APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1976, p. 585-591
Vol. 32, No. 4

Printed in U.S.A.

Quantitative Requirements for Exponential Growth of *Alcaligenes eutrophus*

R. REPASKE* AND A. C. REPASKE[1]

National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 21 April 1976

Quantitative nutrient requirements for unrestricted autotrophic growth of *Alcaligenes eutrophus* were determined. Minimum saturating concentrations of Mg^{2+}, SO_4^{2-}, PO_4^{3-}, Fe^{3+}, and Na^{2+} for an optical density increase of 2 were 10^{-4} M, 8×10^{-5} M, 5×10^{-4} to 6×10^{-4} M, 10^{-5} M, and 10^{-7} to 2×10^{-7} M, respectively. Trace metal requirements for cobalt, chromium, and copper were also demonstrated, but minimum concentrations could not be determined because other reagents contributed a high background of these metals. Under certain conditions an apparent response to zinc was observed, although other experiments suggest the zinc salt contained another metal that was required for growth. Poly-β-hydroxybutyrate biosynthesis was shown to be initiated by a magnesium or sulfate deficiency as well as by a nitrogen or phosphate deficiency.

When large quantities of cells are required, the simplest expedient is to increase normal culture volumes. However, this usually results in a relatively small increase in cell yield. Continuous cultures are another alternative, but considerable time is required to establish proper conditions. In principle it should also be possible to grow high-density cultures in larger volumes than reported by Gerhardt's group (4, 15, 17). *Alcaligenes eutrophus* was eminently suited for these studies because it grows autotrophically in a defined inorganic medium with no significant production of metabolic waste products.

Media currently being used for *A. eutrophus* produce 6×10^9 to 8×10^9 cells per ml in batch cultures before one or more nutrients become growth limiting (13). Arbitrarily increasing the initial concentration of all culture nutrients does not result in a proportional increase in growth; higher concentrations of some nutrients are toxic and others cause precipitate formation. Periodic supplements of specific nutrients are required. If additions of individual nutrients could be made before their concentrations became growth rate limiting, the exponential-growth phase might be extended to higher optical densities. Recognition of an impending nutritional deficiency requires knowing the relationship between a concentration of nutrient and the extent of exponential growth that that concentration would support. This article presents results of experiments designed to determine that relationship. During these studies three new trace metal requirements were found. The application of these data to 23-liter batch cultures is presented in an accompanying article (13); exponential growth with minimum doubling time was extended to optical densities in excess of 40 (8×10^{10} cells per ml).

MATERIALS AND METHODS

Hydrogenomonas eutropha, now designated *Alcaligenes eutrophus* (3, 5), was grown autotrophically as 100-ml liquid cultures in indented 500-ml Erlenmeyer flasks and shaken at 31°C under a gas atmosphere of H_2, O_2, and CO_2 (7:2:1). Conditions for growth and methods for continuously supplying the gas atmosphere have been described (10, 11).

Media. Media were prepared with deionized water and reagent-grade chemicals. The medium used previously was modified by deleting NaCl, changing the concentration of several macronutrients, and replacing the trace metal solution with three required metals. The new medium was prepared with 95 ml of 0.03 M potassium phosphate buffer, pH 6.5, to which the following nutrients were aseptically added to give the indicated final concentrations: NH_4Cl, 1.8×10^{-2} M; $CaCl_2$, 7×10^{-5} M; $NaHCO_3$, 1.2×10^{-2} M; $MgSO_4$, 5×10^{-4} M (or $MgCl_2$ and K_2SO_4, each at 5×10^{-4} M); and $FeCl_3$ in 0.001 M HCl or $Fe(NH_4)_2(SO_4)_2$, 6×10^{-5} M. To these macronutrients were added 3×10^{-7} M $NiCl_2$, 4×10^{-7} M $CuCl_2$, and 5×10^{-7} M $CrCl_3$. The former trace metal mixture (10) provided: $CoCl_2$, 8×10^{-9} M, $MnCl_2$, 2×10^{-5} M; $CuSO_4$, 8×10^{-8} M; Na_2MoO_4, 2

[1] Present address: Department of Bacteriology, American Type Culture Collecton, Rockville, MD 20852.

$\times 10^{-7}$ M; and $ZnSO_4$, 3×10^{-7} M. This mixture minus $ZnSO_4$ was used in several experiments where noted.

Although reagent-grade chemicals contained only small percentages of impurities, the micronutrient contribution from this source was significant when relatively high concentrations of macronutrients were used. Therefore, in specified experiments, NH_4Cl, $CaCl_2$, $MgCl_2$, K_2SO_4, and phosphate buffer were treated with a chelating agent, 8-hydroxyquinoline (8HQ), to reduce trace metal impurities. Concentrated stock solutions of each of the above compounds were adjusted to pH 5.5 (phosphate was prepared from KH_2PO_4 and adjusted with KOH to pH 5.5), and one-fourth volume of 1% 8HQ was added, mixed, and allowed to stand for 2 h. 8HQ metal chelates and unreacted chelator were repeatedly extracted with chlorform until the chloroform was colorless. The pH was then raised to 7.0 (KOH), and residual chelator was removed by additional chloroform extractions. Traces of chloroform remaining in the aqueous phase were vaporized during autoclaving.

Inocula. Inocula were 18-h cultures that had been incubated at 31°C. The same inoculum could be used for several weeks, since stored liquid cultures retained undiminished viability for at least 30 days and no "aging" effects occurred during storage (12). For macronutrient assays, inocula were grown in complete untreated media. For micronutrient tests, inocula were grown in (i) untreated media without added trace metals or (ii) in 8HQ-treated media without added trace metals.

Assay. The minimum saturating concentration of each nutrient was found by determining the minimum concentration of nutrient which supported the same growth as a control culture containing excess nutrient. The assay end point occurred when control cultures had reached an optical density (OD) of 2.0 to 2.5 and were still growing exponentially. This density was reached after 18 h under standardized assay conditions, namely, a washed inoculum resuspended in 0.001 M PO_4, pH 7, inoculated cultures with a starting OD of 0.020, and an incubation temperature of 31°C. Six concentrations of a nutrient were tested simultaneously; four were limiting, one was at or near the minimum saturating concentration, and one, the control, was in excess. Assay results were independent of inoculum size (±threefold) if cultures were removed when the control culture reached the required OD. The background concentrations of CO^{2+}, Cu^{2+}, and Cr^{3+} were too high to determine their minimum requirements. Effects of these micronutrients were based on total culture growth in 24 h as noted.

The OD was determined with a spectrophotometer at 660 nm in a standard 1-cm light path cuvette against medium as a reference. Cell suspensions were diluted as necessary to an OD of 0.020 to 0.400, the range of direct proportionality between OD and cell numbers. Optical density correlated with plate counts in autotrophic or heterotrophic media (2×10^9 to 3×10^9 cells per ml per OD) and cell protein-nitrogen values. Enlarged nutrient-deficient cells containing poly-β-hydroxybutyrate (PHB) had a higher OD than a similar concentration of normal cells, but the spurious contribution to OD was no greater than 10% (18) and did not affect the general validity of OD as a measure of growth.

PHB determination. PHB was determined by a modification of the method of Law and Slepecky (9). The variation between replicate determinations was ±1% when PHB standards were used. When whole cell samples were used, the variation was ±5%, apparently because of difficulty in digesting gram-negative cells. Highest yields and most consistent results were obtained when the washed cell pellet was frozen and thawed several times before Chlorox was added. Chlorox digestion was continued for 1 h at 37°C, after which PHB granules were sedimented by centrifugation. Traces of chlorine were removed with a small amount of sodium thiosulfate (19) added to the PHB resuspended in water. Successive washes in water, acetone, and absolute ethanol were followed by gentle heating to evaporate residual alcohol. Hot-chloroform extractions were not necessary because there was no detectable interference by light-absorbing contaminants in the critical spectral region. Five milliliters of concentrated H_2SO_4 was added to the dry pellet; the tubes were capped with a glass marble and heated in a sand bath at 100°C for 20 min to oxidize PHB to crotonic acid. Samples were then diluted as necessary in concentrated H_2SO_4, and the absorption spectrum of crotonic acid was obtained between 200 and 340 nm with a Cary model 14 spectrophotometer. A molar extinction of 1.55×10^4 at 235 nm was used to determine the concentration of crotonic acid and to calculate the original PHB concentration (9).

Nitrogen determinations. Ammonium nitrogen was determined by the Nesslers reaction (6), and cell protein was determined by the biuret method (8, 16) with bovine serum albumin as a reference standard. Cell protein-nitrogen was calculated from biuret protein values on the assumption that cell protein averaged 16% nitrogen.

RESULTS

The modified medium used in these studies contained sufficient nutrient to permit exponential growth to continue to OD 3.5 and total growth to reach OD 4.5. Subsequent growth was limited by an ammonium-nitrogen deficiency; cultures provided with additional NH_4Cl reached a final OD of about 6 before another nutrient limited growth (13).

If exponential growth could be extended to obtain higher culture densities, it would require making individual supplements of each nutrient before depletion reduced the concentration to a growth-limiting level.

To anticipate a requirement, the amount of exponential growth that can be expected from a given concentration of nutrient must be known. That relationship could be determined by observing culture growth with a series of nutrient concentrations or by selecting an increment of

growth and determining the minimum nonlimiting concentration for that amount of culture growth. The latter approach was chosen. A growth increment of OD 2 to 2.5 (5×10^{-9} to 7×10^{9} cells per ml) was selected because at this density cultures with excess nutrient were still growing exponentially. The minimum concentration of nutrient that duplicated growth of a control culture containing excess nutrient was defined as the minimum saturating concentration for unrestricted growth of 2 OD units of cells. This amount of growth was sufficient to demonstrate nutrient-dependent growth with most nutrients.

Macronutrients. A growth response curve for each macronutrient except NH_4Cl and $CaCl_2$ was determined in complete medium containing various concentrations of the nutrient being tested. Since nitrogen limitation did not occur before OD 3.5, it was not limiting during the assay; calcium had no demonstrable effect on growth, but its presence in most salts compromised any assessment of a requirement.

The effect of iron, sulfate, magnesium, and phosphate concentrations on growth defined by the assay is shown in Fig. 1. In phosphate-limited cultures, the buffer was 0.03 M Tris-chloride, pH 6.5, which had no deleterious effect on growth. A direct relationship between growth and nutrient concentration was shown by plotting the logarithm of culture density against the logarithm of nutrient concentration. The comparibility of the results were seen more readily if the density of each culture in a series was related to the maximum density of its control culture; therefore, the logarithm of the percentage of maximum growth was used. Growth increased proportionally with higher nutrient concentration until saturation occurred; thereafter additional nutrient had no effect. The minimum saturating concentrations were: Fe^{2+} or Fe^{3+}, 10^{-5} M; SO_4^{3-}, 8×10^{-5} M; Mg^{2+}, 10^{-4} M; and PO_4^{3-}, 5×10^{-4} to 6×10^{-4} M.

At the lowest macronutrient concentrations tested, cultures were deficient for the greater part of the incubation period, and secondary effects caused by the deficiencies were observed. One of these effects was culture foaming, which only occurred with an iron deficiency. Several hours after foaming began, iron-limited cultures produced a yellow-green pigment visible in culture filtrates. The pigment increased in intensity with continued incubation. Cells from iron-deficient cultures characteristically were about one-half the size of normal cells, and they stained evenly. In contrast, cells from nitrogen-, phosphate-, magnesium-, or sulfate-deficient cultures were

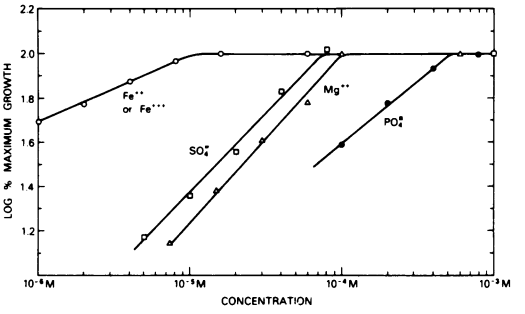

FIG. 1. *Minimum saturating concentration of macronutrients for unrestricted growth of A. eutrophus to OD 2.0 to 2.5. The assay was conducted with the indicated concentrations of each nutrient in an otherwise complete medium containing trace metals. The buffer in the phosphate experiment was 0.03 M Tris-chloride, pH 6.5. Incubation was under a 70% H_2-20% O_2-10% CO_2 atmosphere for 18 h at 31°C.*

large, and large areas in the cytoplasm remained unstained. This appearance suggested PHB inclusions, and it was confirmed by PHB analysis. PHB synthesis resulting from nitrogen or phosphate starvation was reported by Schlegel et al. (14). They found that continued incubation of nitrogen-deficient cultures under H_2, O_2, and CO_2 produced cultures containing as much as 65% dry weight PHB. Until recently we were not aware that any other laboratory had noted PHB synthesis as a consequence of magnesium or sulfate deficiency (7).

PHB synthesized in cultures deficient in phosphate, sulfate, or magnesium was compared with normal- and iron-deficient cultures (Table 1) in medium containing Ni^{2+} and trace metal mixture. No attempt was made to select optimum conditions for the highest yield of PHB. The high OD of the normal culture, no. 1 (OD 4.840), is accounted for by a larger inoculum than was used in nutrient assays and a longer incubation period (20 h). The high OD indicated growth had exceeded the point of nitrogen exhaustion, and the PHB content, although low, was elevated. No more than 1% PHB would be present if the OD were 3.5 or less. The selected concentrations of phosphate, sulfate, and magnesium produced cultures differing in total growth, but the final PHB was uniformly high, ranging between 22 and 34% of the total dry weight. PHB averaged about 50% of the total cell protein, or three to four times the cell protein nitrogen, and was highest in sulfate-limited cultures. Normal and iron-deficient cultures had a 3.6-fold difference in total growth; nevertheless, they had a similar low content of PHB.

TABLE 1. *PHB synthesis and growth of A. eutrophus in relation to nutrient deficiencies*

Culture no.	Growth[a] (OD_{660})	Limiting nutrient	Concn (M)	Residual NH_4-N in medium (mg)	Cells (mg dry wt)	Cell protein-N (mg)	PHB		
							mg	mg/mg of protein-N	% dry wt
Uninoculated		None		26.2					
1	4.840	None		4.5	174	20.3	5.9	0.29	3
2	1.640	PO_4^{3-}	2×10^{-4}	18.6	59	5.9	19.6	3.32	33
3	0.730	SO_4^{2-}	2.5×10^{-5}	24.1	26	2.3	9.0	3.91	34
4	2.580	Mg^{2+}	5×10^{-5}	14.3	93	7.6	20.1	2.64	22
5	1.340	Fe^{3+}	3×10^{-6}	18.1	48	7.1	2.9	0.41	6

[a] Data from 100-ml cultures incubated for 20 h.

Before the association of Mg^{2+} deficiency and PHB synthesis was recognized, Bongers (2) noted that NH_4^+- and Mg^{2+}-starved cultures continued to consume H_2, O_2, and CO_2. Reintroduction of Mg^{2+} inexplicably caused CO_2 assimilation to cease for 30 min, after which the rate of assimilation gradually increased, reaching its full capacity in the third hour. The demonstration of Schlegel et al. (14) of reassimilation of PHB after NH_4^+ was resupplied to NH_4^+-starved cultures and our observation of the same events in Mg^{2+}-deficient cultures suggest that what Bongers observed was the preferential utilization of accumulated PHB at the expense of CO_2 assimilation.

Micronutrients. A nickel requirement for *A. eutrophus* was first demonstrated by Bartha and Ordal (1) with 8HQ-extracted medium supplemented with Hoagland trace metal solution. Bongers (2) reported that nickel increased steady state growth rates in continuous cultures. The dependence of autotrophic growth on nickel, and the difficulty in extracting it to demonstrate the requirement, indicates that appreciable nickel contamination occurs in ordinary medium. Nickel could be depleted by growing cultures successively in the same medium (cumulative OD of about 10) supplemented as necessary with NH_4^+, Mg^{2+}, SO_4^{2-}, and Fe^{2+} and corrected for pH changes. These results incidentally indicated the above reagents were not major contributors of adventitious nickel.

Our results with 8HQ-extracted medium components differed quantitatively from those of Bartha and Ordal (1); extracted medium without added trace metals still permitted growth to OD 1 in 24 h. When 3×10^{-7} M Ni^{2+} and trace metals were included, the OD reached 2, or only about one-half the cell density obtained in untreated medium (see Tables 4 and 5). 8HQ extraction apparently had removed some other necessary but unknown trace metal(s), and this medium was not considered satisfactory for testing the response to Ni^{2+}.

The nickel requirement could be satisfactorily demonstrated in an unextracted medium containing only macronutrients if the inoculum had also been grown in medium without added trace metals. With this inoculum, cultures grew to an OD of 1.5 to 2 when nickel and other trace metals were not provided and to an OD of 3.5 in 24 h when Ni^{2+} was added. Full growth to OD 4.5 in 24 h occurred when copper and chromium (discussed below) were added with Ni^{2+}. The effect of nickel on growth (Fig. 2) was determined by the usual 18-h assay in this medium. Nickel saturation occurred at 10^{-7} to 2×10^{-7} M added nickel, essentially the same concentration found by Bartha and Ordal (1) in a medium showing complete growth dependence on nickel.

Nickelous chloride contained 0.1% cobalt as an impurity and therefore provided 5×10^{-9} M CO^{2+} to the medium when 3×10^{-7} M $NiCl_2$ was used. Some uncertainty existed about the specificity of the Ni^{2+} response, since cobalt at this concentration could conceivably satisfy a cobalt requirement. Nickelous chloride and another nickel compound, nickelous ammonium chloride containing one-fifth less cobalt on a molar basis, were compared at limiting Ni^{2+} concentrations (5×10^{-8} M) in a macronutrient

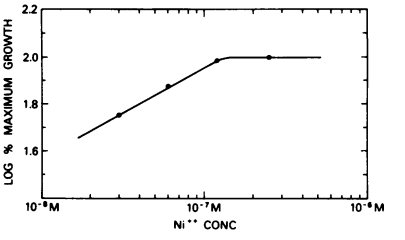

FIG. 2. *Minimum saturating concentration of added nickel for unrestricted growth of A. eutrophus to OD 2.0 to 2.5. The macronutrient medium contained 4×10^{-7} M Cr^{3+} and 5×10^{-7} M Cu^{2+} as micronutrients and the indicated concentrations of $NiCl_2$. Refer to the legend to Fig. 1 for growth conditions.*

medium supplemented with copper and chromium (Table 2). Growth with both nickel salts was the same. When 8×10^{-9} M $CoCl_2$ (contributing 3×10^{-11} M Ni^{2+}) was included, growth was stimulated 180% and was equivalent to growth with 10^{-7} M Ni^{2+} without added cobalt. No significant stimulation was obtained with Co^{2+} alone. These results indicated that both nickel and cobalt were required and suggested the saturating nickel concentration determined in Fig. 2 may be excessive because it represented the amount of $NiCl_2$ needed to supply both Ni^{2+} and Co^{2+}. Further study of the Ni^{2+} and Co^{2+} effects were not made because base line growth without added Ni^{2+} was too high.

Chromium and copper stimulation of *A. eutrophus* growth was first detected indirectly in larger fermentor cultures (13). Supplements of $MgSO_4$ maintained the growth rate more effectively than equivalent concentrations of $MgCl_2$ and K_2SO_4. Analysis of the two magnesium salts (Bureau of Standards) revealed $MgSO_4$ contained between 0.001 and 0.01% of both chromium and calcium as major impurities and less than 0.001% copper. No chromium was detected in the $MgCl_2$, but copper and calcium were present in amounts of 0.01 to 0.1%. The effect of nickel, chromium, and copper singly and together in the macronutrient medium is shown in Table 3. The inoculum was grown without trace metals. Nickel stimulated growth twofold, and Cr^{3+} and Cu^{2+} individually increased growth (with nickel) by an additional 30%. Higher concentrations of Cr^{3+} or Cu^{2+} produced no further stimulation. Neither Cr^{3+} nor Cu^{2+} had any effect without Ni^{2+}. Additive stimulation by Cr^{3+} and Cu^{2+} produced cultures of the same densities as ordinary inocula in medium containing the trace metal mixture. No detectable zinc stimulation (2) was found when zinc was used instead of Cr^{3+} or Cu^{2+} or in conjunction with them.

A reexamination of the 8HQ-extracted medium seemed warranted once Cr^{3+} and Cu^{2+} requirements had been demonstrated. Single replacements of extracted components for unextracted ones showed unextracted phosphate contributed substances responsible for superior growth in the unextracted medium (Table 4). Cr^{3+}, Cu^{2+}, and Zn^{2+} were tested in extracted medium supplemented with Co^{2+} and nickel to determine if these were the critical metals removed by extraction (Table 5). The twofold stimulation by Ni^{2+} discussed previously is shown in line 2. Chromium stimulated growth by an additional 20%, but Cu^{2+} stimulation found previously in nonextracted medium (Table 3) was absent (not shown). Unexpectedly, growth was now influenced by Zn^{2+}. Stimulation by Zn^{2+} was about the same as with unextracted phosphate (Table 4). Neither Cr^{3+} nor Cu^{2+} enhanced Zn^{2+} stimulation. Zinc stimulation of growth was observed only in 8HQ-extracted medium and could not be demonstrated in unextracted medium, even after attempting to deplete Zn^{2+} by growing cultures to an OD of 35 (13) with no phosphate supplements.

Other trace metals contained in the original trace metal solution either were not required for growth or adequate amounts were supplied as impurities.

TABLE 2. *Cobalt stimulation of growth with limiting nickel*

Addition to medium[a]	Growth (Δ OD_{660}/18 h)
None	2.040
Co^{2+} (8×10^{-9} M)	2.190
Ni^{2+} (5×10^{-8} M)	2.490
plus Co^{2+}	3.660
Ni^{2+} (10^{-7} M)	3.750

[a] Medium contained macronutrients and 4×10^{-7} M Cu^{2+} and 5×10^{-7} M Cr^{3+}.

TABLE 3. *Ni^{2+}, Cr^{3+}, and Cu^{2+} stimulation of A. eutrophus growth*

Addition to medium[a]	% Stimulation[b]
None	
+ Ni^{2+} (3×10^{-7} M)	104
+ Cr^{3+} (5×10^{-7} M)	130
+ Cu^{2+} (4×10^{-7} M)	136
+ Cr^{3+} and Cu^{++}	174

[a] Medium contained macronutrients only.
[b] Incubation was for 18 h.

DISCUSSION

The relationship between nutrient concentration and the amount of exponential growth

TABLE 4. *Effect of nickel and unextracted phosphate on growth medium extracted with 8HQ*

Addition to medium[a]	Δ OD_{660}/24 h
None	1.200
$NiCl_2$ (10^{-7} M)	2.520
$NiCl_2$ plus unextracted PO_4^{3-}	4.060

[a] 8HQ-extracted macronutrients plus Fe^{3+}.

TABLE 5. *Cr^{3+} and Zn^{2+} effects in growth of A. eutrophus in 8HQ-extracted medium*

Additions to medium[a]	Δ OD_{660}/24 h
None	1.330
Ni^{++} (3×10^{-7} M)	2.420
+ Cr^{3+} (10^{-7} M)	2.890
+ Zn^{2+} (10^{-7} M)	3.440
+ Zn^{2+} and Cr^{3+}	3.340

[a] 8HQ-extracted macronutrients plus Fe^{3+}.

that could be supported was determined to provide a basis for scheduling additions of nutrients to cultures before the concentration of any nutrient became growth rate limiting. The purpose was to extend the period of exponential growth to increase cell yields in batch cultures. The minimum concentration of nutrient for unrestricted growth to a given population density was determined by an assay which compared growth at various nutrient concentrations with growth in a control culture containing an excess of the nutrient being tested. At the end point (OD 2 to 2.5) the control was still growing exponentially. The resulting growth response curves (Fig. 1 and 2) show a proportional response of growth to nutrient concentration. The relatively small deviation in the fit of experimental points results from the fact that growth in individual cultures in each series was the same until each culture reached nutrient limitation; growth then stopped without passing through extended transitions of gradually decreasing rates.

Since nutrient uptake ultimately is concentration dependent, some residual nutrient must remain when growth stops. It is assumed that growth stopped in all flasks when the same residual nutrient concentration was reached. If that residual concentration were high, the quantity of nutrient consumed would be calculated from the change in concentration. If, however, the residual concentration were very low when growth stopped, the total nutrient added could be considered necessary for the observed cell growth.

The two possibilities may be distinguished by comparing ratios of total growth to nutrient concentration with each nutrient. Constant ratios would indicate growth at each limiting nutrient concentration was proportional to the available nutrient. It would also suggest little residual nutrient remained when growth stopped. If appreciable nutrient had remained, the ratios would increase with increased nutrient concentration. This would follow because proportionally more growth would be obtained at higher nutrient concentrations where the residual nutrient concentration would be a smaller percentage of total nutrient supplied. The ratios for different limiting concentrations of Mg^{2+} and of SO_4^{2-} varied by less than 20%, whereas for PO_4^{3-} and for Fe^{2+} they were greater (50%) and ratios decreased progressively with increasing nutrient concentration. This was contrary to expectations if residual concentrations of nutrient remained high, and it suggested the two latter nutrients caused some inhibition of growth at higher concentrations. The general conclusion, therefore, was that residual nutrient was low and could be discounted when estimating nutrient consumed. The minimum saturating concentrations of nutrients shown in Fig. 1 and 2 were regarded as the quantity required to produce 2 OD units of cells with no restriction on the growth rate.

Detection of required micronutrients was difficult because metal impurities in reagents often exceeded the biological requirements. For example, nickel salts provided sufficient cobalt to meet the growth requirements of the organism, and cobalt stimulation could be demonstrated only when minimum nickel was used (Table 2). Stimulation by such a low concentration of Co^{2+} indicated Co^{2+} itself was the specific active metal. The situation with Cr^{3+} and Cu^{2+} was not as clear. Although both metals apparently were required (Table 3), the fact that either one could independently stimulate growth suggested growth was promoted by a metal contaminant common to both salts. Indeed, zinc could be that contaminant based upon the Zn^{2+} stimulation observed in extracted medium (Table 5). However, if that were true, Zn^{2+} should have replaced or augmented Cr^{3+} or Cu^{2+} in unextracted medium (Table 3). It did not. The identity of the specific required metals from this group or the active metals they contribute remain to be resolved.

The simplified minimal medium containing the macronutrients discussed and micronutrients of nickel (with cobalt), chromium, and copper salts supported growth to OD 4.5, and doubling times were 1.7 to 2 h, which was within the range of minimum doubling times reported by Bongers (2). Nutritional adequacy of the medium is further supported by the fact that 23-liter batch cultures could be grown to OD 60 or 1.2×10^{11} cells per ml with these nutrients alone (13). Exponential growth in those cultures was maintained to OD 40 by scheduling nutrient supplements based upon the requirements presented in this report.

LITERATURE CITED

1. **Bartha, R., and E. J. Ordal.** 1965. Nickel-dependent chemolithotrophic growth of two *Hydrogenomonas* strains. J. Bacteriol. 89:1015–1019.
2. **Bongers, L.** 1970. Some aspects of continuous culture of hydrogen bacteria, p. 241–255. *In* C. J. Corum (ed.), Developments in industrial microbiology, vol. 2. Gareemond-Predemark Press, Baltimore.
3. **Davis, D. H., M. Doudoroff, R. Y. Stanier, and M. Mandel.** 1969. Proposal to reject the genus *Hydrogenomonas*: taxonomic implications. Int. J. Syst. Bacteriol. 19:375–390.
4. **Gerhardt, P., and D. M. Gallup.** 1963. Dialysis flask for concentrated culture of microorganisms. J. Bacteriol. 86:919–929.

5. **Halaus, D., R. Y. Stanier, M. Doudoroff, and M. Mandel.** 1970. Taxonomic studies on some gram negative polarly flagellated "hydrogen bacteria" and related species. Arch. Mikrobiol. 70:1-13.
6. **Johnson, M. J.** 1941. Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem. 137:575-586.
7. **Kesler, T. G., Y. V. Voitovich, N. A. Anistratova, I. N. Trubachev, and N. S. Eroskin.** 1973. The growth of hydrogen bacteria under conditions of biosynthesis blocked by biogenic elements. Mikrobiologiya 41:456-460.
8. **Koch, A. L., and S. L. Putnam.** 1971. Sensitive biuret method for determination of protein in an impure system. Anal. Biochem. 44:239-245.
9. **Law, J. H., and R. A. Slepecky.** 1961. Assay of poly-β-hydroxybutyric acid. J. Bacteriol. 82:33-36.
10. **Repaske, R.** 1962. Nutritional requirements for *Hydrogenomonas eutropha*. J. Bacteriol. 83:418-422.
11. **Repaske, R.** 1966. Characteristics of hydrogen bacteria. Biotech. Bioeng. 8:217-235.
12. **Repaske, R., C. A. Ambrose, A. C. Repaske, and M. L. DeLacy.** 1971. Bicarbonate requirement for elimination of the lag period of *Hydrogenomonas eutropha*. J. Bacteriol. 107:712-717.
13. **Repaske, R., and R. Mayer.** 1976. Dense autotrophic cultures of *Alcaligenes eutrophus*. Appl. Environ. Microbiol. 32:592-597.
14. **Schlegel, H. G., G. Gottschalk, and R. V. Bartha.** 1961. Formation and utilization of poly-β-hydroxybutyrate by knallgas bacteria (*Hydrogenomonas*). Nature (London) 191:463-465.
15. **Schultz, J. S., and P. Gerhardt.** 1969. Dialysis cultures of microorganisms: design, theory and results. Bacteriol. Rev. 33:1-47.
16. **Strickland, L. H.** 1951. The determination of small quantities of bacteria by means of the biuret reaction. J. Gen. Microbiol. 5:698-703.
17. **Tyrrell, E. A., R. E. MacDonald, and P. Gerhardt.** 1958. Biphasic system for growing bacteria in concentrated culture. J. Bacteriol. 75:1-4.
18. **Wilde, E.** 1962. Untersuchungen über Wachstum and Speicherstoff synthese von *Hydrogenomonas*. Arch. Mikrobiol. 43:109-137.
19. **Williamson, D. H., and J. F. Wilkinson.** 1958. The isolation and estimation of the poly-β-hydroxybutyrate inclusions of *Bacillus* species. J. Gen. Microbiol. 19:198-209.