JOURNAL OF BACTERIOLOGY, Aug. 1979, p. 530-536
0021-9193/79/08-0530/07$02.00/0

Vol. 139, No. 2

Response of *Rhodopseudomonas capsulata* to Illumination and Growth Rate in a Light-Limited Continuous Culture

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Received for publication 17 March 1979

Rhodopseudomonas capsulata was grown under anaerobic, photosynthetic conditions in a continuous culture device. Under light limitation, at a constant dilution rate, it was shown that cell composition, including photopigment (bacteriochlorophyll and carotenoids) and ribonucleic acid content, was not affected by incident light intensity; however, steady state culture density varied directly and linearly with light intensity. On the other hand, photopigment and ribonucleic acid levels were affected by growth rate regardless of light intensity. Additional experiments indicated a high apparent K_s for growth of *R. capsulata* with respect to light. These results were interpreted to mean that near the maximum growth rate ($D = 0.45\ h^{-1}$) some internal metabolic process became the limiting factor for growth, rather than the imposed energy limitation. A mathematical expression for the relation between steady-state culture density and dilution rate was derived and was found to adequately describe the data. A strong correlation was found between continuous cultures limited either by light or by a chemical energy source.

The nonsulfur purple bacteria are unusually well-suited for research related to energy conversion. The increasing interest in these organisms (10) is due, in no small part, to the variety of energy transduction systems and resultant growth modes that they exhibit (9). It is surprising, therefore, that to date there has been only limited work reported on the study of these creatures in continuous culture (6). In the few reports on the growth of these organisms in continuous culture, the cells have often been grown in complex medium and within very limited ranges of growth rate, pH, limiting nutrient concentration, and temperature (2, 4-6). An extensive study of the effects of growth rate and incident light intensity on major physiological parameters, such as efficiency of nutrient utilization and cellular composition of nonsulfur bacteria in continuous culture, has not been carried out before the work reported here.

This paper will describe a continuous culture device and culture medium suitable for conducting light-limited continuous culture experiments on *Rhodopseudomonas capsulata*. Special attention will be given to comparison of light limitation with limitation for a soluble energy source, the range of limiting light intensities and growth rates beyond which light ceases to be the limiting factor, mathematical treatment of continuous culture with light as the limiting nutrient, experiments designed to test the mathematical predictions, and comparison of *R. capsulata* with algae grown under similar light-limited conditions (12).

MATERIALS AND METHODS

Organism. Liquid photosynthetic stock cultures of *R. capsulata* ATCC 23782 (strain St. Louis) were maintained by biweekly subculture (14).

Medium. For all continuous culture experiments a "complete," simple salts medium was used, which was a compromise between the regular medium employed for batch culture studies of this organism (14) and a chemostat medium devised for growth of a heterotrophic organism (1). The medium contained: 30 mM L-malic acid, 10 mM NH_4Cl, 5 mM KH_2PO_4, 2.5 mM $(NH_4)_2SO_4$, 0.625 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM $FeCl_3$, 0.05 mM EDTA, 0.05 mM $MnCl_2$, 0.05 mM H_3BO_3, 0.025 mM $ZnCl_2$, 0.01 mM $CoCl_2$, 0.005 mM Na_2MoO_4, 0.005 mM thiamine hydrochloride, and 0.2 μM $CuCl_2$. Strongly acidic (pH 2), 10-liter batches of the medium were autoclaved at 120°C for 1 h. After cooling to room temperature, the separately sterilized (20 min at 120°C) thiamine hydrochloride was added aseptically.

Culture conditions. A continuous culture device, diagrammatically represented in Fig. 1, was built. It consisted of an all-glass 600-ml growth chamber with ports for titrant addition, pH electrode, thermometer, medium plus argon addition, inoculation, overflow, and sampling. It was stirred by a Teflon-coated magnetic stirbar, and the chamber contained baffles to enhance mixing. To keep the entire apparatus anaerobic, sterile argon gas was passed over the culture at a rate of 150 ml/h, forcing excess liquid out via the

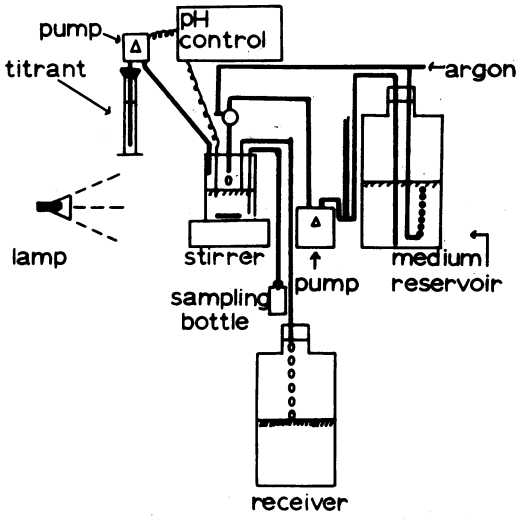

FIG. 1. *Continuous culture device equipped for growth of R. capsulata under conditions of anaerobic, photosynthetic light-limited growth. The 600-ml growth chamber contained ports for titrant addition, pH electrode, thermometer, medium plus argon addition, inoculation, overflow, and sampling.*

overflow tube, thus keeping the culture volume constant. Both the medium and the titrant reservoirs were continuously sparged with sterile argon at a rate of 20 ml/h. The pH of the culture was kept constant at 7.0 ± 0.1 by means of a pH control unit (type 45A; Chemtrix, Inc., Hillsboro, Ore.), which controlled a pump that delivered 1 N NaOH into the culture chamber as a titrant. The medium in the reservoir contained L-malic acid which was not neutralized before it entered the growth chamber. This kept osmolarity to a minimum since only one pH adjustment was required to keep the medium in the growth chamber at a constant pH 7.0 ± 0.1. Incidentally, this arrangement also served to minimize contamination problems since the pH in the unneutralized medium reservoir was approximately 2.0.

Though the titrant volume never exceeded 70 ml/liter of medium, calculations of dilution rate were corrected for the added volume. The temperature of the culture was maintained at 31°C by placing the entire apparatus in a constant temperature environment. Regular monitoring of the culture showed that the temperature never varied more than 0.5°C. Illumination was provided by 150-W flood lamps. A reflecting surface was placed at the side of the growth chamber opposite the lamp. To attain different intensities of illumination on the culture, the distance between the lamp and the culture was varied, except in the experiment detailed in Fig. 4, in which a set of four identical 150-W flood lamps was placed at a distance of 165 cm from the culture vessel. The lamps were arranged in a vertical array and individually aligned to each give an incident light intensity of 2,150 lx ± 5% at the center of the culture.

Sampling. Measurements of cell density taken at various times were used to assure that steady states were achieved before 100- to 200-ml samples were taken directly from the growth chamber. At a growth rate of 0.1 h^{-1} it was shown that samples taken after 48 h of operation were always in a steady state, while with a growth rate of 0.2 h^{-1} or higher, samples taken after 24 h of continuous running were in steady state without exception.

Assays. Contents of bacteriochlorophyll and carotenoids were estimated immediately upon sampling by the method of Cohen-Bazire et al. (3). For dry-weight estimation, at least 80 ml of sample was centrifuged at 12,100 × *g* for 10 min at 0°C, washed once with distilled water, recentrifuged, and immediately frozen, lyophilized, and weighed. The freeze-dried cells were subsequently used for assay of RNA, DNA, carbohydrate, and protein as described by Herbert et al. (8). L-Malate was assayed as described by Stahl and Sojka (15), in samples taken from the media and in the supernatants from the dry-weight samples. After a correction for dilution by titrant, the yield values were calculated (grams of dry bacteria formed per gram of malate consumed). Light intensity was measured with a Weston illumination meter, model 756; foot candle values obtained with this instrument were converted to lux by calculation.

RESULTS

Influence of illumination on the steady-state density of the culture. At a constant dilution rate of 0.2 h^{-1} (slightly less than half the maximum growth rate), the steady-state dry weight of the culture was determined at several different intensities of incident light. The different light intensities were achieved by diminishing the distance between the culture chamber and the light source. The relationship between illumination and dry weight proved to be linear (Fig. 2), indicating that geometrical factors did not influence the results and that the cell mass

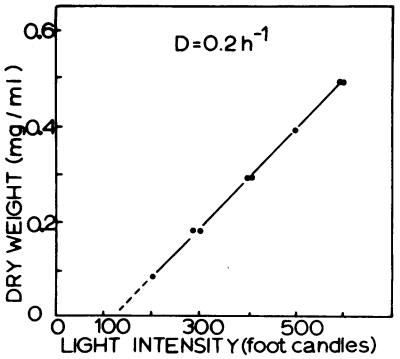

FIG. 2. *Relation between incident illumination on the culture and steady-state density achieved at a constant dilution rate of 0.2 h^{-1} at 31°C and pH 7.0. (100 foot candles = 1,080 lx; 300 foot candles = 3,230 lx; and 500 foot candles = 5,380 lx).*

produced per photon was not dependent upon incident light intensity over the range of intensities employed at that growth rate. It should be noted that this situation is different from that observed in batch cultures, because in a light-limited batch culture the growth rate will vary with the incident light intensity (14).

In a light-limited continuous culture maintained at a constant dilution rate, increasing light intensity results in increasing culture density, up to the point where each cell in the culture receives exactly the minimum amount of light required to grow at the prescribed rate, which results in a culture density characteristic of that illumination and that dilution rate. In other words, "self-shading" is the only and ultimate process that limits the density and imposes a specific, finite culture density on a light-limited culture resulting in a steady state. In fact, it is the process of self-shading in such a culture that insures the maximal efficiency possible with regard to utilization of photons entering the culture chamber. Therefore, it is self-shading that makes a light-limited continuous culture comparable to a continuous culture limited by a soluble, chemical nutrient; for in such a chemostat the limiting soluble nutrient is also taken up from the medium by the cells to the maximum possible extent and utilized with utmost efficiency.

Thus, a chemostat limited for a soluble energy source such as glucose (at a constant dilution rate) would yield a density-versus-nutrient concentration curve similar to that in Fig. 2, which plots density versus light intensity. There is one significant difference between Fig. 2 and the analogous curve found with a chemostat limited for a soluble energy source. Whereas the chemostat curve extrapolates through the point corresponding to zero nutrient-zero density, the light-limited curve does not extrapolate through this point (see dotted line in Fig. 2). This may be ascribed partly to the difference between incident light on the surface of the growth chamber (the place of measurement) and the effective light intensity actually falling on the cells in the culture and partly to the light passing through the growth chamber unabsorbed (see Discussion).

Influence of illumination on the steady-state cell composition. Table 1 supports the conclusion reached above that self-shading results in all cells in a light-limited continuous culture at constant dilutions rate receiving the same actual number of photons, irrespective of light intensity. From Table 1 it can be seen that light-limited cells from cultures with the same dilution rate have similar bacteriochlorophyll and carotenoid contents as well as very similar overall compositions, regardless of incident light intensity. This situation is analogous to that seen in a chemostat limited for a soluble energy source. In such a device, cells grown at the same dilution rate have very similar compositions, independent of the concentration of the limiting nutrient in the medium reservoir. From this observation we can conclude that increasing the illumination incident upon a culture of *R. capsulata* at a given dilution rate will serve only to increase the steady-state culture density; a situation exactly analogous to that seen when the medium reservoir concentration of a limiting soluble energy source is increased in a chemostat operated at a constant dilution rate. This reinforced our notion that light may be considered a nutrient directly analogous to a soluble, chemical nutrient.

Influence of dilution rate on the steady-state density of the culture. Figure 3 shows the steady-state culture densities obtained at several different dilution rates when the illumination was held constant. It can be discerned from this figure that the maximum growth rate obtainable in this medium in this continuous culture device was ca. 0.45 h^{-1} (which corresponds to a doubling time of 90 min). This value is very close to that obtainable with batch cultures, employing a similar medium (14). It should also be noted that the shape of the curve in Fig. 3 is significantly different from the familiar "plateau curve," obtained in a chemostat limited for a soluble energy source, operated at

TABLE 1. *Influence of increasing illumination on the content of the main cell constituents in R. capsulata, grown in a light-limited continuous culture at constant dilution rate ($D = 0.2\ h^{-1}$)*

Illumination (lx)	Contents (g/100 g of dry cells)					
	Bacteriochlorophyll	Carotenoids	Carbohydrate	Protein	DNA	RNA
2,150	1.62	0.557	7.38	57.5	5.04	8.95
3,230	1.57	0.503	7.92	55.1	4.87	9.25
4,300	1.88	0.564	7.93	55.8	4.64	7.92
5,380	1.73	0.546	8.39	57.8	4.82	8.84
6,460	1.84	0.541	8.15	56.2	4.75	8.03

different dilution rates with a constant concentration of the limiting nutrient (7). The explanation for this difference is rather straightforward, however. In a chemostat limited for glucose (for example), if the dilution rate is doubled, the number of cells washing out of the culture vessel per unit of time is doubled, but so also is the total amount of limiting soluble nutrient entering the vessel per unit time; as a result the density of the culture will remain nearly constant over a range of dilution rates (the "plateau"). In a light-limited continuous culture device, however, doubling the dilution rate still doubles the washout rate of cells from the culture, but the total number of photons entering the vessel per hour does not increase but, rather, remains constant. This results in a reduction of culture density in a fashion reciprocal with D, and, indeed, the data in Fig. 3 can be described by the equation $\bar{x}$ = constant/D, where $\bar{x}$ equals the steady-state culture density. A similar situation has been observed in light-limited continuous cultures of algae (12).

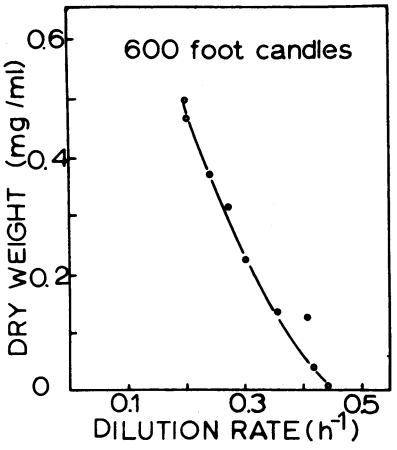

FIG. 3. *Relation between dilution rate and steady-state density of light-limited cultures of R. capsulata, grown with constant incident illumination of 6,460 lx (600 foot candles) at 31°C and pH 7.0.*

Influence of dilution rate on the steady-state cellular composition. The data in Table 2 show that the composition of cells grown at constant illumination varies strongly with dilution rate; this is in contrast with the composition of cells grown at a constant dilution rate, which remained constant with altering illumination (Table 1). RNA content increased with increasing growth rate, whereas bacteriochlorophyll and carotenoid content decreased. These results are similar to those reported in batch cultures and in previous continuous culture experiments (2).

Effect of concurrent changes in illumination and growth rate on the steady-state cell density of the culture. To achieve a situation analogous to the typical plateau curve (nearly rectangular $D - \bar{x}$ curve; [7]) found under carbon/energy limitation in a chemostat, it was necessary to compensate for the "dilution of light" effect ($\bar{x}$ = constant/D) described above. This was done by increasing the illumination in parallel with the dilution rate; Fig. 4 presents the results of such an experiment. Four identical light bulbs were aimed at the culture from the same distance, giving an incident light intensity on the culture vessel of 2,150 lx each; at $D = 0.1\ h^{-1}$ one lamp was switched on, at $D = 0.2\ h^{-1}$ two lamps were used, etc. The resultant curve had a relatively large initial slope at low dilution rates (Fig. 4). In a glucose-limited chemostat, a curve of similar shape would indicate a high apparent K_m for glucose. By analogy, *R. capsulata*, grown in a light-limited continuous culture device, seemingly exhibited a high apparent K_m with respect to light. This represents an unexpected result since one would assume that when light is limiting, the cells would be at their maximal efficiency in capturing the available light energy.

DISCUSSION

The use of continuous culture techniques can be a powerful tool when studying the physiology of microorganisms in general and with regard to

TABLE 2. *Influence of increasing dilution rate on the content of the main cell constituents in R. capsulata, grown in a light-limited continuous culture at constant illumination (6,460 lx)*

Dilution rate (h^{-1})	Contents (g/100 g of dry cells)					
	Bacteriochlorophyll	Carotenoids	Carbohydrate	Protein	DNA	RNA
0.199	1.84	0.541	8.15	56.2	4.75	8.03
0.244	1.65	0.495	8.59	57.9	5.03	9.76
0.304	1.15	0.362	7.92	55.6	5.13	11.5
0.360	0.87	0.265	7.73	54.8	5.41	13.2
0.421	0.64	0.214	ND[a]	ND	ND	ND

[a] ND, Not determined.

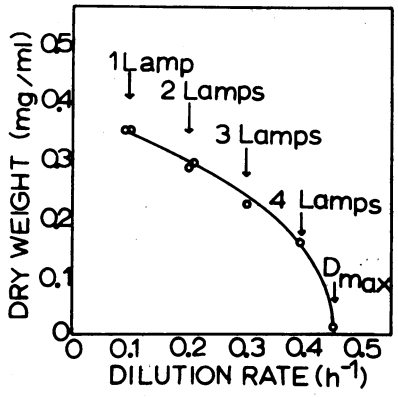

FIG. 4. *Relation between dilution rate and steady-state density of light-limited cultures of R. capsulata, grown with an illumination that varied in proportion with the dilution rate (31°C; pH 7.0). The four identical lamps employed in this experiment were focused on the culture in such a way as to provide 2,150 lx ± 5% of incident illumination each. As the dilution rate was increased incrementally from 0.1 to 0.4 h^{-1}, one to four of the lamps were turned on at the points indicated, thus providing an equivalent incremental increase in incident light intensity.*

problems related to energy metabolism in particular (1). Therefore, the study of nonsulfur purple bacteria under light-limited conditions would seem to be ideally suited for such an approach, and it is very surprising that extensive study of anaerobic, photosynthetic growth of these organisms has only recently been attempted (6), whereas similar studies on algae have been performed more than 15 years ago (12).

Introductory experiments (Fig. 2) showed that cultures of *R. capsulata* could be brought easily into steady-state growth in a simple salts medium under closely defined, light-limited conditions. At least under limiting conditions, light could be treated as a soluble, chemical nutrient because a linear relationship was found between illumination incident on the culture and steady-state density, a situation similar to the linear relationship which is found between the concentration of a chemical energy source and the density of a culture (7). This linear relationship also indicated that the utilization of light for growth was constant and independent of the incident intensity at a constant dilution rate.

Our study of the effect of increased dilution rate on the steady-state density of the culture (at constant illumination) made clear that the relationship between the dilution rate D and the steady-state cell density $\bar{x}$ (Fig. 3) was very different from the one observed in the case of a similar (carbon and) energy limitation in a heterotrophic organism grown in a continuous culture. A tentative explanation has already been given in Results; however, the concept of dilution of light, as we have named the phenomenon, may be clarified by considering an energy-limited continuous culture device equipped with two medium pumps: one for the energy source and one for the remainder of the medium. If we were to increase the dilution rate in such a device by increasing the flow rate through both pumps simultaneously by the same factor, this would have almost no effect on the steady-state density of the culture, because the increased washout rate of cells would be balanced by the increased inflow rate of the growth-limiting energy source. However, if we did not change the setting of the energy source pump, but increased the rate of the other pump, delivering the bulk of the medium, this balancing effect would not take place; the energy source would be diluted, and the steady-state density would drop. It is apparent that the shape of the $D - \bar{x}$ curve obtained in Fig. 3 can be explained in this way if a light bulb can be considered an energy pump delivering photons at a constant rate.

When Pipes and Koutsoyannis performed an experiment on *Chlorella* (12) similar to that shown in Fig. 3, they derived a mathematical expression showing that, due to this dilution of light effect, the steady-state density of the culture should vary inversely with dilution rate: $\bar{x} = \text{constant}/D$. However, they assumed complete (100%) utilization of light under all circumstances and did not take into account that at higher growth rates the relative amount of unused photons will increase, due to saturation of some (growth) rate-limiting process (either energy conversion or some other process) that would ultimately determine the maximum growth rate of the organism (7). Thus, there will never be a 100% utilization of the limiting nutrient (be it light or a water-dissolved nutrient), but there will always be a small steady-state residual concentration $\bar{s}$, left over from the original input concentrations S_R (7). Therefore, the steady-state density $\bar{x}$ will only by approximation be linearly related to the input concentration of the limiting nutrient: $\bar{x} = Y \cdot S_R$ (where Y is the yield constant for the limiting nutrient). The actual relation will be: $\bar{x} = Y\ (S_R - \bar{s})$ (7). For most purposes, however, $\bar{s}$ will be so small as to be negligible and the simpler equation will be valid; but it should be kept in mind that $\bar{s}$ is growth-rate dependent: $\mu = \mu_{max} \cdot \left(\frac{\bar{s}}{K_s + \bar{s}}\right)$, which (in steady-state $\mu = D$) gives: $\bar{s} = K_s \cdot \left(\frac{D}{\mu_{max} - D}\right)$, where μ is the growth rate, μ_{max}

is the maximum growth rate, and K_s is a Michaelis-Menten-like constant (7). It is the increase in $\bar{s}$ very close to the maximum growth rate that produces the sharp drop in the plateau-shaped $D - \bar{x}$ curve mentioned earlier (7):

$$\bar{x} = Y \cdot \left[S_R - K_s \left(\frac{D}{\mu_{max} - D} \right) \right] \quad \text{(equation 1)}$$

To describe the situation depicted in Fig. 3, we now only have to insert a factor accounting for the dilution of light effect and we arrive at:

$$\bar{x} = Y \cdot \left[\frac{c}{D} - K_s \left(\frac{D}{\mu_{max} - D} \right) \right] \quad \text{(equation 2)}$$

which adequately describes the curve found in Fig. 3 (c is a constant describing the incident illumination by photons of a utilizable wavelength).

To separate the effects on the steady-state density $\bar{x}$ of the dilution of light effect (c/D) from the decreased utilization of limiting nutrient (high $\bar{s}$) found near μ_{max}, we devised the experiment detailed in Fig. 4. This situation is completely analogous to a glucose-limited culture of a heterotrophic organism and therefore is adequately described by equation 1, since the rate of photons entering the culture was equalized with the dilution rate. From the shape of the curve it can be supposed that the apparent K_s value is rather unexpectedly high. For instance, it can be seen from Fig. 4 that at a dilution rate of 0.2 h^{-1}, $\bar{s}$, the steady-state amount of unused photons, has already become appreciable. Extrapolation to zero dilution rate in Fig. 4 shows that at a dilution rate of 0.2 h^{-1} approximately one-fourth of the utilizable light was not effective in the production of cell mass. This amount of illumination is equivalent to the number of utilizable photons in a measured intensity of 1,080 lx (100 foot candles) of incident white light. In this connection reference to Fig. 2 will show that extrapolation of the dry weight versus light intensity line does not pass through the origin, but rather intercepts the abscissa at a value slightly greater than 1,080 lx. This intercept is very likely the sum of the unutilized light (equivalent to approximately 1,080 lx at a dilution rate of 0.2 h^{-1}) plus the small amount of incident light reflected from the surface of the vessel and which thus never enters the culture. This correlation serves to emphasize the striking similarity of continuous cultures limited either by light or by a chemical energy source.

Although the actual number of photons entering the culture in the experiment detailed in Fig. 4 was increased at each increment of D by strictly identical amounts (identical lamps), we cannot rule out the possibility that the light arrangement per se (illumination from one side) may have tended to affect the shape of the curve. It should be kept in mind that competition for available photons in the form of self-shading is the principle upon which a light-limited continuous culture device must operate. Yet, any continuous culture device must be a uniform reactor with each cell receiving identical treatment to that received by all others in the culture. In our arrangement uniformity of treatment was achieved by stirring, in conjunction with a series of baffles that caused the culture to be thoroughly and randomly mixed, assuring that each cell received the same average amount of illumination over a very short time period. Experiments (data not given) showed that the culture density did not vary significantly with several widely different stirring rates when light intensity and D remained constant. This strongly suggests that the mixing arrangement employed maintained uniform conditions in the cultures with regards to shading. However, we are aware that the cells in a stirred, light-limited culture in steady state will experience fluctuations in illumination which over time represent an average illumination level. This is of course different from the prevailing situation in a nutrient-limited chemostat in steady state, where all the cells are exposed to a constant concentration of limiting nutrient over time. Nevertheless, the linear relationship found in Fig. 2 indicates that such fluctuations of intensity in a stirred light-limited continuous culture device do not constitute a problem, at least over the range of incident light intensities employed in the present study (14).

It was pointed out in Results that cell composition proved to be independent of illumination at constant dilution rate. In fact, apart from changes in pigments and RNA which correlated with changes in dilution rate, the contents of other cell polymers were remarkably constant, and the total of constituents for which assays were performed always accounted for about 80% of the dry weight, leaving 20% for lipids and inorganic ions. It is also interesting that under all light-limited conditions (independent of illumination and D) the yield value for malate was 0.30 (grams, dry weight, of bacteria synthesized per gram of malate consumed), which indicated a certain lack of efficiency in the incorporation of carbon-containing compounds into cell material, since under carbon-limited conditions, on the other hand, this value was at least 0.45. The bacteriochlorophyll/carotenoid ratio (mole/mole) always remained constant at 2.06 ± 0.15, independent of illumination and dilution rate. Thus, under conditions of light limitation in continuous culture, at least, it appears that syn-

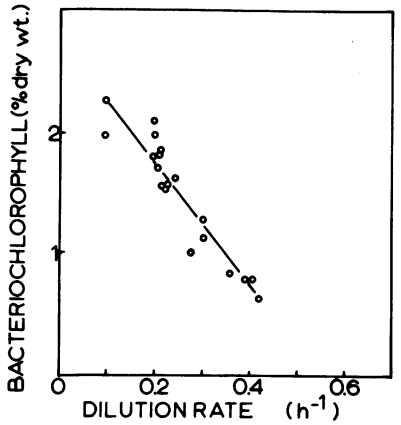

FIG. 5. *Relation between bacteriochlorophyll content and dilution rate of R. capsulata cells that were grown under light limitation with various different intensities of incident light (in the range 1,080 lx to 8,600 lx).*

thesis of bacteriochlorophyll and carotenoids may be coordinately regulated.

There has been much speculation concerning the inverse relationship between RNA and bacteriochlorophyll content (2, 13, 14), which indeed, we also found (Table 2). However, in agreement with others (11), we think this relationship may be fortuitous and due simply to the linear responses of RNA content and bacteriochlorophyll content to growth rate (Fig. 5). It is obvious why RNA increases linearly with growth rate, but why bacteriochlorophyll should decrease linearly with growth rate under conditions of limiting light (Fig. 5) is far from clear. It would seem reasonable that energy requirements (and thus the content of photopigments) would increase rather than decrease with growth rate, just as the cytochrome content of heterotrophic organisms increases with growth rate under conditions of carbon and energy limitation (1). Obviously this perplexing behavior requires further attention.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant PCM 77-05618.

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