* (Moyse *et al.* 1957). Nitrogen fixation has been demonstrated in several members of the genus *Anabaena*, including *A. cylindrica* (Fogg 1942, Allen and Arnon 1955), *A. variabilis*, *A. gelatinosa*, and *A. naviculoides* (De 1939).

A blue-green alga was isolated in unialgal culture from a mixed stock culture designated A-37 which was obtained from a local oxidation pond. This blue-green alga was purified of all bacterial contaminants which can be detected by usual laboratory examination (Tischer 1965). The alga was identified by Dr. G. W. Johnston, Department of Botany, Mississippi State

Dry weight determinations

Dry weight determinations were obtained by filtering 10-15 ml of the blended cell suspension onto asbestos mats in tared Gooch crucibles and drying at 105° for 1 hour. The weights of the crucibles were obtained with a five place balance.

Nitrogen determinations

Nitrogen determinations were made on 5-15 ml sub-samples of the blended cell suspension, employing the semi-micro Kjeldahl method. Nitrogen determinations were expressed as mg in terms of dry weight. All values are the mean of two determinations.

Procedure

Culture chambers containing 120 ml of the basal medium were inoculated with a blended cell suspension of A. flos-aquae A-37 (10 % by volume) and grown under conditions for nitrogen fixation. The control, an uninoculated bottle of the culture, was aerated in series according to the method of Fay and Fogg (1962). The control served as a measure of the nitrogen contaminants which might have escaped the traps. The cultures were assayed for cellular dry weight and cellular nitrogen.

Results and Discussion

Anabaena flos-aquae A-37 was able to grow and fix nitrogen in the basal medium aerated with washed air-5 % CO_2 mixture as shown in Table 1. The mean per cent increase in cellular nitrogen ranged from 285 per cent

Table 1. *Nitrogen fixation by Anabaena flos-aquae A-37 in cultures aerated with the washed air-5 % CO_2 mixture in relation to growth.* The control for all experiments consisted of an uninoculated bottle of medium and, in all cases, it showed insufficient nitrogen to be detected with the Kjeldahl method. Three repetitions and average values.

Experiment	Cellular nitrogen mg/mg of inoculum	Dry weight mg		Cellular nitrogen mg		Increase in cellular nitrogen %	
I	$\frac{0.532}{7.5} = 0.07$	81.68		4.41		727	
		86.44	83.60	4.28	4.16	703	682
		82.74		3.81		616	
II	$\frac{0.280}{4.0} = 0.07$	16.91		0.99		254	
		21.94	19.25	1.18	1.07	323	285
		16.88		1.03		280	
III	$\frac{0.820}{11.5} = 0.07$	51.00		3.56		457	
		33.30	34.00	3.01	3.86	377	371
		27.30		3.12		320	
IV	$\frac{0.850}{12.0} = 0.07$	97.07		5.07		496	
		80.36	87.31	4.78	4.97	432	485
		85.88		5.07		496	

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up to 682 per cent. Since elemental nitrogen was the only nitrogen source present in sufficient quantity to account for the increase in cellular nitrogen, the observed increase in cellular nitrogen and the accompanying increase in cell mass could only have been the result of nitrogen fixation.

There was not a sufficient amount of nitrogenous substance liberated into the medium or control during the short growth period (about 5 days) to be detected by the Kjeldahl method.

Conclusion

From these results, it can be concluded that a pure culture of the high temperature heteropolysaccharide producing blue-green alga, *Anabaena flos-aquae* A-37, possesses the ability to fix atmospheric nitrogen. It may be conjectured that A. flos-aquae A-37 fixed nitrogen in its natural environment.

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CARBOHYDRATE METABOLISM IN HYDROGENOMONAS EUTROPHA[1]

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Fructose was found to be the only carbohydrate of the 12 tested that can be utilized as a carbon and an energy source by *Hydrogenomonas eutropha* although glucose, ribose, xylose, and arabinose were isolated from the spent medium on which this organism had grown. This organism can adapt to fructose under an air atmosphere as well as under an H_2-O_2-CO_2 atmosphere.

Fructose-grown cells of *H. eutropha* oxidize fructose almost completely to CO_2 and water whereas autotrophically grown cells exhibit a respiratory quotient of about one-half of the theoretical value.

H. eutropha grew on "autoclaved glucose" medium although the cells failed to oxidize glucose in resting cell experiments. Apparently, these cells were growing at the expense of fructose formed from the glucose during autoclaving.

Introduction

Brown, Cook, and Tischer (1964) demonstrated the presence of carbohydrate material in the spent inorganic medium on which the chemoautotrophic microorganism, *Hydrogenomonas eutropha*, had grown. Radioautograms of the chromatographically separated ^{14}C-labeled carbohydrate material indicated that several sugars were present in the spent medium. One of these sugars was identified as ribose.

The utilization of carbohydrates by *H. eutropha* has not been studied extensively. Bovell (1957) reported that *H. eutropha* utilized glucose and sucrose as both a carbon and an energy source, but that this organism did not ferment glucose, sucrose, lactose, or mannitol to acid and gas. Bovell did not elaborate on the procedures used to sterilize the sugars or the medium employed. Repaske and Lizotte (1965) reported the use of cells of *H. eutropha* grown on 0.1% glucose in an inorganic medium under an air atmosphere but they did not state the methods used to sterilize the medium.

The studies reported herein are concerned with the identification of three sugars, in addition to ribose, found in the spent medium on which *H. eutropha* had grown, the inability of this organism to utilize the sugars tested except fructose, and certain changes that occur in glucose autoclaved with phosphate buffer.

Materials and Methods

Basal medium.—The basal inorganic salts medium employed in these investigations was Respaske's (1962), modified by deleting sodium bicarbonate and doubling buffer concentration.

"Autoclaved glucose" medium.—Glucose in a concentration of 0.5% was

[1]Material in this paper was taken in part from the dissertation of D. W. Cook, presented to the Faculty of Mississippi State University in partial fulfillment of requirements for the Ph.D. degree in Microbiology. This work was performed with the assistance of a grant from the National Aeronautics and Space Administration and a NASA predoctoral traineeship to the senior author.

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autoclaved (121 °C for 15 minutes) in 0.05 *M* phosphate buffer. After the medium was cooled, the other components of the basal medium were added.

H_2-O_2-CO_2 gas mixture.—The mixture used contained approximately 66% hydrogen, 22% oxygen, and 11% carbon dioxide. This was chosen because hydrogenomonads were reported to use these gases in a ratio of 6:2:1 (Lechtman, Goldner, and Canfield 1964).

Measurement of carbohydrates.—Reducing sugars were measured by the procedure of Nelson (1944), using a modification of Somogyi's arsenomolybdate method, and by the anthrone method (Morris 1948). Standard concentration curves for these procedures were prepared using reagent grade α-D-glucose.

Chromatographic procedures.—Chromatographic separation of sugars was made on Whatman No. 1 chromatographic paper employing one-dimensional descending paper chromatograms. The solvent systems used were phenol–water–NH_3 (water-saturated phenol containing 1% ammonium hydroxide), 1-butanol–acetic acid–water (60/15/25, v/v/v), 1-butanol–pyridine–water (80/80/40, v/v/v). The detection reagents were silver nitrate reagents, triphenyltetrazolium chloride reagents, and aniline phthalate reagent (Hais and Macek 1963).

Production and preparation of cell-free spent medium.—*H. eutropha* was grown in 500 ml of basal medium in 1-liter Erlenmeyer flasks on a New Brunswick rotary action shaker (200 r.p.m.) at 30 ± 2 °C. The flasks were connected to a gas reservoir containing the H_2–O_2–CO_2 mixture and kept at a pressure of 0.5–1.0 p.s.i. (Cook 1966). The optical density[3] of the culture was checked periodically and when the rate of growth began to diminish at the end of the log phase, growth was terminated. The medium was freed of cells by centrifugation with a Sharples super centrifuge and passage through a Seitz filter. The cell-free spent medium was desalted by passage through a Dowex 50W-X8 strong cation exchange column and then through a Dowex 3 weak anion exchange resin. The desalted medium was then concentrated on a rotary evaporator under vacuum at 60 °C. Normally 6 liters of cell-free spent medium yielded 10 ml of desalted concentrated material which contained the neutral organic compounds (carbohydrates).

Manometric studies.—Manometric studies with washed resting cell suspensions of *H. eutropha* were carried out at 30 °C by standard Warburg manometric techniques as outlined by Umbreit *et al.* (1964). All cells were grown in liquid medium and harvested after 12 to 14 hours incubation.

Results and Discussion

Three sugars in addition to ribose were found in the cell-free spent medium on which *H. eutropha* had grown. From the color reaction with the aniline phthalate reagent, one of the spots was identified as an aldohexose and two of the spots identified as aldopentoses. The data in Table I show that the sugars found in the spent medium were glucose, xylose, ribose, and arabinose.

The carbohydrates were then analyzed quantitatively by two different procedures, with glucose as the standard. The procedure of Nelson (1944), in which Somogyi's reagent is used, led to an average estimate of 4 mg of

[3]Determined using a Coleman Model 9 nephocolorimeter.

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TABLE I

Identification of sugars found in cell-free spent medium by paper chromatography
Solvent A: phenol – water – NH_4OH
Solvent B: butanol – acetic acid – water
Solvent C: butanol – pyridine – water

Sample	Solvent A, $R_f \times 100$	Solvent B, $R_{Rham} \times 100$	Solvent C, $R_G \times 100$
From spent medium			
Spot 1	37	55	102
Spot 2	52	65	123
Spot 3	45	74	141
Spot 4	63	84	159
Standards			
Rhamnose	—	100	—
Glucose	37	52	100
Arabinose	52	65	120
Xylose	44	74	141
Ribose	61	83	158
Deoxyribose	78	111	—

carbohydrates per liter. Quantitative analysis by the anthrone method (Morris 1948) showed an average of 11 mg of carbohydrate per liter. It was speculated that the actual amount of carbohydrates in the medium lies somewhere between these two figures.

It seemed of interest to determine whether *H. eutropha* metabolized these sugars. Glucose was the first sugar to be studied.

Little or no oxygen uptake was observed with α-D-glucose (dissolved in distilled water) by resting cells prepared from cultures grown either autotrophically or heterotrophically (nutrient broth).

When a normal air atmosphere (no H_2) was employed, *H. eutropha* failed to grow on the basal medium to which was added either 0.5% filter-sterilized glucose or 0.5% glucose autoclaved in distilled water.

Uniformly labeled ^{14}C-glucose was used to determine if *H. eutropha* utilized glucose when growing under a H_2–O_2–CO_2 gas mixture. Filter-sterilized glucose at a concentration of 0.5% containing uniformly labeled ^{14}C-glucose was added to the basal medium. The medium was inoculated and incubated under air or under a H_2–O_2–CO_2 gas mixture. Samples were taken periodically and assayed for radioactivity after removal of cells by centrifugation. During the 40-hour incubation period normal growth occurred in the samples incubated under the H_2–O_2–CO_2 gas atmosphere; however, no growth occurred in the samples incubated under the air atmosphere. The decrease in the radioactivity after incubation was less than 0.98% in all samples. The manufacturer of the labeled glucose (Nuclear-Chicago) states that its purity as determined by paper chromatography ranges from 98.2 to 99.1%. Therefore, it was concluded that this slight reduction in radioactivity was insignificant.

H. eutropha grew on "autoclaved glucose" medium although the cells failed to oxidize glucose in resting cell experiments.

Changes which occurred in the glucose autoclaved in the buffer were noted as the acquisition of a brownish-yellow cast to the colorless medium and the ability of the medium to support the growth of *H. eutropha* under an air

TABLE II.

Oxidation of glucose autoclaved in various pH buffers by *Hydrogenomonas eutropha*

Initial pH of buffer and glucose*	Color change during autoclaving	Oxygen consumed in 90 minutes, μl†
3.0	None	18
4.0	None	20
5.0	None	37
6.0	Light yellow	228
7.0	Brownish-yellow	372
8.0	Brownish-yellow	377
9.0	Brownish-yellow	376
10.0	Brownish-yellow	367

*Phosphate buffer (0.05 molar) with 0.5% glucose.
†28 μmoles glucose added/flask; 0.4 mg protein N/flask; endogenous of 60 μl (for 90 min) substracted.

atmosphere when the other components of the basal medium were added. To demonstrate that these changes were due to the hydroxyl ion concentration rather than the presence of phosphate ions, glucose was autoclaved in 0.05 *M* phosphate buffer formulated to pH values ranging from 3.0 to 10.0 (Table II) by the addition of acid or base where necessary. It is realized that above pH 8.0 and below pH. 5.0, there is little or no buffering capacity. After autoclaving, a change in the color was noted only in the samples ranging from pH 6.0 to pH 10.0. All samples were neutralized to pH 7.0 and supplied to cells grown on the "autoclaved glucose" medium in manometric studies. Table II shows that little oxygen consumption occurred in cells supplied with glucose autoclaved at pH 6.0 or lower. When glucose was autoclaved in distilled water which had been adjusted to pH values of 7.0 to 10.0 with NaOH, the changes in the medium were not as pronounced. Discoloration of the medium occurred only at pH values above 9.0. In the medium with an initial pH of 8.0 or lower, the final pH was always 4.0 or lower.

The concentration of the buffer was relatively unimportant in changing the glucose during autoclaving as long as the buffer concentration was able to hold the pH above 6.0 during autoclaving.

From these results, it was concluded that the buffer or added base in higher concentrations held the hydroxyl ion concentration sufficiently high to allow alteration of the glucose at the extreme temperature of autoclaving. With the low concentrations of buffer or base, apparently slight changes in the medium quickly lowered the hydroxyl ion concentration below the critical point so that degradation was stopped.

The other sugars found in the spent medium in which *H. eutropha* had been grown were tested to determine if they could support the growth of *H. eutropha*. None of these sugars (ribose, xylose, or arabinose) were able to support growth when the filter-sterilized sugar was added to the basal medium. Likewise, little or no oxygen consumption was observed when these sugars were supplied to autotrophically grown or heterotrophically (nutrient broth) grown cells of *H. eutropha*.

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TABLE III

Growth of *Hydrogenomonas eutropha* in the presence of various sugars

Medium	Measurements	Arabinose	Fructose	Galactose	Glucose	Lyxose	Mannose	Rhamnose	Ribose	Sedoheptulose	Sorbose	Sucrose	Xylose	Control
Basal medium with sugar under air	Final O.D.*	0.05	1.25	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
	Initial sugar (mg/ml)	3.60	5.00	2.10	4.80	4.10	2.70	3.50	3.20	—	4.00	—	5.10	0.00
	Final sugar (mg/ml)	3.70	0.00	2.10	4.90	4.10	2.60	3.50	3.20	—	4.00	—	5.50	0.00
Basal medium with sugar under H_2–O_2–CO_2	Final O.D.	1.45	2.65	1.45	1.50	1.45	1.45	1.60	1.50	1.50	1.40	1.45	1.40	1.50
	Initial sugar (mg/ml)	3.70	5.10	2.20	4.90	4.20	2.70	3.00	3.30	—	4.00	—	5.10	0.00
	Final sugar (mg/ml)	3.70	0.00	2.10	5.00	4.20	2.80	3.10	3.40	—	3.90	—	5.00	0.00
Nutrient broth with sugar under air	Final O.D.	2.30	3.00	2.35	2.25	2.30	2.30	2.25	2.25	2.35	2.35	2.25	2.[illegible]	2.35
	Initial sugar (mg/ml)	3.20	4.90	2.30	5.00	4.10	2.70	2.90	3.50	—	4.50	—	5.00	0.00
	Final sugar (mg/ml)	3.30	0.00	2.40	4.90	4.00	2.70	3.00	3.50	—	4.40	—	4.90	0.00

*Initial optical density = 0.05 in all cases.
†These sugars gave no color reaction when Nelson's (1948) method was used.

Unlike glucose, these sugars would support only sparse growth of *H. eutropha* when they were autoclaved in the presence of the buffer after the addition of the other components of the basal medium; however, all of these sugars exhibited a color change in the medium from colorless to brownish-yellow during autoclaving.

A number of other sugars (rhamnose, fructose, galactose, lyxose, mannose, sorbose, sedoheptulose, and sucrose) were studied to determine if they could be utilized by *H. eutropha*. All sugars were filter-sterilized and added to the medium at a concentration of 0.5%. Each sugar was placed in basal medium and incubated under air or under the H_2–O_2–CO_2 gas mixture. Each sugar was also used to supplement nutrient broth. These media were inoculated and incubated for 36 hours. Before and after incubation, the sugar concentration of each sample was determined, by the method of Nelson (1944). Table III exhibits the inability of *H. eutropha* to attack any of the sugars except fructose. It should also be noted that none of the sugars except fructose affected the growth at a concentration of 0.5%.

Manometric studies were conducted to demonstrate the utilization of fructose. Figure 1 illustrates its use by both autotrophically grown and fructose-grown cells of *H. eutropha*. As shown, cells grown on fructose use fructose immediately; however, autotrophically grown cells require an adaptation period of about 20 minutes before they show any oxygen consumption.

Table IV shows the oxygen utilization, carbon dioxide production, and respiratory quotient for both types of cells that use fructose. The respiratory quotients of the cells grown under the various conditions were vastly different.

TABLE IV

Oxygen consumption, carbon dioxide production, and respiratory quotients of *Hydrogenomonas eutropha* cells metabolizing fructose

Cell type*	Time, min	O_2 consumed,† µl	CO_2 produced,† µl	R.Q.
Cells grown autotrophically	10	0	0	0
	20	2	0	0
	30	6	0	0
	40	13	0	0
	50	26	8	0.37
	60	42	17	0.41
	70	65	33	0.50
	80	75	34	0.45
	90	89	36	0.40
Cells grown on fructose	10	29	20	0.69
	20	55	41	0.75
	30	85	66	0.78
	40	114	94	0.88
	50	133	115	0.86
	60	153	132	0.86
	70	172	169	0.99
	80	196	183	0.93
	90	216	193	0.90

*Both cell suspensions contained approximately 0.25 mg of protein nitrogen per milliliter.

†Corrected for endogenous respiration, which was 35 µl O_2 consumed and 19 µl CO_2 produced by autotrophically grown cells and 81 µl O_2 consumed and 46 µl CO_2 produced by fructose-grown cells during the 90 minutes.

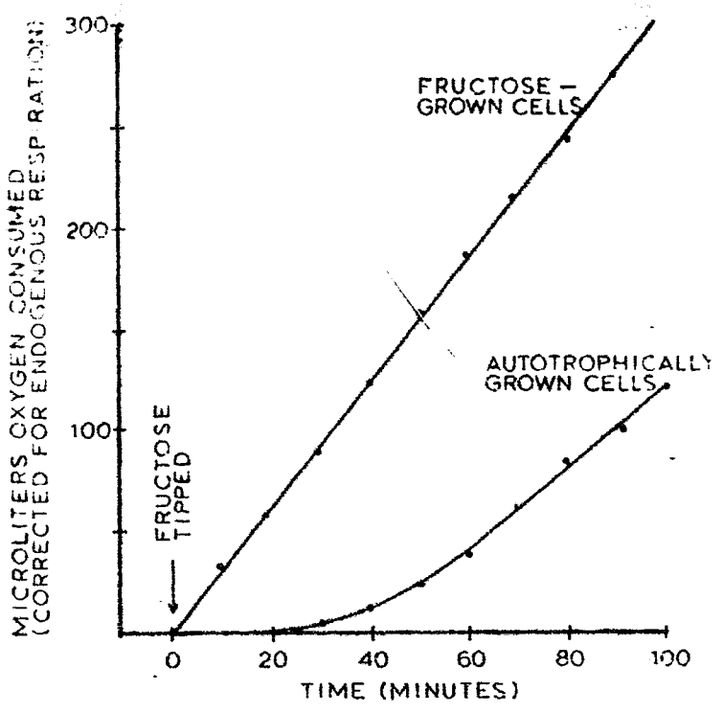

FIG. 1. Oxidation of fructose by resting cells of *Hydrogenomonas eutropha*.

With the fructose-grown cells the respiratory quotient rose steadily until it approached the theoretical value of one. This indicated that the fructose was almost quantitatively oxidized to CO_2 and water. With the autotrophically grown cells the respiratory quotient remained at zero until the cells were adapted to fructose and then quickly rose to a value of about one-half of theoretical and remained constant. These results indicated either that the autotrophically grown cells were not oxidizing the fructose completely to carbon dioxide and water or that the cells were fixing about one-half of the carbon dioxide which they produced.

When cells grown on the "autoclaved glucose" medium were supplied fructose in manometric experiments, they utilized it without lag, as if the cells had been grown on fructose.

The "autoclaved glucose" medium was desalted and analyzed on paper chromatograms. Upon the development of the chromatograms two spots appeared which had R_f values corresponding to those obtained with glucose and fructose. These results led to the conclusion that during autoclaving of the glucose in the buffer some glucose was transformed into fructose, probably by a mechanism similar to the Lobry de Bruyn-Albeida von Ekenstein transformation. Thus it can be seen how the autoclaving of carbohydrates in a medium containing a buffer can lead to inaccurate conclusions, as in this case where the organism was growing on the fructose rather than the glucose. The apparent discrepancy between the work reported herein and that of Repaske and Lizotte (1965) may be clarified by Repaske, for he stated (personal communication, 1966) that although the glucose he used for growing *H. eutropha* was filter-sterilized, an adaptive period of 2 to 4 weeks was required before growth began; additionally, the culture became pigmented (bright yellow) and he suspected that a mutant had developed.

Autotrophically grown cells of *H. eutropha* were shown to adapt to and utilize fructose under a H_2–O_2–CO_2 atmosphere as well as under an air atmosphere. When autotrophically grown cells were given fructose under the H_2–O_2–CO_2 atmosphere, the fructose disappeared without affecting the rate of gas utilization as compared with a control flask containing no fructose. This may indicate that *H. eutropha* can use fructose and hydrogen simultaneously.

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Addendum[1]

Recent studies have indicated that *Hydrogenomonas eutropha* uses the carbohydrate derivative, gluconic acid. As demonstrated by manometric studies with autrotrophically grown resting cells, this organism requires an adaptive period of about 20 min before it oxidizes fructose or gluconic acid. When the autotrophically grown resting cells were adapted to fructose, they utilized both fructose and gluconic acid without an additional lag period. When the cells were adapted to gluconic acid, they could not utilize fructose without an additional lag period.

Since the phosphate ester of gluconic acid is a major intermediate in the Entner–Doudoroff pathway, enzyme studies were performed to determine whether the enzymes associated with this pathway were present in the organism. Studies were conducted on cell extracts from sonically ruptured cells which had been grown in the basal medium containing fructose. After the cells were ruptured, the cell extract was centrifuged to remove whole cells and cell fragments. Enzyme assays were performed as described by Gottschalk, Eberhardt, and Schlegel (1). With the use of these assay procedures, the presence of the following enzymes was demonstrated in fructose grown cells: phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydratase, and phospho-2-keto-3-deoxy gluconate aldolase. These enzymes were also found in extracts from autotrophically grown cells, but the concentrations were much less than in the fructose grown cells.

[1]Addendum received January 1, 1967.

These studies did not exclude other metabolic pathways for the metabolism of fructose from being operative in *Hydrogenomonas eutropha*. The fact that this organism can use gluconic acid, that cells adapted to fructose were sequentially induced to use gluconic acid, and that the concentration of the enzymes associated with the Entner–Doudoroff pathway were increased when the organism was adapted to fructose, suggested the conclusion that *Hydrogenomonas eutropha* metabolized fructose by the Entner–Doudoroff pathway.

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XVI. Davis, Edith B., and Tischer, R.G. Photochemical Reduction of Elemental Nitrogen by Anabaena flos-aquae A-37. Nature 212:302-303. 1966.

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Photochemical Reduction of Elemental Nitrogen by *Anabaena flos-aquae A-37*

Anabaena cylindrica has been shown to reduce elemental nitrogen photochemically[1,2]. *Anabaena cylindrica* is readily cultivated at room temperature, and it was of interest to determine if a blue-green alga with an optimum temperature of 39° C possessed this ability.

Anabaena flos-aquae A-37 was isolated from an oxidation pond and obtained in unialgal bacteria free culture[3]. This alga was identified by Dr. G. W. Johnston, Department of Botany, Mississippi State University.

Anabaena flos-aquae A-37 has been shown to possess the ability to fix atmospheric nitrogen[4] and to be an abundant producer of an extracellular polysaccharide[5]. The assets of this alga could be increased if it were capable of reducing elemental nitrogen according to the following assumed equation[1]:

$$N_2 + 3H_2O \xrightarrow{\text{Light}} 2NH_3 + 1{\cdot}5\ O_2$$

and was therefore capable of producing oxygen other than by photosynthesis.

To establish whether *Anabaena flos-aquae A*-37 was capable of reducing elementary nitrogen photochemically, manometric techniques were used. The cells to be investigated were grown on a modification of the mineral salts medium of Dyer and Gafford[6]. The modifications consisted of omitting all nitrogen except for that present as contaminants in the chemicals used, and substituting ferric citrate (0·030 g/l.) for ferric chloride. The trace elements used were a modification of those recommended by Gafford[7]. The modifications consisted of substituting other salts of the desired element for those which contained nitrogen and raising the molybdenum content from 0·9 p.p.m. to 10·0 p.p.m. The mixture was aerated with 5 per cent carbon dioxide at 40 ± 2° C for 5 days. The cells were collected by centrifugation and were washed twice in sterile basal medium under aseptic conditions and, in most cases, were depleted of nitrogen by aerating for 12 h with a 5 per cent carbon dioxide–90 per cent argon mixture. The cells were blended in a Waring blender and then centrifuged. Next, the cells were resuspended in 9 ml. of the basal medium, and the percentage light transmittance was obtained on a 1/10 dilution using white light. The percentage light transmittance was correlated with standard curves in order to obtain the cellular dry weight and cellular nitrogen corresponding with that value.

The cells were nitrogen-depleted in an effort to increase nitrogen uptake. During the period of depletion, carbon

Table 1. PHOTOCHEMICAL REDUCTION OF ELEMENTAL NITROGEN BY NORMAL CELLS OF *Anabaena flos-aquae A*-37

Time (min)	Nitrogen fixed* (μl.)	Oxygen produced* (μl.)	O_2/N_2	Argon control change in gas volume (μl.)
10	3	4	1·34	0
25	5	7	1·40	3
40	10	14	1·40	3
55	11	15	1·37	3
70	12	18	1·50	3
85	9	15	1·67	2
100	14	20	1·43	3
115	12	18	1·50	2
130	15	23	1·53	3
			$\bar{x}$ 1·46	

$Q_{N_2}^{N_2}(N) = 104$; $Q_{N_2}^{N_2} = 7{\cdot}5$; $Q_{O_2}^{N_2}(N) = 156$; $Q_{O_2}^{N_2} = 11{\cdot}3$.

* These are cumulative totals.

was still assimilated and stored as a carbohydrate reserve which allowed for substantial nitrogen uptake when the cells were exposed to free nitrogen.

The Warburg flasks used had double side arms with one side arm connected to the centre wall. The main compartment of the flask contained 2·3 ml. of the basal medium and 0·5 ml. of cells. The flasks were flushed for 10 min with either nitrogen or argon and 0·4 ml. of chromous chloride was then added to the side arm connected to the centre well of half the flasks. 0·4 ml. of distilled water was added to the remaining flasks. The flasks were then flushed for another 5 min. The gassing was carried out in the light. Readings were begun after 15 min and continued at 15 min intervals for the duration of the experiment, usually about 2 h.

Manometric measurements were carried out using a conventional Warburg respirometer equipped with four 20 W white fluorescent lamps. Chromous chloride (prepared and handled as described in a communication with Dr. R. M. Brown as a modification of the technique developed by M. Cramer as reported by Myers[8]) was used as the oxygen absorbent.

The data obtained from the manometric studies are expressed as follows. (The superscript refers to the atmosphere and the subscript to the gas being measured.)

O_2/N_2; ratio of μl. oxygen produced to μl. nitrogen fixed.

$Q_{N_2}^{N_2}(N)$; μl. nitrogen fixed in an atmosphere of nitrogen per mg of cellular nitrogen per hour.

$Q_{N_2}^{N_2}$; μl. nitrogen fixed in an atmosphere of nitrogen per mg dry weight of cells per hour.

$Q_{O_2}^{N_2}(N)$; μl. oxygen produced in an atmosphere of nitrogen per mg cellular nitrogen per hour.

$Q_{O_2}^{N_2}$; μl. oxygen produced in an atmosphere of nitrogen per mg dry weight of cells per hour (ref. 9).

Nitrogen fixation and oxygen production are plotted in relation to time.

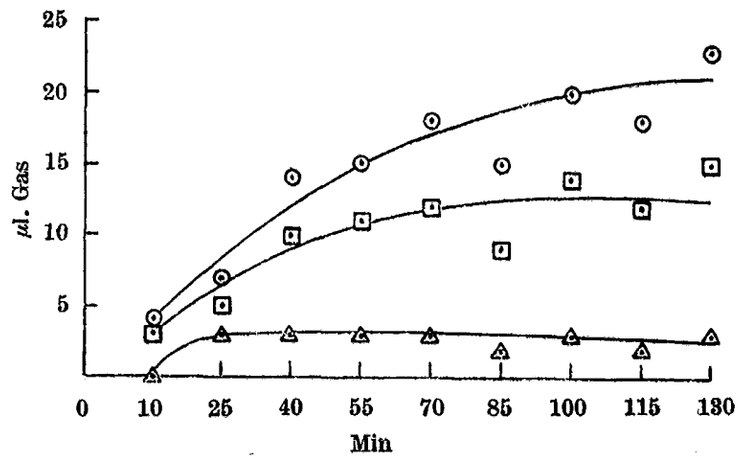

Fig. 1. Photochemical reduction of elemental nitrogen by normal cells of *Anabaena flos-aquae A*-37. △, Argon control; ⊡, nitrogen uptake; ⊙, oxygen produced.

The results obtained from the manometric studies with *Anabaena flos-aquae A*-37 indicate that this alga is capable of reducing elemental nitrogen with concomitant oxygen production.

Table 1 contains the results found when normal cells not depleted of nitrogen were used as the inoculum. The ratio of oxygen produced to the nitrogen fixed ranges from 1·34 to 1·67 with a mean value of 1·46. This is very close to the theoretical value of 1·50 (ref. 1). Fig. 1 contains these data plotted in relation to time.

The results in Table 2 were obtained when nitrogen depleted cells were used as the inoculum. Here the ratio of oxygen produced to nitrogen fixed ranges from 1·45 to 1·72 with a mean value of 1·58. The response from the nitrogen depleted cells was much greater than that of the normal cells, which is to be expected as the nitrogen depleted cells would have a carbohydrate reserve which would enable the cells to assimilate nitrogen at a more rapid rate. The carbohydrate reserve would be used for the manufacture of protein and other nitrogenous substances as the required nitrogen became available. Fig. 2 contains the data plotted in relation to time.

The indication that *Anabaena flos-aquae A*-37 is capable of reducing elementary nitrogen photochemically would have great value in a system which required more oxygen than could be derived from photosynthesis alone.

Table 2. Photochemical Reduction of Elemental Nitrogen by Nitrogen Depleted Cells of *Anabaena flos-aquae A*-37

Time	μl. nitrogen fixed	μl. oxygen produced	O_2/N_2
35	5	14	–
50	29	42	1·45
65	32	48	1·50
80	35	54	1·54
95	36	57	1·58
110	36	59	1·64
125	36	62	1·72
140	37	60	1·62
			$\bar{x}$ 1·58

$Q^{N_2}_{N_2}(N) = 188$; $Q^{N_2}_{N_2} = 12{\cdot}1$; $Q_{N_2}(N) = 283$; $Q^{N_2}_{O_2} = 18{\cdot}1$.

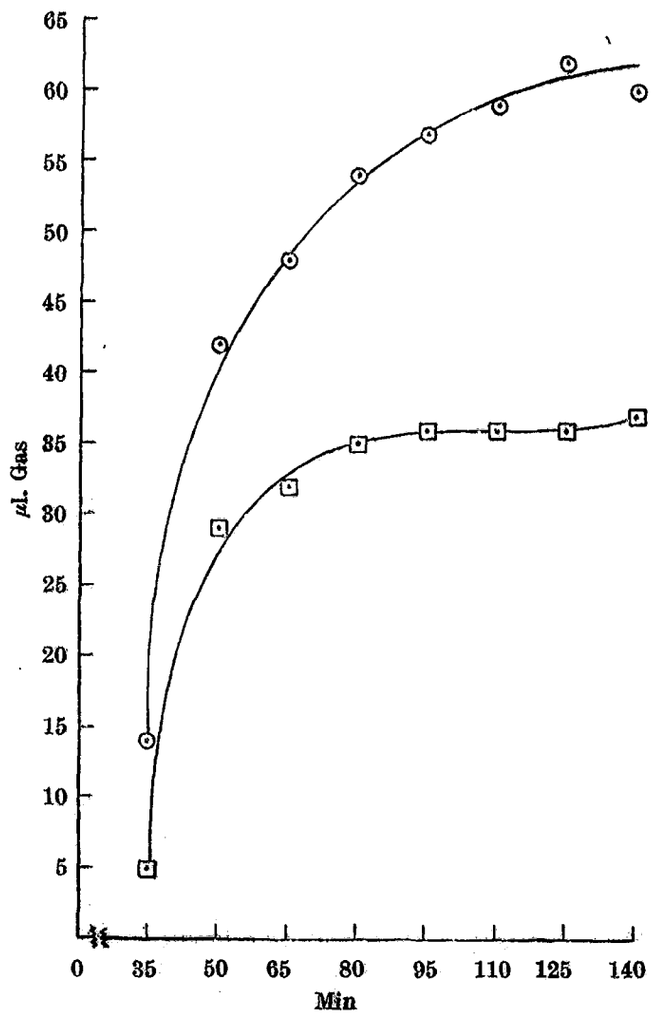

Fig. 2. Photochemical reduction of elemental nitrogen by nitrogen depleted cells of *Anabaena flos-aquae A-37*. ⊡, Nitrogen uptake; ⊙, oxygen produced.

To make use of basic equations to illustrate this fact, let us assume that the following reactions occur:

$$6\ CO_2 + 6\ H_2O \text{ —— } C_6H_{12}O_6 + 6\ O_2$$
$$3\ H_2O + N_2 \text{ ——— } 2\ NH_3 + 1{\cdot}5\ O_2$$

$$6\ CO_2 + 9\ H_2O + N_2 \text{ —— } C_6H_{12}O_6 + 2\ NH_3 + 7{\cdot}5\ O_2$$

A 25 per cent increase in oxygen production could be gained using an alga with this ability.

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XVII. McGee. J.M., L.R. Brown, and R.G. Tischer. A High-Temperature Hydrogen-Oxidizing Bacterium--*Hydrogenomonas thermophilus*, n.sp. Nature. 214:715-716. 1967

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A High-temperature, Hydrogen-oxidizing Bacterium—*Hydrogenomonas thermophilus*, n.sp.

WE have succeeded in isolating in pure culture from petroliferous soil a thermophilic bacterium which utilizes hydrogen. The isolation was accomplished by streaking material from a soil enrichment culture on to Repaske's mineral salts medium prepared by mixing equal volumes of a sterile 4 per cent agar solution in water and a sterile, double-strength solution of mineral salts containing ammonium chloride, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, potassium dihydrogen phosphate, sodium monohydrogen phosphate, and several trace elements. On cooling to about 50° C, 1 ml. of a solution of ferrous ammonium sulphate was added, the plates poured, allowed to solidify and immediately streaked. The plates were incubated in an atmosphere containing 70 per cent hydrogen, 20 per cent oxygen, and 10 per cent carbon dioxide at 50° C. By picking the minute, flat, smooth, translucent colonies into liquid mineral salts medium and incubating in the 70–20–10 gas mixture, a pure culture was obtained.

It seems that early attempts to obtain colonies of this organism by streaking failed because of the method used to prepare the Repaske's agar plates. This organism apparently does not grow on Repaske's medium when all the ingredients are autoclaved together, or if the plates are held for 24 h before streaking.

The organism has been characterized as follows. It is a Gram-negative rod about 1·3μ by 0·6μ, which occurs in pairs, or occasionally singly, or in short chains. It does not form spores, does not possess a capsule, nor is it acid-fast. It propels itself by means of a single polar flagellum.

The organism produces an alkaline reaction when it is grown in glucose, lactose, or sucrose broths with peptone as a nitrogen source. No growth was observed on gelatine medium, glucose–phosphate–peptone water, or citrate broth; no nitrites were detected in heterotrophic nitrate broth. The bacterium grew in tryptophan broth but no indole was produced. We have not detected the production of any hydrogen sulphide by the organism. These studies in heterotrophic media were conducted at both 30° C and 50° C. Growth at 30° C was always much slower than at 50° C.

It can be seen from Table 1 that the optimum temperature for growth of this organism under autotrophic conditions is about 50° C. This experiment was carried out with modified Sohngen units; static conditions were used. Like certain other members of the genus *Hydrogenomonas*,

Table 1. EFFECT OF TEMPERATURE ON GAS CONSUMPTION BY A HIGH TEMPERATURE HYDROGEN-UTILIZING BACTERIUM (STATIC CONDITIONS)

Incubation time	Percentage of gas in Sohngen unit consumed at 20° C	30° C	40° C	50° C	60° C
3 days	0	Trace	33	100	0
7 days	0	60	100	–	0
14 days	0	75	–	–	0

the new isolate appears to be sensitive to oxygen. Under stationary conditions of incubation, the organism will grow in an atmosphere containing 10, 20, 30 or 40 per cent oxygen. When the medium is incubated with constant shaking, no growth occurs at either 30 or 40 per cent oxygen, but good growth is obtained at 10 per cent oxygen. The results obtained at 20 per cent oxygen are questionable, and more definitive investigations in this region are being carried out at present.

The organism described here has been tentatively classified as a species of *Hydrogenomonas*. It does not fit any of the descriptions for species listed in *Bergey's Manual* nor does it agree with all the characteristics of *H. eutropha*. At the present time, we regard it as a new species of *Hydrogenomonas*, and suggest the name *Hydrogenomonas thermophilus* n.sp.

The newly isolated hydrogenomonad, besides being something of a biological oddity and having potential value in fixing hydrogen gas in a closed ecological system such as would exist in long space flights, is of interest for several other reasons. First, the bacterium, as well as other hydrogen bacteria, may be a missing link in the chain of events that led to the evolution of aerobic forms of life from anaerobes, which by current theory are placed as the first living organisms on Earth[2–4]. It is thought that anaerobes evolved first because there is evidence that the Earth had a reducing atmosphere rather than the oxidizing atmosphere which we know today. According to Oparin[4], the Earth lost most of its free hydrogen at the time the planet was formed. A number of processes, including radioactive decay, could have liberated free hydrogen gas into warm bodies of water at a time when the atmosphere was becoming oxidized, however, thus providing an environment suitable for the development of an organism identical with, or at least similar to, our *H. thermophilus* n.sp. The fact that hydrogen gas is usually included (and is therefore apparently necessary) in the reaction mixture for the abiogenic synthesis of biological compounds[1,2] seems to indicate that hydrogen in the free state may have played an important part in the earliest stages of the evolution of life. Furthermore, it is possible that thermophilic organisms utilizing hydrogen (or hydrocarbon) may exist on other planets in the Solar System. This possibility should not be overlooked when samples from such planets are analysed for life.

This work was carried out in co-operation with the U.S. National Aeronautics and Space Administration.

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XVIII. Mickelson, J.C., E.B., Davis, and R.G. Tischer. The Effect of Various Nitrogen Sources Upon Heterocyst Formation in Anabaena flos-aquae A-37. Journal of Experimental Botany. 18:397-405.

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The Effect of Various Nitrogen Sources upon Heterocyst Formation in *Anabaena Flos-Aquae* A–37[1]

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WITH THREE FIGURES IN THE TEXT

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ABSTRACT

Heterocysts are formed abundantly in cultures of *Anabaena flos-aquae* A–37 which are fixing nitrogen. Fewer are formed in the presence of NO_3^- ions, whereas none occurred in the presence of NH_4^+ ions. The results indicate that heterocysts may have a role in nitrogen metabolism.

INTRODUCTION

HETEROCYSTS are found in a large number of blue-green algae which fix free nitrogen. However, they are also found in some which do not, for example, *Aphanizomenon flos-aquae* (Williams and Burris, 1952). *Chlorogloea fritschii* (*Chroococcales*) fixes nitrogen (Fay and Fogg, 1962) and was first thought to lack heterocysts; however, their presence was later demonstrated (Fay, Kumar, and Fogg, 1964). Heterocysts are known to increase in numbers throughout the growth period in non-continuous culture. Fogg (1944) demonstrated that metabolic depletion of nitrogen salts from the medium was a factor accompanying the increase of heterocysts in *Anabaena cylindrica*. He showed further that ammonia, and to a lesser extent nitrate, inhibited heterocyst formation which was greatest when the alga was fixing nitrogen (Fogg, 1949; 1951).

Several hypotheses have been advanced with respect to the function of heterocysts. They attracted attention as early as 1856 when Carter suggested a role in spore formation. Subsequently it has been suggested that they induce spore formation (Wolk, 1965), that they are storage vessels for food reserves (Hieronymus, 1892; Hegler, 1901; Fritsch, 1904), that they are vestigial reproductive bodies (Brand, 1901; Spratt, 1911; Geitler, 1921) and that they are the source of a stimulus to cell division and growth (Schwabe, 1947; Fritsch, 1951). On the other hand, Fogg (1944) and Geitler (1960) suggest that an inverse relationship exists between heterocysts and the frequency of

[1] This work was done in co-operation with the National Aeronautics and Space Administration, Washington, D.C.

cell division and growth. Fogg (1951) suggested that heterocysts serve as a mechanism for releasing materials, especially nitrogenous substances, to adjacent cells when the nitrogen available in the medium is at a minimum. Fogg (1944, 1949) also suggested that the heterocyst may be involved in nitrogen fixation; however, Fay and Walsby (1966) have shown that little or no $^{15}N_2$ was fixed by the isolated heterocysts of *A. cylindrica*.

Our objectives were to determine if the nitrogen source affected heterocyst production in a bacteria-free culture of *Anabaena flos-aquae* A–37 (Tischer, 1965), whether a relationship occurred between heterocysts and nitrogen metabolism (especially on free nitrogen) (Davis, Tischer, and Brown, 1966), and to attempt to shed further light on some of the hypotheses advanced previously concerning heterocysts.

METHODS

The basal medium and growth conditions employed were as described by Davis, Tischer, and Brown (1966). The pH was adjusted to 7·5–8·5 with KOH before autoclaving. The various nitrogen sources, prepared as sterile stock solutions, were added in the concentration necessary to provide the same amount of nitrogen as in KNO_3 (1 g/l) which was selected as the standard. The inoculum was prepared as described by Davis *et al.* (1966) and size was expressed in ml/100 ml. The purity of the culture was determined as described by Tischer (1965) and by microscopic examination. The cultures were aerated with an air-5 per cent CO_2 mixture, except where free nitrogen was omitted in which case these cultures were aerated with an argon-5 per cent CO_2 mixture.

Heterocyst frequency was determined either by Fogg's method (1944) which consisted of examining sufficient filaments, regardless of fields, to obtain about 400 cells and counting the heterocysts on those filaments or by using a Howard mould (counting) chamber and counting a sufficient number of fields to provide a representative estimate of cells and heterocysts. Only heterocysts attached to filaments were counted and the number was expressed as per cent to obtain the frequency of occurrence. Cells/ml and heterocysts/ml were obtained by multiplying the average number per field by a microscope factor calculated for 450× magnification. Nitrogen and dry-weight determinations were performed as described by Davis *et al.* (1966).

Heterocyst formation in relation to the nitrogen source

A. flos-aquae A–37 (1 per cent by volume) was grown in bottles which contained 100 ml of the basal medium each with a different nitrogen source. Fogg's method (1944) was employed for one experiment and the Howard mould counting chamber for others.

Heterocyst formation on different nitrogen sources in relation to time.

An inoculum (8 per cent by volume) prepared from adapted cells, i.e. cells previously grown on the nitrogen source, was placed in the basal medium

either with or without nitrate or ammonium chloride. The cultures were aerated as above except that the air-5 per cent CO_2 mixture was washed with 1 per cent $NaHCO_3$ and 25 per cent H_2SO_4 to remove all nitrogenous compounds except free nitrogen (Fay and Fogg, 1962). Samples were removed aseptically at 24-h intervals and the following determinations were made: cells/ml, heterocysts/ml, and heterocyst frequency. These determinations were made with the Howard mould-counting chamber.

Unadapted inoculum (10 per cent by volume), i.e. cells not previously grown on the experimental nitrogen source but grown on the basal medium with aeration by the washed air-5 per cent CO_2 mixture, was employed for the basal medium with and without nitrate or ammonium chloride. The cultures were aerated and the determinations made as for the adapted cells.

RESULTS AND DISCUSSION

Table 1 shows the heterocyst frequency (determined by Fogg's method) under both aerated and static conditions when different nitrogen sources or

TABLE 1

Heterocyst frequency in Anabaena flos-aquae *A-37 cultures when grown on various nitrogen sources as determined by Fogg's method*

These were obtained by counting as many fields as necessary to obtain over 400 cells; they are not based upon any particular volume; and they are, therefore, only relative. All values are the mean of five replication.

Nitrogen source	Conditions	Filaments no.	Cells no.	Heterocysts no.	Frequency %
N_2	Aerated[1]	17·0	447	34	7·56
NO_3	Aerated[2]	19·6	431	7	1·60
$NO_3 + N_2$	Aerated[1]	10·2	446	4	0·88
N_2	Static[3]	19·0	421	43	10·20
None	Static[4]	15·0	427	58	14·00

[1] Washed air-5 per cent CO_2 mixture.
[2] Argon-5 per cent CO_2.
[3] Gassed with a mixture of 4 per cent N_2, 5 per cent CO_2, and a balance of helium.
[4] Gassed with a mixture of 89 per cent H_2, 5 per cent CO_2, and a balance of helium.

no nitrogen source was employed. There were large differences among the aerated cultures; those fixing nitrogen had the greatest heterocyst frequency. Of the static cultures, those without a nitrogen source had the larger frequency. We should point out that the values in Table 1 are relative and are not to be taken as a measure of growth. For example, it required several times as many fields to obtain the counts for the cultures with no nitrogen as it did for any of the other cultures.

The results in Table 2 were obtained by growing *A. flos-aquae* A-37 on various nitrogen sources. It is observed that the cultures fixing nitrogen had the greatest heterocyst frequency in all replications. The cultures with nitrate had substantially fewer heterocysts, thus indicating partial inhibition in comparison with the cultures grown on free nitrogen; complete inhibition was observed in all cultures containing ammonia.

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TABLE 2

Effect of nitrogen source on growth and heterocyst production

Each value is the mean of two determinations made using the Howard mould counting chamber. R-1, R-2, and R-3 designate the replication.

Nitrogen source	Cells/ml × 10^{-7}	Heterocysts/ml × 10^{-6}	Heterocyst frequency %
N_2			
R-1	2·9	1·4	4·75
R-2	1·7	1·6	9·20
R-3	2·4	1·6	4·70
Mean	2·3	1·5	6·21
KNO_3			
R-1	2·9	2·1	1·00
R-2	1·9	1·0	5·30
R-3	3·1	1·3	2·55
Mean	2·3	1·5	2·95
NH_4NO_3			
R-1	3·5	0·0	0·00
R-2	1·2	0·0	0·00
R-3	3·3	0·0	0·00
Mean	2·7	0·0	0·00
NH_4Cl			
R-1	2·7	0·0	0·00
R-2	2·2	0·0	0·00
Mean	2·4	0·0	0·00
Na_2NO_3			
R-1	3·2	1·2	1·53
R-2	2·2	0·87	4·75
R-3	1·5	0·37	1·40
Mean	4·0	0·76	2·36
$Mg(NO_3)_2$			
R-1	7·1	1·8	2·58
R-2	0·8	0·35	4·35
R-3	5·7	1·1	2·00
Mean	4·5	1·1	3·04
$NH_4H_2PO_4$			
R-1	0·18	0·0	0·00
R-2	1·3	0·0	0·00
R-3	6·0	0·0	0·00
Mean	2·3	0·0	0·00

Fig. 1 presents heterocyst frequencies in relation to time in adapted cells. There was an important difference between the effects of nitrogen source on heterocyst frequency and heterocysts/ml. For cultures on free nitrogen (A) the linear regression between heterocyst formation and time was significant at the 5 per cent level of probability, whereas that for cultures on nitrate (B)

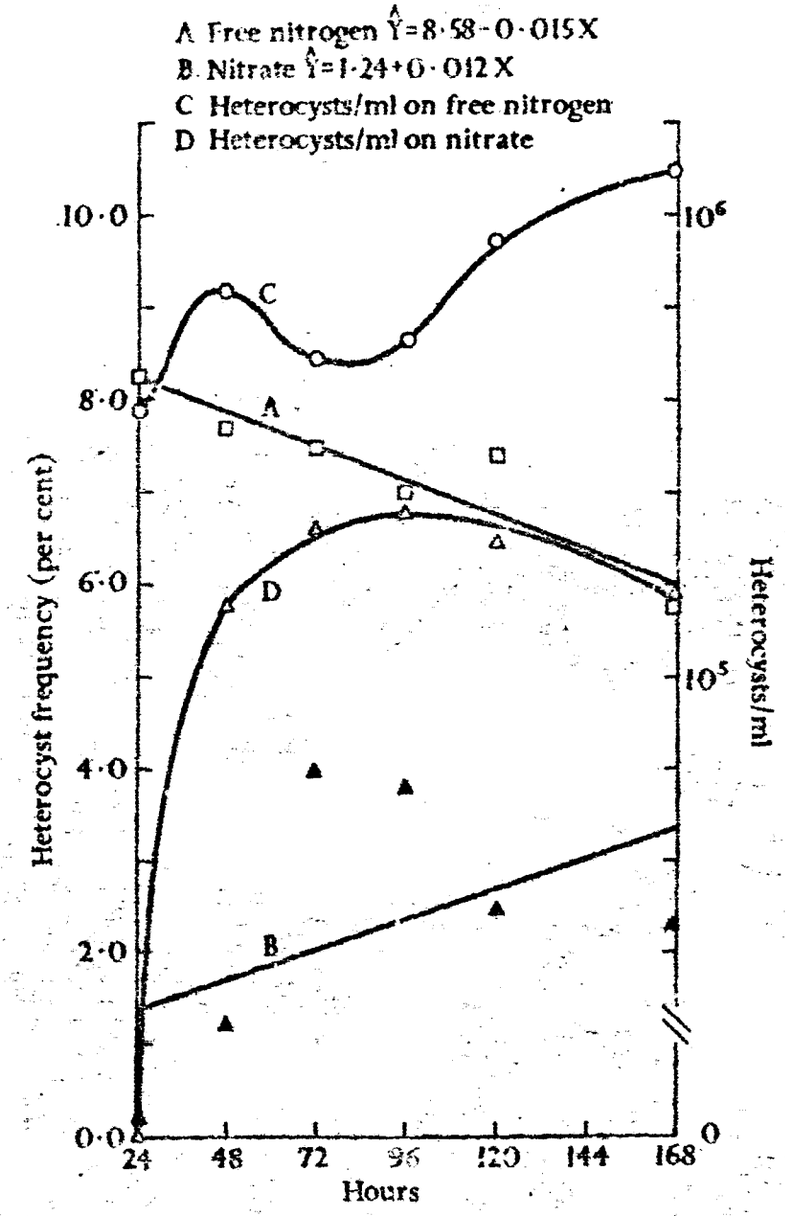

FIG. 1. Heterocysts/ml and linear regressions for heterocyst frequencies in adapted cells. The equations are valid only for the values of X shown on the graph. X equals hours.

was non-significant. Heterocyst formation was completely inhibited by ammonium chloride. There were more heterocysts/ml in the cultures which were fixing free nitrogen (C) than in those utilizing nitrate (D). The values of heterocysts/ml are observed after 72 h to increase on free nitrogen and decrease on nitrate. Heterocyst frequency also decreases with time because the increase in the number of heterocysts does not keep pace with the number of cells.

Fig. 2 includes the linear regressions of heterocyst formation with time both on free nitrogen (A) and on nitrate (B) when non-adapted cells were employed for the inoculum. Both regression lines have negative slopes. Analyses of variance show these regressions to be highly significant at the 1 per cent level of probability for both nitrogen sources. It is also observed that both the heterocyst frequency and heterocyst/ml were greater in the

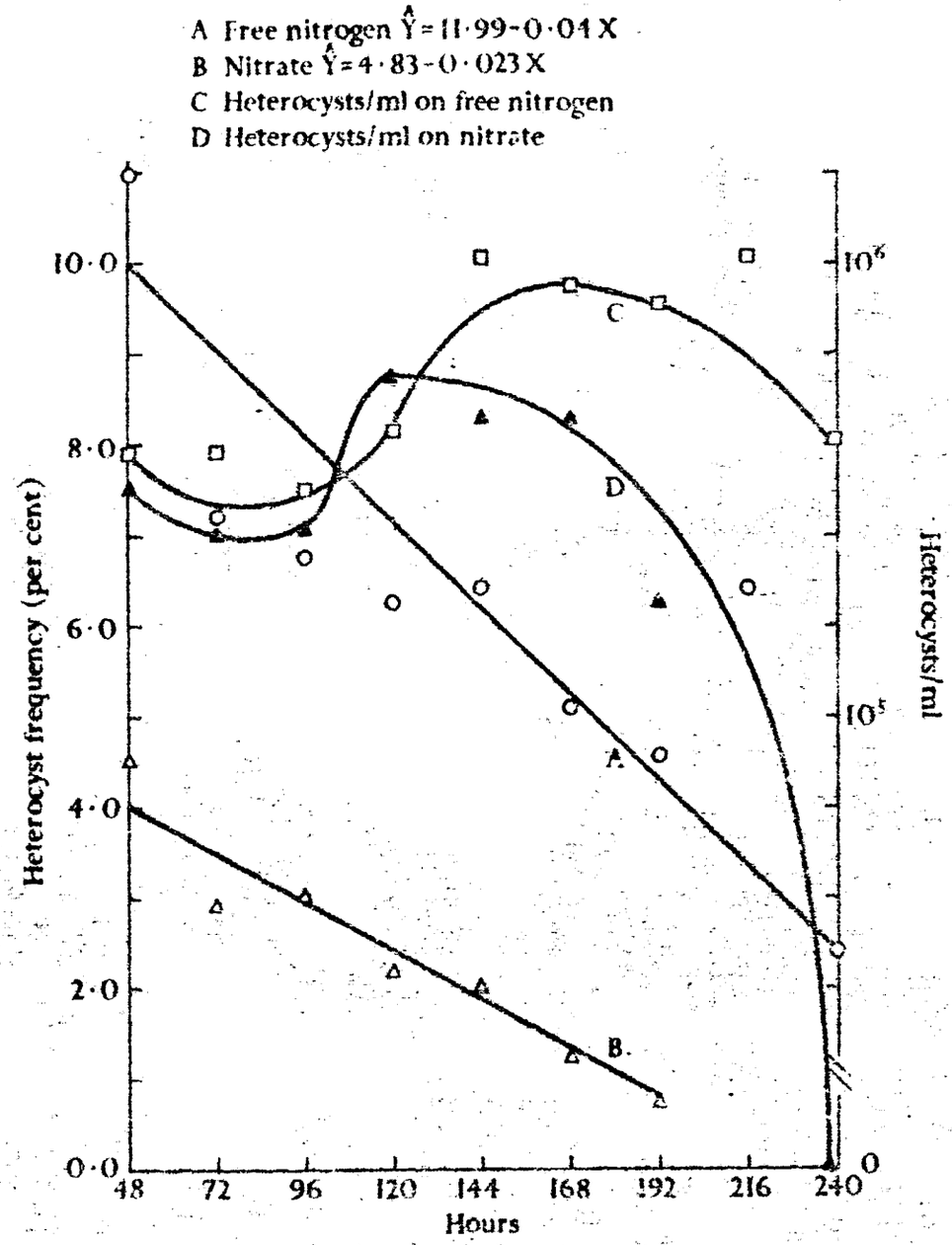

FIG. 2. Linear regression for heterocyst frequencies in non-adapted cells. The equations are valid only for the values of X on the graph. X equals hours.

cultures supplied with free nitrogen (A), (C). The heterocyst frequency decreased with time, and at the end of 240 h, showed a frequency of 2·4 per cent in the cells fixing nitrogen and zero in the cells utilizing nitrate. Here again heterocyst formation was completely inhibited by ammonium chloride. Heterocysts/ml increased and then remained fairly constant on free nitrogen (C), whereas after 144 h on nitrate (D) numbers dropped rapidly to zero.

Fig. 3 shows the linear regressions of heterocyst frequency and cellular nitrogen, expressed as mgN/mg dry weight, when either free nitrogen or nitrate was employed as the nitrogen source. It is of interest to note that the heterocyst frequency decreased as the nitrogen content of the cells increased. Analyses of variance for these regressions indicated that the relationship

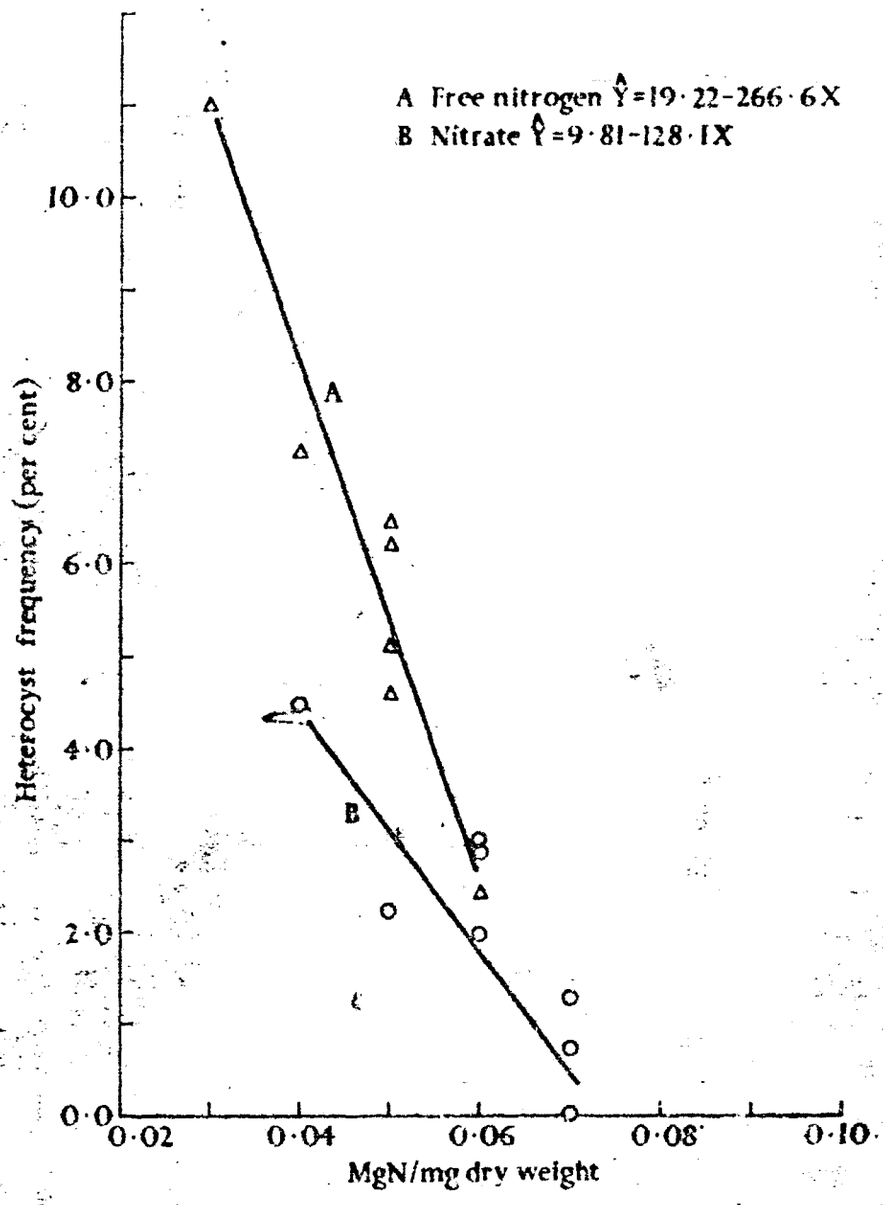

FIG. 3. Relationship between heterocyst frequency and mgN per mg dry weight when free nitrogen or nitrate was employed as the nitrogen source. The equations are valid only for the values of X on the graph. X equals mgN/mg dry weight.

between heterocyst frequency and mgN/mg dry weight was highly significant at the 1 per cent level of probability for the cultures fixing nitrogen, whereas that on nitrate was only significant at the 5 per cent level of probability.

CONCLUSIONS

Several conclusions may be drawn from the data presented herein. First, the nitrogen source appears to have a direct influence upon heterocyst formation. More heterocysts are formed when the culture is fixing free nitrogen

than when growing on any combined nitrogen source tested. Heterocyst formation was completely inhibited by any nitrogen source which contained ammonia. These results agree with those obtained by Fogg with *Anabaena cylindrica*. Second, it was observed that the absence of any nitrogen source resulted in the greatest frequency of heterocysts, indicating that heterocysts may have some role in nitrogen metabolism. Third, excellent growth was obtained in those cultures which did not produce heterocysts. This seems to discount the hypotheses and suggestions put forth by Schwabe (1947), Fritsch (1951) and Fogg (1951) that heterocysts perform various secretory and regulatory functions. However, an inverse relationship does appear to exist between heterocyst numbers in and growth of those cultures which contain them, which agrees with the observations of Fogg (1944) and Geitler (1960).

The following hypothesis relating the heterocyst to nitrogen metabolism may be put forth. It has been proposed (Wilson and Burris, 1947) that ammonia is a key intermediate in the pathway by which nitrogen is fixed. If such is the case, one would expect ammonia and salts containing ammonium ions to be assimilated more easily than free nitrogen or nitrates. It stands to reason that the ease with which the nitrogen source is utilized would have a bearing on the energy required for assimilation. Therefore, the heterocysts may be an indication of the work and energy required for the cell to utilize a particular nitrogen source.

It seems reasonable to speculate that heterocysts might play a controlling role both in economizing and recycling nitrogen under conditions of its virtual absence from the medium. In accord with this hypothesis, Wildon and Mercer (1963) asserted that a protoplasmic connexion exists between heterocysts and filament cells. An added point in support of this hypothesis is the observation that cultures which contain heterocysts tend to clump and aggregate much more than the cultures which do not contain heterocysts, e.g. cultures fixing free nitrogen or utilizing nitrate aggregate more than those growing on ammonium salts.

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Some Observations on the Sodium and Potassium Interactions in the Blue-green Alga *Anabaena flos-aquae* A-37

By

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Abstract

The growth of *Anabaena flos-aquae* A-37 has been shown to be severely limited by the absence of either sodium or potassium from the culture medium. Neither element is capable of replacing the other. The addition of sodium to sodium-starved cells restores growth while potassium-starved cells are not affected by the addition of potassium.

Introduction

It has been indicated by a number of workers (1, 2, 5) that the *Cyanophyta*, or blue-green algae, are unique in that sodium is required for normal growth. From reports in the literature it also appears that just as certain species of blue-green algae are unable to grow in the absence of sodium and the presence of adequate potassium, growth is also repressed by the absence of potassium from a medium rich in sodium. The studies described in this paper were initiated in order to demonstrate similar effects of sodium and potassium deficiences in *Anabaena flos-aquae* A-37.

Methods and Materials

Anabaena flos-aquae A-37 was isolated in unialgal, bacteria-free culture by Tischer (7). This alga has been shown to fix atmospheric nitrogen (3) and to produce abundant quantities of an extracellular polysaccharide (6).

The culture medium employed was similar to that described by Hughes, Gorham, and Zehnder (4). The modifications of the medium were as follows: Sodium-deficient

medium was prepared by replacing all sodium salts with the corresponding potassium salts on an equimolar basis. Potassium-deficient medium was prepared by similarly replacing all potassium salts with the corresponding sodium salts. The sodium and potassium content of the control medium was 40.5 mg/l and 188 mg/l, respectively. Extreme care was taken to avoid contamination of all glassware, culture vessels, *etc.*, with the cation in which the medium was deficient. All chemicals were of reagent grade and the water used for preparing culture medium and rinsing was of the highest purity (ca. 50 ppb as NaCl) produced from Consolidated Model EX2-V2 still. Cultures were incubated for seven days under constant illumination provided by four forty-watt daylight fluorescent bulbs (13,455 lux) at 40±2°C. The culture vessels consisted of six-ounce prescription bottles, each containing 100 ml of medium. Cellular dry weights were obtained by collecting cells onto tared Millipore filters and drying overnight at 110°C before weighing to constant weight.

The original inocula of one ml each for each transfer in these experiments were taken from a homogeneous cell suspension containing less than 0.5 mg dry weight of cells per milliliter in order that the initial cell concentration for each transfer would be the same. Prior to these experiments the algal culture had been carried in a normal mineral salts medium which contained a full complement of Na and K. Subsequent one ml inocula were prepared after pooling 5 ml from each sodium-deficient culture. The cells contained in the pooled 15 ml sample were washed, resuspended and employed as the inoculum in the subsequent trials using one third of the inoculum for each flask.

The rates of inoculation were 0.5 mg to Transfer I; 3.0 mg to Transfer II, and 1.0 mg to Transfer III, which represents 5 % of the cells grown in each previous culture.

Results and Discussion

The effect of the absence of sodium on the growth of *A. flos-aquae* A-37 is shown in Table 1. From these data it may be observed that when the alga

Table 1. *The effect of sodium-deficient medium on the growth of Anabaena flos-aquae A-37* (dry weight mg/100 ml).

Medium	Reps	Transfer I	Transfer II	Transfer III
Sodium-deficient	1	56.5	18.0	12.5
	2	72.0	21.5	8.0
	3	66.0	19.0	7.5
	$\bar{x}$	64.8	19.5	9.3
Sodium-deficient after NaCl addition	1	90.0	112.5	100.5
	2	110.0	97.5	89.0
	3	92.0	105.0	93.5
	$\bar{x}$	97.3	105.0	94.3
Control	1	92.5	95.0	82.5
	2	98.5	89.5	105.0
	3	103.0	91.0	97.0
	$\bar{x}$	98.0	91.8	94.8

Table 2. *The effect of potassium-deficient medium on the growth of Anabaena flos-aquae A-37* (dry weight mg/100 ml).

Medium	Reps	Transfer I	Transfer II
Potassium-deficient	1	34.0	18.0
	2	36.5	21.5
	3	43.0	27.5
	$\bar{x}$	37.8	22.3
Potassium-deficient after added KCl	1	82.5	21.5
	2	80.0	30.0
	3	89.0	30.5
	$\bar{x}$	83.8	27.3
Control	1	90.0	81.5
	2	110.0	90.5
	3	92.0	88.5
	$\bar{x}$	97.3	86.8

was carried for three consecutive transfers in sodium-deficient medium, growth for Transfers I, II and III was 66.6, 18.6, and 9.3 per cent respectively, of that supported by the control medium. The decrease in growth with each transfer may be explained by the fact that the sodium originally contained within the cells is depleted by utilization as the cells are grown in the medium deficient in sodium. The growth which did occur on the third transfer is attributed to sodium contamination from the chemicals, glassware, *etc.* From Table 1 it is also evident that the addition of sodium (5 mg/l) to the cells which had been sodium-starved for 7 days restored growth to the cultures equal to that of the controls following four days further incubation.

Statistical analysis reveals that the differences in growth among the sodium-deficient cultures previous to the addition of sodium, and the controls were significant at the 95 % level. After the addition of sodium to the medium statistical analysis failed to show a significant difference among the sodium-deficient cultures and the controls.

From Table 2 it may be observed that when the alga was cultured in potassium-deficient medium normal growth, as compared to controls, failed to occur. It should be noted here that in the case of potassium-deficient medium, it was not possible to restore growth to the cells by addition of potassium to the medium following seven days potassium starvation. From these data it is opined that potassium is required for normal growth of the alga and the absence of potassium from the medium irreversibly affects some necessary metabolic growth process.

Conclusions

From these data it is suggested that both sodium and potassium are required for the growth of *Anabaena flos-aquae* A-37 and that neither cation is able to replace the other. Moreover, further evidence is given in support

of the hypothesis that a sodium requirement is a characteristic of the blue-green algae.

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THE ELECTRODIALYSIS OF *ANABAENA FLOS-AQUAE* A-37

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(Received October 13th, 1967)

SUMMARY

A method for the electrolytic depletion of ions from *Anabaena flos-aquae* A-37 was described. The electrolytic depletion of Na^+, K^+, Ca^{2+}, and Mg^{2+} was determined to be 96.4%, 64.4%, 50.0%, and 5.4%, respectively. The alga survived the electrolytic treatment to the extent of from 40 to 50%.

From these data it was suggested that the electrodialysis method described is a workable research tool for the removal of ions from the organism.

INTRODUCTION

The concept of removing charged particles from plant tissues with the aid of an electrical current has been investigated by a number of workers. NELLY[1] makes reference to the work of LISBONNE AND VULQUIN[2] in 1912 as being among the first to successfully employ an electric current for the depletion of substances from plant cells. MAKSIMOW[3] later employed electrolytic techniques to determine the effectiveness of electrodialysis for cation depletion of tobacco leaves, grain, straw, and various seed types. COLLINS AND GRIMMETT[4] have reported on the electrolytic removal of salts from the concentrated extracts of a variety of plant tissues. MULLISON[5] and NELLY[1] have described procedures for electrolytically desalting pea seeds.

The studies described in this paper were initiated in order to demonstrate a useable method for the electrolytic demineralization of the blue-green alga *Anabaena flos-aquae* A-37 and to study some of the effects of the electrodialysis treatment on the alga.

MATERIALS AND METHODS

The organism used for these studies was isolated in unialgal, bacteria-free culture by TISCHER[6]. This organism has been shown to fix actively, atmospheric nitrogen[7] and to produce abundant quantities of an extracellular heteropolysaccharide[8]. Optimum growth occurs at 40° and under constant illumination of approx. 13455 lux.

The culture medium employed was a mineral salts medium similar to that described by HUGHES, GORHAM AND ZEHNDER[9]. Nitrates served as the source of nitrogen.

The electrodialysis apparatus consisted of a clear plexiglass cell containing six compartments separated by Permaplex ion-exchange membranes as shown in Fig. 1. The electrodes were of high purity platinum with platinum wire connections. Current

was supplied by a well-filtered d.c. power supply capable of producing from 0 to 7500 V.

Prior to the electrolytic treatment, the cells were harvested and washed by centrifugation with deionized-distilled water. The water was produced from a Consolidated Model EX 2V-2 still, the distillate having an electrical resistance in the range of 12 to 15 MΩ.

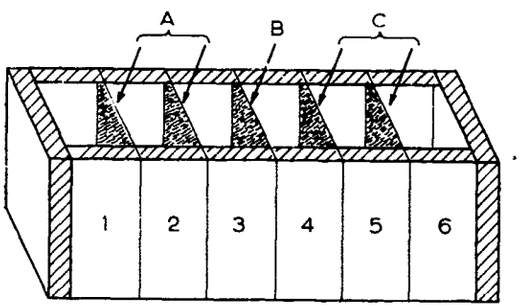


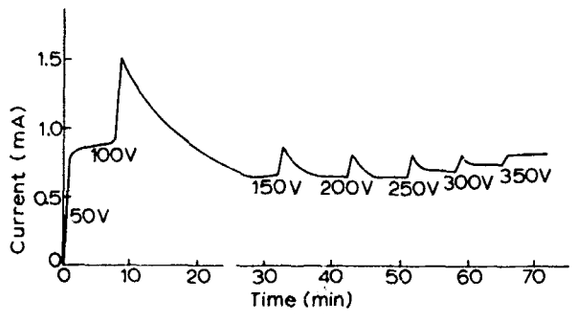
Fig. 1. Diagram of plexiglass cell used for electrolysis treatments. A, anion-exchange membranes; B, non-ionic semipermeable membrane; C, cation-exchange membranes. 1, anode compartment; 2, water compartment; 3, sample compartment; 4, sample compartment; 5, water compartment; 6, cathode compartment.

Electrodialysis was carried out by placing 5.0 ml of a dense cell suspension into 10.0 ml of water contained in the sample compartment of the apparatus. Voltage was applied in 50-V step-up intervals and current conducted through the apparatus was measured at 1-min intervals. It was found that this gradual increase in voltage prevented the large temperature rises associated with large initial voltage increases. Following each 50-V increase during the treatment the sodium, potassium, calcium, and magnesium content of the cells was measured. Measurement of cations was carried out by rupturing the cells in a Raytheon sonic oscillator for 45 min at 10000 Hertz. The cell-free extract thus obtained was then analyzed for cation content with a Beckman Model DU-2 flame spectrophotometer.

RESULTS AND DISCUSSION

The current conducted through the apparatus containing $1.05 \cdot 10^9$ cells per ml is shown in Fig. 2. From this figure it may be noted that the two initial 50-V increases were responsible for the highest peaks in current. It was then postulated that a majority of the ions released from the cells were released during the first 100 V of the treatment. Individual cation analyses, shown in Figs. 3–6, support this assumption. From Fig. 3 it may be noted that during the first 100 V of the treatment, 94.1 % of the cells' Na^+ was released. Further voltage increases failed to remove additional substantial amounts of Na^+ from the cells. The K^+ content of the cells, shown in Fig. 4, was decreased to the extent of 35.6 % during the first 100 V of the treatment, with an additional 28.8 % removal of the cation during the five subsequent voltage increases. Ca^{2+} depletion, shown in Fig. 5, was 33.3 % during the first 100 V. Further voltage increases were responsible for the removal of an additional 16.7 %. The Mg^{2+} content of the cells was hardly affected by the treatment, as may be observed in Fig. 6. Removal of Mg^{2+} was 3.0 % during the first 100 V and 2.4 % as a result of the subsequent increases in voltages.

It was observed that during the initial 50 V of the treatment, the cells rapidly migrated to the anode side of the sample compartment of the electrolysis apparatus. This behavior is attributed to the possibility that cations removed from the cells



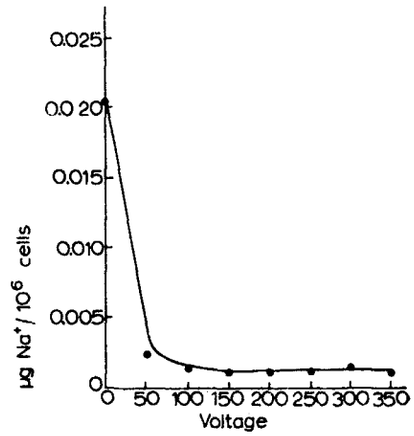
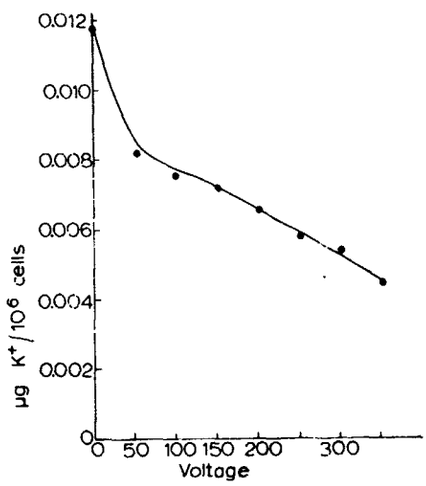


Fig. 2. The relationship of current to time and voltage during electrolysis treatment.

Fig. 3. The effect of electrolysis on the Na^+ content of *A. flos-aquae* A-37.



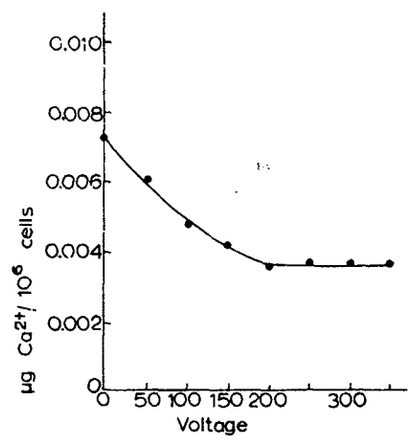


Fig. 4. The effect of electrolysis on the K^+ content of *A. flos-aquae* A-37.

Fig. 5. The effect of electrolysis on the Ca^{2+} content of *A. flos-aquae* A-37.

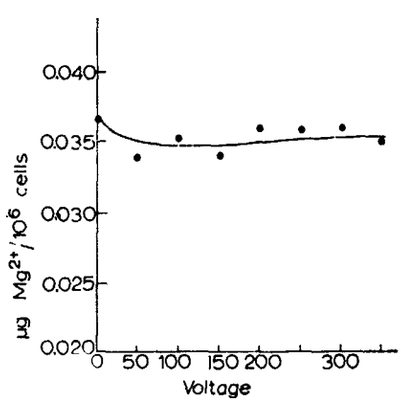


Fig. 6. The effect of electrolysis on the Mg^{2+} content of *A. flos-aquae* A-37.

are pulled away from large, bound organic molecules leaving the cells with a negative net charge.

Survival of the cells during the treatment was determined to be from 40 to 50 % regardless of the amount or duration of the voltage applied.

From Fig. 1 it appears that in the absence of cells, the flow of current will deplete compartments 3 and 4 of salt by electrodialysis. When cells are present, they lose ions to the ion-depleted medium and not as a result of current going through the cells. Both cations and anions are lost and the reason for cell movement to the anode is the negative surface charge at the shear plane.

The values in Fig. 2 suggest that water is often electrolysed in the medium rather than ions removed.

The question of the origin of the ions removed by electrodialysis, while it is not critical to the value of the work presented, is at the very least an intriguing question.

Since it is the belief of the authors that very special methods would be required to differentiate with certainty between adsorbed and absorbed ions and further to delineate their source and the actual mechanism of their extraction, research endeavouring to prove these points was not undertaken.

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XXI. Wright, E.L., and R.G. Tischer. Pure Culture of Plectonema boryanum. Submitted to the Journal of Phycology, 1969.

PURE CULTURE OF PLECTONEMA BORYANUM

Purification of Plectonema boryanum, host of the blue-green algal virus LPP-1, has been accomplished by irradiation techniques. Wu, Lewin, and Werbin[4] and Shugarman[2] employed ultra violet irradiation. Kraus[1] used gamma irradiation. Although the methods mentioned above were successful, they seem fairly drastic.

An impure culture of Plectonema boryanum was obtained from the American Type Culture Collection. This culture was purified using the 'Vibration Technique'. A sample of the pure culture was sent to the ATCC and was assigned ATCC No. 18200.

The equipment used to produce cibration and acceleration consisted of a model 200 CD wide range oscillator, an MB Electronics Model 2250 MB power amplifier, an MB Electronics Model EA 1500 exciter fitted with an MB type 100 vibration pickup, and an MB Electronics Model M3 vibration meter. Attached to the vibration pickup was a special encasement made to hold 4 oz prescription bottles in which algae were vibrated.

The method consists of (1) washing a 20 ml sample of Plectonema boryanum with two drops of a sterile 1% sodium lauryl sulfate solution, (2) washing the algal sample five times with sterile distilled water, (3) vibrating the sample at 700 hertz, 10 gravities (G's) for 30 minutes, (4) washing the sample three times in sterile distilled water, (5) preparing streak plates on modified Chu #10 agar at the alga's limiting dilution less one, (6) picking with sterile cotton plugged Pasteur pipettes, filaments farthest from contaminating bacteria and other algal colonies and filaments after 24 hours growth; and (7) inoculating each filament into a tube of modified Chu #10 broth. Upon the first appearance of growth, cultures were checked for purity by inoculation of 1 ml of the algal suspension into nutrient broth. The absence or presence of bacteria after 96

hours was observed. In some instances, additional study by microscopic examination was employed.

Comparisons were made between control samples (treated in the same manner with the exception of step three) and vibrated samples. The 'Vibration Technique' was more than three times as effective as the control technique.

Microscopic examination of streak plates indicated less contamination on plates prepared from algae vibrated at 700 hertz, 10 G's for 30 minutes, but not on those prepared from algae vibrated at 700 hertz, 30 minutes at 9, 8, or 7 G's. To determine whether vibration did reduce the amount of contamination and at what point between 9 and 10 G's the effect was exerted, standard plate counts were made of vibrated and control samples. Samples of 5 ml of impure algae were vibrated at 700 hertz for 30 minutes at 9.0, 9.2, 9.4, 9.6, 9.8, and 10 G's. A 10^{-5} dilution of each vibrated sample and its control were used to prepare pour plates. After incubation, bacterial colonies were counted. Data were analyzed using the t test for paired observations. Significant differences existed between control samples and samples vibrated at 9.6, 9.8, and 10 G's. The amount of contamination was significantly reduced in this range.

Essentially all of the methods employed to obtain axenic cultures of blue-green algae, with the exception of the 'Positive operator bias' technique of Tischer[3], are based on the destruction or partial destruction of contaminating organisms. The 'Vibration Technique' renders itself useful by significantly reducing the amount of contamination, thereby increasing the probability of obtaining pure cultures.

This work was done in co-operation with the National Aeronautics and Space Administration, Washington, D. C.

Esther L. Wright

R. G. Tischer

XXII. Wright, E.L. and R.G. Tischer. Effects of Spacecraft Level Vibrations and Gravities on Plectonema boryanum. Submitted to Aerospace Medicine, 1969.

EFFECT OF SPACECRAFT LEVEL VIBRATIONS AND GRAVITIES ON PLECTONEMA BORYANUM

Wright, E., Brown, L. R., and Tischer, R. G.

ABSTRACT

This study was designed to determine if spacecraft level vibrations and G's caused changes in the blue-green alga Plectonema boryanum. The proprosed use of unicellular algae as a medium of gas exchange, food and water supply, and waste disposal during prolonged space flight prompted this investigation. Exposure to vibration or gravity (G) ranges for 15, 30, and 60 minutes failed to cause a change in algal colonial morphology. A critical G range (9.6 to 10 G's) at constant vibration of 700 hertz for 30 minutes caused a significant decrease in the amount of contamination in impure cultures. Algae cells exhibited swelling when exposed to 9.6 and 9.8 G's at the same vibrational frequency and time period as mentioned above. An increase in extracellular polysaccharide production was exhibited in pure cultures inoculated with algal samples vibrated at 700 hertz, 10 G's for 30 minutes. Chromatographic studies indicated that the composition of the polysaccharide was not changed as a result of vibration of the inoculum at 700 hertz, 10 G's for 30 minutes.

INTRODUCTION

With the advent of manned space flight, increasing numbers of investigations on space flight conditions and their effects on the astronauts have been undertaken. Comparatively little, however, has been done to discover what effects are exerted on microorganisms which may accompany man on space journeys. A few published reports deal with the effect of altitude stress as a space flight parameter capable of causing changes in a host's susceptibility to infection (2, 5, 6, and 7).

Russian scientists have studied the effect of simulated spacecraft conditions as well as actual space flight conditions on the lysogenic intestinal rod, Escherichia coli K-12 (λ)(13). Induction of phage was greatly increased in actual flight conditions. Ground-base experiments indicated that vibration alone

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did not cause the greater viral induction. It was found that induction increased when E. coli K-12 (λ) was exposed to vibrations before but not after irradiation. Ground-base conditions consisted of vibrations at frequencies of 18, 35, 75, 100, and 700 cycles per second for 15, 30, and 60 minutes. A constant acceleration of 10 G's was used with all vibrations. Gamma irradiation at a rate of 21 roentgens per minute up to a dose of 100 roentgens was combined with other experimental papameters (14). The effect of vibrations on chromosomes has been studied by Knepton (8) and Antipov et. al. (1).

Prolonged space flight produces the problems of oxygen supply, carbon dioxide removal, water and food supply, and waste disposal. For some time algae have been considered as gas exchangers in life support systems (4). A man has been sustained for thirty days in an enclosed atmosphere with Chlorella vulgaris without detrimental effect to either organism (9). It has been shown that Chlorella pyrenoidosa 71105 was able to grow on a medium produced from electrochemically treated human feces and urine (3). Anabaena flos-aquae A-37 has been shown to produce an abundant supply of extracellular polysaccharide (11).

In view of the proposed important role of algae in prolonged space flight, it is surprising to find that no studies to date have been reported concerning possible effects exerted on algae by various space flight conditions. The possibility that stresses which occur during space journeys might cause changes in algae have prompted the investigations contained in this paper. These studies were concerned with the effect of spacecraft level vi-

brations and gravities (G's) on the colonial morphology and cell size of the blue-green alga Plectonema boryanum as well as the effect on the amount and composition of the extracellular polysaccharide. In addition, studies were made to determine the effect of spacecraft conditions on the contaminant in impure cultures.

MATERIALS AND METHODS

Equipment

Equipment used to produce vibration and acceleration was a model 200 CD wide range oscillator, an MB Electronics model 2250 MB power amplifier, an MB Electronics model EA 1500 exciter fitted with an MB type 100 vibration meter. Attached to the vibration pickup was a special encasement made to hold 4 oz prescription bottles in which algae were vibrated. The vertical displacement, velocity, and acceleration were read from the vibration meter. All vibrations were carried out at room temperature.

Culture

An impure culture of the blue-green alga Plectonema boryanum was obtained from the American Type Culture Collection, Rockville, Maryland. The culture was purified in this laboratory. Cultures were checked for contamination before and after use and at the end of experiments by inoculation of 1 ml of the algal suspension into nutrient broth. The absence or presence of growth after 96 hours was observed. In some instances additional study by microscopic examination was employed.

Growth Medium and Cultural Conditions

Modified Chu #10 basal mineral salts medium was employed for the cultivation of Plectonema boryanum. Slants and plates were prepared by the addition of 1.5% agar to the liquid medium.

Growth was carried out in three types of apparatus. For the most part, 200 ml prescription bottles fitted with aerators were used. Static cultures were grown in 250 ml screw cap Erlenmeyer flasks. For the larger cultures, 1,000 ml capacity bottles fitted with aerators for the supply of an air-5% CO_2 mixture were employed. Aerators were plugged with glass wool to prevent contamination from the gas mixture or from air in the incubator. The temperature was maintained at 30±2°C. Three forty watt cool-white fluorescent lamps provided illumination. Small cultures in prescription bottles and Erlenmeyer flasks were cultivated for five days, while larger cultures were allowed to grow ten days.

Because Plectonema boryanum adheres in sheets to the walls of culture vessels, it was necessary to gently scrape the algae from the walls with a rubber policeman. Blending in a sterile Waring blendor for 15 to 30 seconds yielded a uniform suspension of cells.

Colonial Morphology Study

To determine if various levels of vibrations and G's caused changes in the colonial morphology of Plectonema boryanum, 5 ml samples of scraped and blended cells were subjected to a range of vibrations and G's for 15, 30, and 60 minutes. Algal samples were vibrated in 4 oz prescription bottles sealed with stoppers. Exposure to the following eight experimental conditions was studied: (1) 18 hertz, 5 G's; (2) 35 hertz, 5 G's; (3) 25 hertz, 8 G's;

(4) 100 hertz, 6 G's; (5) 700 hertz, 10 G's; (6) 700 hertz, 9 G's; (7) 700 hertz, 8 G's; and (8) 700 hertz, 7 G's. In the first five cases an attempt was made to exert an acceleration of 10 G's in all vibrational frequency ranges. Because of extreme vibration of the equipment, this was not possible. In each case the maximum G's possible approaching 10 were used. In each experiment, three treatments were used: (1) exposure to vibrations and G's for 15 minutes, (2) exposure for 30 minutes, and (3) exposure for 60 minutes. There were three replicates per treatment. Samples from each replicate were streaked on modified Chu #10 agar plates and studied with a low power, wide-field microscope.

Effect of Vibrations and G's on the Amount of Contamination

Microscopic examination of the plates prepared in the algal colonial morphology study indicated less contamination on the plates prepared from algae vibrated at 700 hertz, 10 G's for 30 minutes, but not on those prepared from algae vibrated at 700 hertz for 30 minutes at 9, 8, or 7 G's. To determine whether vibration did reduce the amount of contamination and at what point between 9 and 10 G's the effect was exerted, standard plate counts were made of vibrated and control samples. Samples of 5 ml of impure algae were vibrated at 9.0, 9.2, 9.4, 9.6, 9.8, and 10 G's. A 10^{-5} dilution of each vibrated sample and its control were used to prepare three pour plates. After incubation, bacterial colonies were counted.

Cell Size Study

In an attempt to discover whether vibrations and G's effected the size of algal cells, 5 ml samples of scraped and blended

Plectonema boryanum were subjected to various vibration and G levels. Experimental conditions to which algal samples were exposed were 700 hertz, 10 G's; 700 hertz, 9 G's; 700 hertz, 8 G's; and 700 hertz, 7 G's. Three treatments (15, 30, and 60 minutes) per experiment and three replicates per treatment were employed. Samples from each replicate were measured. In a subsequent study, 5 ml algal samples were vibrated at 700 hertz, 9.6 G's for 30 minutes and 700 hertz, 9.8 G's for 30 minutes. Cells were measured using a Spencer micrometer eye-piece with a vernier scale. All measurements were made using the 100x objective and the 8x ocular. Cell length and width were measured.

Polysaccharide Study

To determine the effects of vibration and G's on extracellular polysaccharide production and polysaccharide composition, 20 ml samples of scraped and blended algal cells were exposed to vibration at 700 hertz, 10 G's. These samples were used to inoculate 800 ml of the basal medium. Controls were prepared using 20 ml of non-vibrated algae. Vibrated and control cultures were incubated for ten days during which they were aerated with an air-5% CO_2 mixture. Large cultures were prepared of pure as well as impure cultures.

The algae were scraped from the walls of the culture vessel, and the entire culture blended for 15 to 30 seconds in a sterile Waring blendor. A Baush and Lomb Spectronic 20 was used to determine the amount of growth in each large culture by measuring the optical density at 630 mμ of scraped and blended algal samples. Spent medium from these cultures was concentrated to 1/10 the original volume in a rotary flash evaporator. Samples of con-

centrate were removed and used to determine the amount of extracellular polysaccharide by the phenol-sulfuric acid method of Montgomery (10). The optical density was referred to a standard curve in order to obtain glucose equivalents. The polysaccharide was precipitated with two to four volumes of 95% ethanol from the concentrated spent medium. The alcoholic solution and precipitate were centrifuged and the polysaccharide removed and dried in a 47 C oven. Samples of 50 mg of dried polysaccharide and 4 ml of 1N H_2SO_4 were placed in a screw cap tube and boiled in a 100°C water bath for 6 hours. Hydrolysates were neutralized with $BaCO_3$ to the congo red endpoint and the $BaSO_4$ was removed by centrifugation. The supernatant was filtered and concentrated to 0.5 ml in a vacuum oven at 47°C.

The solvent systems used for the chromatographic separation of sugars were phenol saturated water (114:25-V:V) and n-butanol, pyridine, and water (10:3:3-V:V:V). Whatman No. 1 filter paper was used. Sugars were separated by descending chromatography at room temperature. Sugar spots were located by spraying the dried chromatogram with p-anisidine-phthalic acid spray and heating to 105°C. Identification was made by comparison with standard reference sugars.

RESULTS

Colonial Morphology Study

Microscopic examination of the streak plates prepared from algae vibrated at the ranges previously indicated were normal with one exception. Examination of the plate prepared from the third re-

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plicate of algae vibrated at 700 hertz, 10 G's for 30 minutes revealed a deeper green elongated colony. The colony was picked with a wire loop and inoculated into modified Chu #10 broth. After growth, streak plates were prepared and incubated. Only normal colonies resulted. Results indicate that no effect or at least no prolonged effect is exerted on the colonial morphology of Plectonema boryanum.

Effect of Vibration and G's on the Amount of Contamination

Table I is a composite of data obtained from nine separate experiments in which standard plate counts were preformed on vibrated and control algal samples. Data were analyzed using the t test for paired observations. Significant differences existed between control samples and samples vibrated at 9.6, 9.8, and 10 G's. The amount of contamination was significantly reduced in this range. It was difficult to keep the G force exactly on the 0.2 G increment due to slight fluctuations of current to the machinery. This could account for the discrepancies in the results at 9.6 and 10 G's. In both experiments, the difference in the amount of contaminant was significant at 9.8 G's.

Cell Size Study

Cell measurement data from algae vibrated at 700 hertz, 10 G's; 700 hertz, 9 G's; 700 hertz, 8 G's; and 700 hertz, 7 G's were analyzed by Dunnett's procedure (12). Vibration and G's at the above mentioned levels did not cause any change in algal cell size. Exposure of algae to 700 hertz, 9.6 G's for 30 minutes and to 700 hertz, 9.8 G's for 30 minutes did result in a significant change in cells. Cell length was not affected, but cell width of vibrated

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TABLE I: Standard Plate Counts of Control Algal Samples and Algal Samples Vibrated at 700 Hertz, 30 Minutes at Various G's.

Treatment	Number of Bacterial Colonies Sample 1	Sample 2	Sample 3	$\bar{x}$	$\bar{d}$
9.0 G's	278	280	147	235	25.3
Control	287	313	231	260.3	
9.2 G's	287	300	281	289.3	8.3
Control	291	295	257	281	
9.4 G's	377	348	388	371	13.6
Control	362	413	379	384.6	
9.6 G's	382	380	368	376.6	25.0
Control	446	374	385	401.6	
9.8 G's	122	190	212	175.3	>1400**
Control	>1900	>1500	>1500	>1600	
10 G's	234	273	289	265.3	83.7**
Control	332	356	359	349	
9.6 G's	24	44	50	39.3	48.7*
Control	82	94	88	88	
9.8 G's	31	33	33	32.3	27.7*
Control	63	66	51	60	
10 G's	53	52	55	53.3	16.0
Control	69	78	61	69.3	

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

Each set of paired observations was obtained from experiments using different cultures and performed on different days.

samples increased significantly. Results are shown in Table II. In this experiment, data were analyzed using the t test for paired observations. Results of this study indicate that there is a critical G range at which cell size of Plectonema boryanum is affected. It is important to note that this critical G range is the same as the critical G range in the reduction of bacterial contamination.

Polysaccharide Study

The results of a study to determine whether vibration at 700 hertz, 10 G's for 30 minutes changes the amount of extracellular polysaccharide produced by two pure and two impure cultures are found in Table III. In both cases of pure cultures there is less growth of vibrated algae. There is at the same time a greater production of extracellular polysaccharide as indicated by glucose equivalents. In the case of pure algal culture B, the control is more than twice as dense as the vibrated sample; yet there was less extracellular polysaccharide produced. The impure cultures do not exhibit the same relationship. Since the rate of utilization of polysaccharide by the contaminant is not known, the significance of this data cannot be evaluated.

Phenol saturated water did not prove completely effective in the separation of sugars. Butanol, pyridine and water was quite successful. Components of the polysaccharide samples are shown in Table IV. Vibration and G's to which the algae were exposed did not cause a change in polysaccharide composition in pure or impure cultures.

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TABLE II: Cell Width and Length of *Plectonema Boryanum* Vibrated at 700 Hertz, 9.[illegible] G's and 700 Hertz, 9.[illegible] G's for 30 Minutes.

Treatment	Cell Width			
	Sample 1	2	3	$\bar{x}$
	μ	μ	μ	μ
700h, 9.[illegible]'s 30 min	1.6698	1.8612	1.7490	1.76000
Control	1.5543	1.4982	1.1913	1.41460
difference	0.1155	0.3620	0.5577	$\bar{d}$ 0.35540 **
700h, 9.8G's 30 min	1.6896	1.6632	1.7622	1.70500
Control	1.3398	1.4916	1.5675	1.46630
difference	0.3498	0.1716	0.1947	$\bar{d}$ 0.23870 **

Treatment	Cell Length			
	Sample 1	2	3	$\bar{x}$
	μ	μ	μ	μ
700h, 9.6G's 30 min	1.8711	1.3497	1.4454	1.55540
Control	1.3497	1.4949	1.6764	1.50700
difference	0.5214	-0.1452	-0.2310	$\bar{d}$ 0.04840
700h, 9.[illegible]G's 30 min	1.6236	1.5048	1.7974	1.64193
Control	1.6599	1.7259	1.4454	1.61040
difference	-0.0363	-0.2211	0.3520	$\bar{d}$ 0.03153

**Significant at the 0.01 probability level.

TABLE III: Growth and Extracellular Polysaccharide Production by Pure and Impure *Plectonema boryanum* Vibrated at 700 Hertz, 10 G's for 30 Minutes and Pure and Impure Control Cultures.

Culture	Glucose Equivalents per ml Unconcentrated Medium	OD at 620mμ of blended alga sample
	μg/ml	
Medium	0	0
Vibrated Pure Culture A*	4.98	0.270
Control Pure Culture A	3.37	0.365
Vibrated Pure Culture B	2.10	0.045
Control Pure Culture B	1.90	0.095
Vibrated Impure Culture A	1.30	0.990
Control Impure Culture A	1.60	1.180
Vibrated Impure Culture B **	1.27	0.740
Control Impure Culture B **	1.90	0.950

*Cultures identified as vibrated are those which grew from inoculation of a 20ml vibrated algal sample into 800ml of modified Chu #10 medium.

**For an undetermined reason, the color reactions were not normal.

TABLE IV: Separation and Detection of Sugar Spots by Butanol, Pyridine, and Water and p-Anisidine-Phthalic Acid Spray.

Sample	Spot	Spot Color	Calculated Rg Value	Identification of Spot
Standard	glucuronic acid	yellow	0.05	..
	galactose	yellow	0.86	..
	glucose	yellow	1.00	..
	mannose	yellow	1.17	..
	arabinose	pink	1.31	..
	xylose	pink	1.44	..
	fucose	yellow	1.50	..
	ribose	pink	1.59	..
	rhamnose	yellow	1.76	..
Impure Alga Vibrated	1	yellow	1.03	glucose
	2	yellow	1.25	mannose
Impure Alga Control	1	yellow	1.03	glucose
	2	yellow	1.26	mannose
Pure Alga Vibrated	1	yellow	0.83	galactose
	2	yellow	1.03	glucose
	3	yellow	1.22	mannose
	4	pink	1.34	arabinose
	5	pink	1.46	xylose
	6	yellow	1.87	rhamnose
Pure Alga Control	1	yellow	0.84	galactose
	2	yellow	1.03	glucose
	3	yellow	1.22	mannose
	4	pink	1.34	arabinose
	5	pink	1.46	xylose
	6	yellow	1.83	rhamnose

SUMMARY

The area of study surrounding the effects exerted on living organisms by space flight conditions is a relatively new branch of science. Most of the work done to date has been attempted in the Soviet Union or the United States. American research has been done primarily at Aerospace Medical Institutes or NASA space centers. Only recently have actual space flight parameters been employed in experiments. At Mississippi State University a program of study on the effects of spacecraft level vibrations and G's on the alga Plectonema boryanum was undertaken. Studies were made using a range of vibrations and G's which occur during space flight.

Variation of vibrations and G's did not cause a change in colonial morphology of Plectonema boryanum. Subsequent experiments indicate that a critical level exists within the studied range. Cultures of pure and impure algae showed significant changes when subjected to vibrations at a frequency of 700 hertz and G's in the range of 9.6 to 10. It was shown that both the algae and the contaminant are affected. Whether there is a cause and effect relationship between the affected algae and bacteria is not evident. The amount of contamination is significantly reduced after treatment at the conditions noted above.

Cells of the algae vibrated at 700 hertz, 10 G's for 60, 30, and 15 minutes; 700 hertz, 9 G's for 60, 30, and 15 minutes; 700 hertz, 8 G's for 60, 30, and 15 minutes; and 700 hertz, 7 G's for 60, 30, and 15 minutes did not differ significantly in cell length or cell width when compared to non-vibrated controls. Cells vibrated at 700 hertz, 9.6 G's for 30 minutes and 700 hertz, 9.8 G's

for 30 minutes were wider than controls. The increase in width in both cases was significant at the 0.01 level of probability. There was not a significant difference in cell length.

Pure cultures whose inoculum had been vibrated at 700 hertz 10 G's for 30 minutes showed less growth but greater production of extracellular polysaccharide as compared with non-vibrated controls. It is possible that the swelling effect on algal cells increased the permeability of cell walls which in turn allowed greater release of polysaccharide.

The composition of extracellular polysaccharide was not changed by exposure to vibration at 700 hertz, 10 G's for 30 minutes. Polysaccharide from pure cultures, vibrated and control, was composed of galactose, glucose, mannose, arabinose, xylose, and rhamnose. Sugars detected in hydrolysates from impure cultures, vibrated and control, were glucose and mannose.

This research is important in itself as having found a set of critical conditions which exist during space flight. Even more important are the questions which have become obvious from this investigation. If algae are to be used in spacecraft more detailed studies must be undertaken.

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