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Studies of Pigments and Growth in *Chloroflexus aurantiacus*, a Phototrophic Filamentous Bacterium

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Abstract. 1. The chlorophyll pigments of *Chloroflexus aurantiacus* were separated by column chromatography on powdered sugar and were identified by spectrophotometry in various solvents as BChl *a* and BChl *c* (chlorobium chlorophyll 660). The bacteriopheophytins were also prepared and characterized spectrophotometrically. The identity of the BChl *a* is tentative because of its anomalous phase test behavior and because of changes in its absorption spectrum observed under different conditions of preparation.

2. Growth rates of *Chloroflexus* at 55° C and synthesis of the 2 chlorophylls were compared in cells growing under anaerobic conditions at different light intensities. Growth rates increased with increasing light intensity to a saturation level of about 0.30 doublings/hr at 20000 lux and above during the second exponential phase of growth. The rate of the first exponential phase continued to increase, at least up to 50000 lux. The specific content of both chlorophylls decreased with increasing light intensity but to different extents. A linear relationship between specific chlorophyll content and growth rate for either chlorophyll was only observed over a limited range of growth rates. The ratio of BChl *c*/BChl *a* decreased with increasing light intensity. The greatest change occurred between 300 and 5000 lux. The differential responses of BChl *c* and BChl *a* to light intensity were also demonstrated by shifting highly pigmented cells (grown at low light intensity) to high light intensity. In these cases different rates of synthesis of the 2 pigments followed initial adjustments. Carotenoid synthesis did not decrease with increasing light intensity under anaerobic conditions.

3. Chlorophyll synthesis was suppressed under fully aerobic conditions in darkness and light. In either case the pigments were diluted out by continued cell growth. At least 1 carotenoid pigment, however, was synthesized under aerobic conditions. Other carotenoids characteristic of anaerobic growth were not observed.

4. The chemoheterotrophic aerobic growth rate at 55° C in darkness (0.14 to 0.22 d/hr) was less than the maximum second phase phototrophic rate under anaerobic conditions (0.30 d/hr). Aerobic growth rate in the light was the same as in darkness if chlorophylls were lacking, but was enhanced if these pigments were still present. The oxygen consumption rate was partially suppressed in the light only when chlorophylls were present in the cells.

5. A light-minus-dark difference spectrum revealed the presence of a light-induced reversible decrease in absorbance of BChl *a* with a maximum effect at 860 nm, tentatively identifying a reaction center complex.

Key words: Chloroflexus — Thermophilic — Photosynthetic Bacterium — Bacteriochlorophyll — Pigment Regulation — Respiration in Light — Carotenoids.

A general description of the characteristics of *Chloroflexus aurantiacus* has been published (Pierson and Castenholz, 1974). Several features combine to give uniqueness to this genus. The organism is a thermophilic gliding filament containing chlorobium vesicles and the BChl *c* and BChl *a* characteristic of green bacteria. However, *Chloroflexus* is primarily an anaerobic photoheterotroph and is capable of aerobic chemoheterotrophic growth, resembling the purple nonsulfur bacteria in these respects.

This paper describes in more detail the chlorophyll pigments of *Chloroflexus* in comparison with those of other phototrophic bacteria. In addition, the relationship of growth rate, pigment content, and light intensity is described in some detail. The effects of high oxygen tension on the same properties are also characterized.

Materials and Methods

Growth of Cells for Pigment Studies

Chloroflexus aurantiacus, strain *J-10-fl*, was used throughout this investigation. *Chloroflexus* was grown in 900 ml screw cap Roux culture bottles filled to the neck with medium *D* (Castenholz, 1969) containing 0.2% powdered yeast extract and 0.1% glycylglycine, pH 8.2. Cultures were exposed to a light intensity of 1000 to 2000 lux at 45° C. Cells used for the pigment extraction and identification were harvested by centrifugation, washed once in Tris-Mg^{2+} buffer (0.05 M Tris, 0.001 M Mg^{2+}), pH 7.55, and the pellets were stored frozen at −20° C until needed. Cells for the *in vivo* spectra and light-minus-dark difference spectrum were harvested by centrifugation, washed twice in TSM buffer (Sistrom and Clayton, 1964) and broken by ultrasonic disruption as described previously (Pierson and Castenholz, 1971).

For comparative purposes, *Chlorobium limicola* forma *thiosulfatophilum*, strain *B* (kindly provided by Dr. N. Pfennig) was grown anaerobically in a medium modified from Larsen and Pfennig (see Pierson, 1973) at 23—27° C in a light intensity of 100—400 lux. Cultures were harvested after 6 days of growth and washed and frozen as described for *Chloroflexus*.

Separation of Pigments by Chromatography

The pigments were prepared using techniques modified from Stanier and Smith (1960) and Sistrom (1966). Washed wet pellets (*ca.* 4 g wet weight) were extracted 3 times in 60 ml volumes of 85% methanol saturated with $CaCO_3$. The pigments were transferred to ethyl ether or petroleum ether (b.r. 30—60) from the methanol with the addition of water or saturated NaCl solution. These solutions were then washed with water and dried over Na_2SO_4 and taken to dryness at 36° C under a stream of dry nitrogen. Powdered sugar columns were prepared using petroleum ether (b.r. 60—110). Pigments were applied to the columns in a solution of ethyl ether with petroleum ether (1:1). Columns were first developed with petroleum ether (b.r. 60—110) to remove most of the carotenoids. The BChl *a* and BChl *c* were separated from each other using a series of mixtures of n-propranol in petroleum ether ($^1/_4$, $^1/_2$, 1, 2%) or a series of mixtures of ethyl ether in petroleum ether ranging from 1 to 25%. After the columns were dried (under a stream of nitrogen) and extruded, the bands were carefully cut out and eluted with ethyl ether. Pigments were re-chromatographed 2 or 3 times until spectrally pure. All manipulations were done in dim light at room temperature. Only freshly opened cans of ethyl ether were used.

Preparations of Pheophytins

Pheophytins were prepared by mixing concentrated solutions of purified pigments (Abs_{770} or Abs_{660} of about 5 in ethyl ether) with an equal volume of 20% w/w HCl (Sistrom, 1966) in an ice bath followed by an incubation of 1 hr. The addition of water was necessary to force bacteriopheophytin *c* back into the ether layer. The ether layers were then washed 5 times with water and dried with Na_2SO_4.

Molisch Phase Tests

Phase tests were performed on small volumes (0.5—1.0 ml) of the purified pigments in ether by underlaying with approximately an equal volume of 30% w/v KOH in methanol (Smith and Benitez, 1955). The *Abs* of the pigment solutions used in these tests ranged from 1 to 2 at the red absorbance maximum.

Difference Spectrum of BChl *a*

The cell-free preparation in TSM buffer, pH 7.8 (Sistrom and Clayton, 1964), used for the infrared difference spectrum was equivalent to 12.5 mg cell dry wt/ml. The difference spectrum was determined at room temperature, point by point a 5 or 10 nm intervals. The actinic light source was a 1000 watt DXN quartz iodine lamp. The focused actinic beam was passed through a Corning 4-97 blue filter and 1.5 cm of 5% aqueous $CuSO_4 \cdot 5H_2O$. The sample and reference beams passed through Corning 2-64 red filters before reaching the photomultiplier.

Culture Conditions for Determining Rates of Growth and Pigment Synthesis

Cells were grown in medium *D* containing 1.0 g/l yeast extract, 2.0 g/l vitamin-free casamino acids (Difco) and buffered with 0.8—1.0 g/l glycylglycine, pH 8.2 ("Roux" medium). The growth vessels were 900 ml capacity Roux culture bottles containing 600—700 ml medium. The bottles had silicone rubber stoppers with fittings allowing for the continuous gassing of the medium through a sintered glass air stone and for periodic sterile sampling. For anaerobic conditions, cultures were constantly gassed with 99.5% N_2 + 0.5% CO_2. For aerobic conditions, 99.5% air + 0.5% CO_2 or line air was used. The gases were first passed through 30 cm of sterile cotton, followed by a humidifier of sterile water, and sometimes through an additional 10 cm of sterile cotton. The Roux bottles were incubated at 55°C in a glass aquarium. Constant temperature was maintained by a Haake ED Unitherm heater circulator unit. Light intensities were measured with an EEL (Evans Electroselenium Ltd.) "Lightmaster" photometer. Light sources were 75, 150, and 300 watt tungsten photo-flood lamps centered on the bottles and spaced for appropriate intensities, or a G.E. 500 watt "Quartzline" filament lamp. For dark conditions, bottles were wrapped in 2 layers of aluminum foil.

Growth Rates

Growth rates were determined by increase in cell mass. Dry weights were determined as described previously (Pierson and Castenholz, 1974), with the exception that General Electric Nuclepore filters (GE-60, 0.6 μm pore size) in later experiments replaced other membrane filters.

Oxygen Consumption

The rate of oxygen consumption was measured at 50°C with a Clark type membrane-covered polarographic probe and a YSI Model 54 meter reading directly in

p.p.m. The probe was sealed in a glass vessel filled to capacity with culture (66 ml), and a magnetic stirring bar was used for constant mixing. A single culture was never used for more than 30 min. Initially, exponential phase cells were spun down and resuspended in new "Roux" medium. The medium with cells was then saturated with oxygen by aeration prior to each experiment or when required.

Pigment Estimation

Spectra and absorbancies were determined with a modified Cary 14R spectrophotometer using an EMI Electronics type 9659B photomultiplier in the range of 340 nm to 900 nm. Routine absorbancies at 668 and 770 nm were determined by a Coleman-Hitachi Model 101 spectrophotometer with a diffraction grating monochromator and a constant bandpass of 10 nm. Usually, cuvettes with a 2 cm pathlength were used.

Pigments were quantified by extracting wet cells collected on Whatman glass fibre (4.25 cm GF/C) or Nuclepore filters with absolute methanol (stored over $CaCO_3$ or $MgCO_3$). Extractions were carried out at 4° C in the dark for a minimum of 1 hr, but were complete in a much shorter time. Extracts were cleared by pressure filtration through 24 mm GF/C filters. BChl *a* was determined at 770 nm using the extinction coefficient, 60 mM^{-1} cm^{-1} (Cohen-Bazire and Sistrom, 1966) and BChl *c* was determined at 668 nm using the specific absorption coefficient 86.0 $l\,g^{-1}$ cm^{-1} (Stanier and Smith, 1960).

Results

Pigment Separation and Identification

The crude methanolic extract of the pigments of *Chloroflexus* (Pierson and Castenholz, 1971, 1974) indicated the presence of BChl *a* and *c*, and

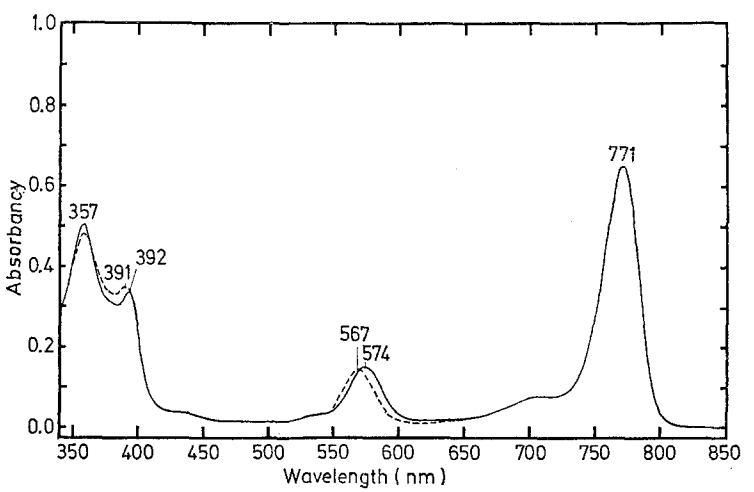

Fig. 1. Absorption spectrum in ethyl ether of purified BChl *a* from *Chloroflexus*. The solid line is the spectrum of BChl *a* prepared by transferring the pigments from methanol to ethyl ether in the initial purification step. The dashed line represents the shifted peak positions obtained when the pigment was prepared using petroleum ether

Table 1. Positions and relative absorbancies of the absorption maxima of BChl *a* from *Chloroflexus*

Solvent	Wavelength (nm) and relative absorbancy[a] (A)				
Ether	λ	771—772	574	392	357
	A	100	23.6	52.0	77.6
	Shoulders:	600—610			
Acetone	λ	770	580	393	359
	A	100	30.3	58.3	96.0
	Shoulders:	700			
Methanol	λ	771	608	366	
	A	100	32.1	106.9	
	Shoulders:	700, 340			
Chloroform	λ	780	582	390—400	363
	A	100	28.1	53.1	83.6

[a] The absorbancies (A) are relative to that at the longest wavelength peak.

carotenoids. After extraction into methanol, the pigments were transferred to ethyl ether and separated on powdered sugar columns. The faster moving blue pigment was separated in 0.5% n-propanol in petroleum ether. Its spectrum in ethyl ether (Fig. 1) and absorption maxima and relative absorbancies in different solvents (Table 1, Fig. 2) show it to be nearly identical to BChl *a* isolated from *Rhodopseudomonas sphaeroides* (Raymond and Sistrom, 1967). The spectrum of its pheophytin in ether (Fig. 3) and the absorption characteristics in ether and chloroform

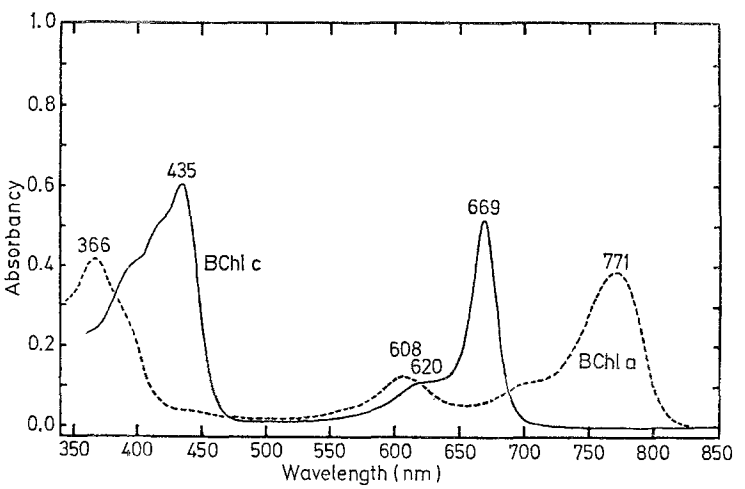

Fig. 2. Absorption spectra in methanol of purified BChl *a* (----) and BChl *c* (——) isolated from *Chloroflexus*

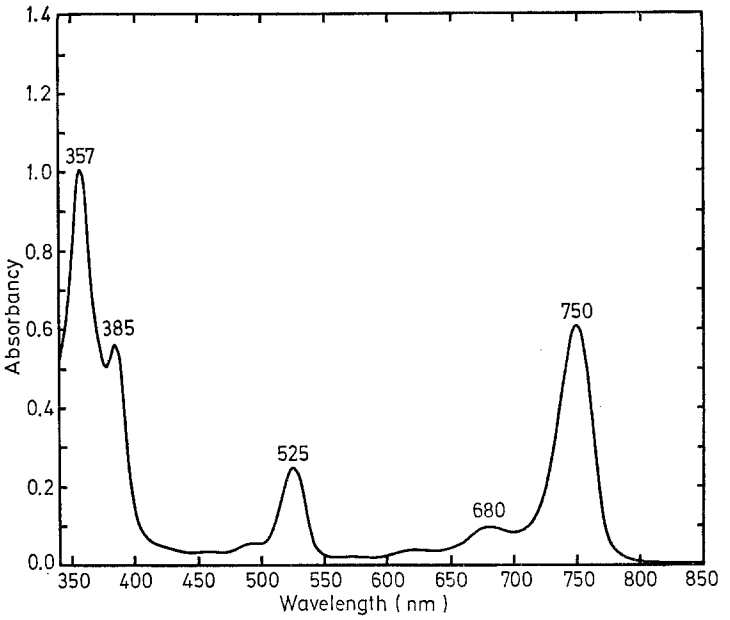

Fig. 3. Absorption spectrum in ethyl ether of bacteriopheophytin *a* from *Chloroflexus*

Table 2. Positions and relative absorbancies of the absorption maxima of bacteriopheophytin *a* from *Chloroflexus*

Solvent	Wavelength (nm) and relative absorbancy[a] (A)					
Ether	λ	750	680	525	385	357
	A	100	15.5	40.8	92.1	165.8
	Shoulders: 620, 490					
Chloroform	λ	757	686	531	390	362
	A	100	18.9	42.6	86.1	164.8
	Shoulders: 620—630, 495					

[a] The absorbancies (A) are relative to that at the longest wavelength peak.

(Table 2) are also identical with those of bacteriopheophytin *a* isolated from *R. sphaeroides* (Raymond and Sistrom, 1967). The pigment isolated from *Chloroflexus*, however, produced an anomalous Molisch phase test. When an ethereal solution of the BChl *a* was underlaid with 30% KOH in methanol, an immediate green color was observed at the interface with no preceding red, yellow, or blue. The same test performed on BChl *a* isolated from *R. sphaeroides* produced a transient yellow color typical of the positive phase test for BChl *a* (Smith and Benitez, 1955).

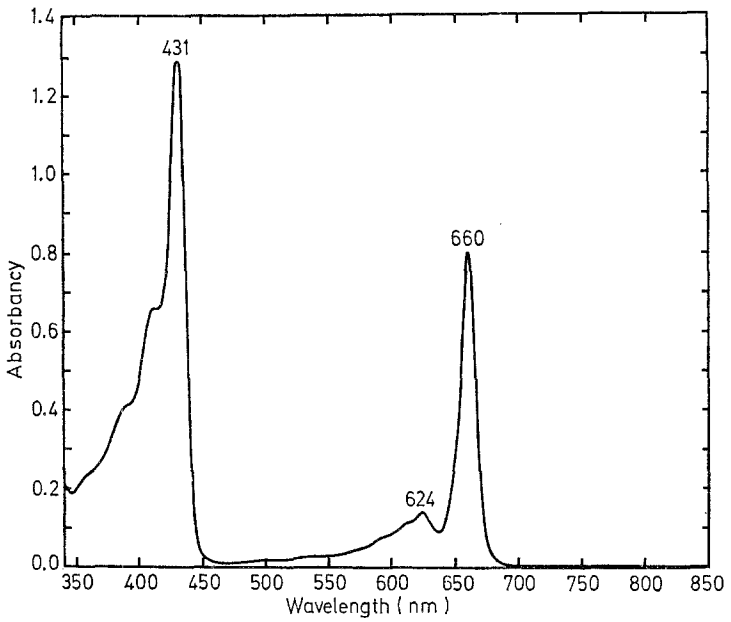

Fig. 4. Absorption spectrum in ethyl ether of purified BChl *c* from *Chloroflexus*

In an alternative preparation of the BChl *a* from *Chloroflexus*, the pigments were transferred from the crude methanolic extract into petroleum ether and separated on powdered sugar. The purified BChl *a* had a slightly different spectrum in ethyl ether than in the previous preparation. The orange peak occurred at 567 nm rather than at 574 nm (Fig. 1). This pigment produced a green color when underlaid with KOH/methanol in the phase test.

The slower moving green pigment from *Chloroflexus* was separated on powdered sugar in 2% n-propanol in petroleum ether. The spectrum in ethyl ether (Fig. 4) and the absorption characteristics in other solvents are nearly identical with those of BChl *c* isolated from *Chlorobium limicola* (Table 3, Fig. 2). The pigment prepared from both organisms had a fine structure in the 624 nm peak in ethyl ether that may be seen in greater detail in Fig. 5.

The spectrum of bacteriopheophytin *c* from *Chloroflexus* is nearly identical with that of the same pigment obtained by Stanier and Smith (1960) and that of *Chlorobium limicola* examined here (Fig. 6, Table 4).

The Molisch phase test was performed on BChl *c* isolated from *Chloroflexus* and *Chlorobium*. Pigment solutions with a peak absorbance of 1—2 in ethyl ether were used and there were no detectable color changes.

Table 3. Positions and relative absorbancies of the absorption maxima of BChl *c* from *Chloroflexus* and *Chlorobium*

Organism	Solvent	Wavelength (nm) and relative absorbancy[a] (A)					
Chloroflexus	Ether	λ	660	623 (4)	431	412	
		A	100	17.1	160.9	82.1	
		Shoulders: 612, 590, 390, 358					
	Acetone	λ	662	627	433		
		A	100	19.3	152.3		
		Shoulders: 615, 415, 390					
	Methanol	λ	668–669	620	435		
		A	100	21.8	117.4		
		Shoulders: 420, 395					
	Chloroform	λ	668	633	435		
		A	100	21.5	152.3		
		Shoulders: 618, 416, 390–395					
Chlorobium	Ether	λ	660	624	431	412	
		A	100	16.6	151.6	74.4	
		Shoulders: 612, 590, 390, 358					
	Acetone	λ	663	627	434		
		A	100	19.9	143.3		
		Shoulders: 615, 415, 390					
	Methanol	λ	669	620	435		
		A	100	21.4	109.8		
		Shoulders: 420, 395					
	Chloroform	λ	668	633	436		
		A	100	20.3	144.6		
		Shoulders: 618, 416–417, 395					
	Ether	λ	624	612–613	593–595	535–536	503
	(Fine structure of 624 nm peak)						

[a] The absorbancies (A) are relative to that at the longest wave-length peak.

Growth and Chlorophyll as Functions of Light Intensity

The specific growth rate (dry wt doublings/hr) for anaerobic cultures of *Chloroflexus* increased with increasing light intensity at 55° C (Fig. 7). At intensities above 10000 lux two growth rates are plotted. This is because of two exponential phases being evident, a faster rate before cell densities of about 0.03 to 0.05 mg/ml were reached and a second slower phase at densities above this (Fig. 8). The first rate continued to increase with light intensity to at least 54000 lux, at which point 0.63 to 0.80 doublings/hr were obtained. A linear extrapolation of the first rate curve of Fig. 7 to this intensity also results in a rate within the above range. The reason for the two rates is not known, but a switch by the organism

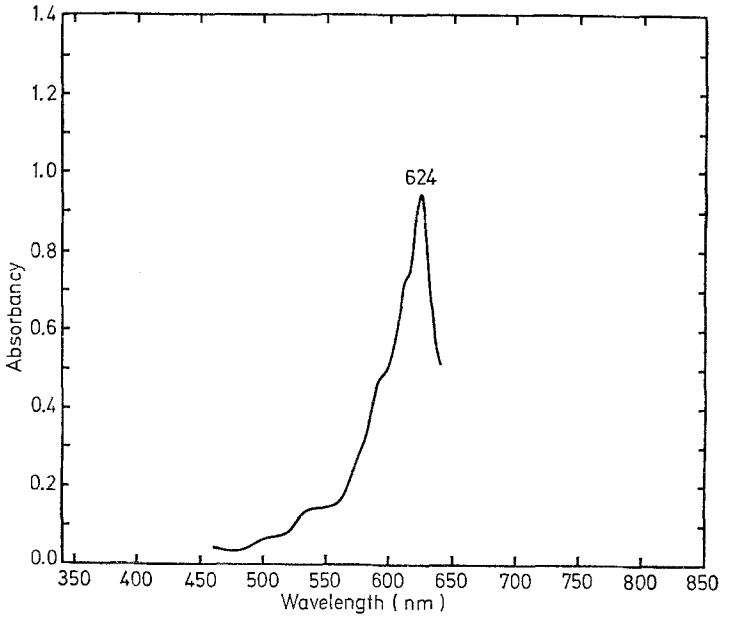

Fig. 5. Absorption spectrum in ethyl ether of concentrated BChl *c* from *Chlorobium limicola* showing the substructure of the 624 nm peak. Shoulders are at 612—613 nm, 593—595 nm, 535—536 nm, and 503 nm. The same substructure was observed in BChl *c* from *Chloroflexus*

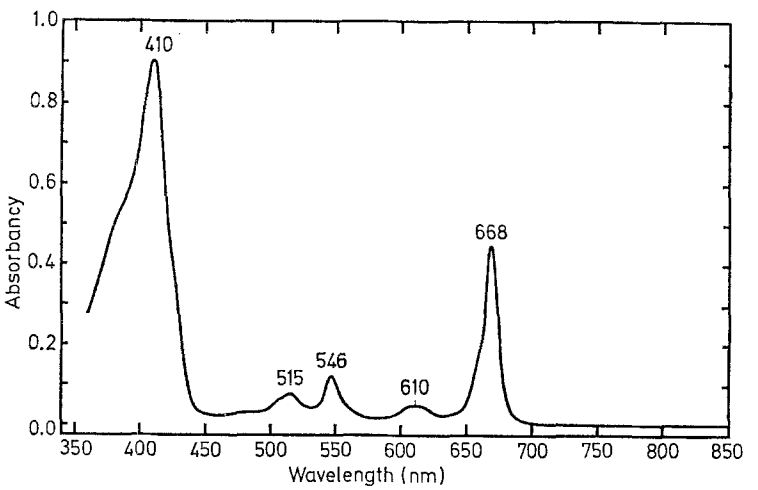

Fig. 6. Absorption spectrum in ethyl ether of bacteriopheophytin *c* from *Chloroflexus*

Table 4. Positions and relative absorbancies of the absorption maxima of bacteriopheophytin *c* from *Chloroflexus* and *Chlorobium*

Organism	Solvent	Wavelength (nm) and relative absorbancy[a] (A)					
Chloroflexus	Ether	λ	668	605–615	546	515	410
		A	100	11.2	27.2	17.8	203.6
		Shoulders: 505, 480, 380					
Chlorobium	Ether	λ	667–668	605–615	547	516	410
		A	100	11.9	29.0	17.9	196.4
		Shoulders: 505, 480, 380–385					

[a] The absorbancies (A) are relative to that at the longest wavelength peak.

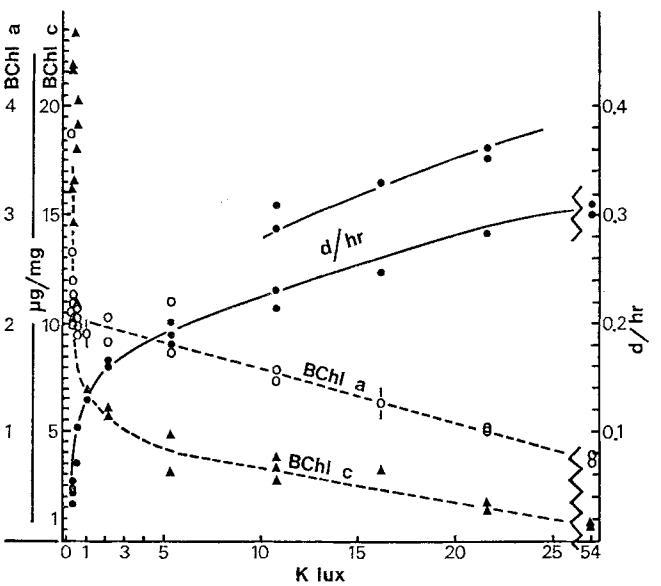

Fig. 7. The anaerobic growth rates at 55°C in doublings/hr (•) and specific contents of BChl *c* (▲) and BChl *a* (○) in µg/mg dry wt as related to light intensity (kilo lux). The 2 growth rates above 10 K lux are explained in the text. The lower represents the second exponential phase rates. The curves are drawn by eye

from one organic substrate to another is possible, considering the complex nature of the "Roux" medium. Unless otherwise specified, growth rate will refer to the second, slower rate.

The second exponential rate appeared to saturate approximately at 20000 lux with a rate of about 0.3 d/hr (Fig. 7). The upper portion of the light-dependent curve (*i.e.*, from 5000 to 20000 lux) approaches linearity, but with lower intensities there is a very steep drop in growth rate with

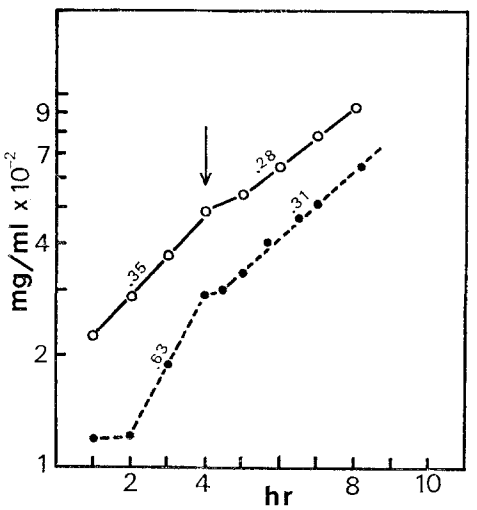

Fig. 8. Two representative anaerobic growth curves at 55° C in mg dry wt/ml, one at 22000 lux (o) with an early exponential phase (0.35 d/hr) somewhat higher than the second (0.28 d/hr). The curve at 54000 lux (•) shows a much steeper early growth phase (0.63 d/hr)

decrease in light intensity (Fig. 7). In fact, in this range it appears that the growth rate decreases linearly with a logarithmic decline in light intensity.

The specific pigment content (μg chlorophyll/mg dry wt) of both BChl *c* and BChl *a* in anaerobic cultures decreased with increasing light intensity (Fig. 7). The pigment contents were determined near the end of exponential phase or at several points of a growth curve. The pigment contents remained fairly constant during much of the exponential phase (the second phase at higher intensities). The decrease in specific pigment content with increasing light intensity is far more dramatic for BChl *c*, particularly between 250 and 5500 lux. In this range the BChl *c* content dropped 70—90%, whereas the BChl *a* content showed a drop of only 10—30% over the same intensity range (Fig. 7, Table 5). The decline in BChl *a* appears fairly linear over the entire range of light intensities. BChl *c*, after the initial 70—90% drop, below 5400 lux decreased as a possible linear function of light intensity up to 54000 lux, the highest intensity used. At the lowest intensities there appeared to be great variability in the specific content of either pigment, and anomalously high values for BChl *a* (3.7 μg/ml) and BChl *c* (27 μg/mg) occasionally occurred.

Table 5. Specific pigment contents (μg BChl/mg dry wt) and pigment ratios at selected light intensities of growth in *Chloroflexus*

Lux	BChl *c*	BChl *a*	BChl *c*/*a*
210–270	23.42	2.27	10.3
	21.96	2.04	10.8
320–540	19.20	2.04	9.4
	16.44	2.03	8.1
	10.96	1.97	5.6
1080	6.67	2.07	3.2
2150	5.60	1.83	3.1
5400	2.90	1.73	1.7
10800	2.69	1.47	1.8
21500	1.72	1.09	1.6
54000	0.63	0.78	0.8

When the specific pigment content is plotted as a function of growth rate (second exponential phase), BChl *c* content appears to fall with increasing growth rate in a linear fashion at growth rates greater than 0.1 d/hr (Fig. 9). Below 0.1 d/hr the drop is considerably steeper. BChl *a* content also appears to decrease linearly above rates of about 0.15 d/hr. A plot of growth rate versus pigment content in *Rhodopseudomonas sphaeroides* is linear for BChl *a* (the sole chlorophyll) with respect to

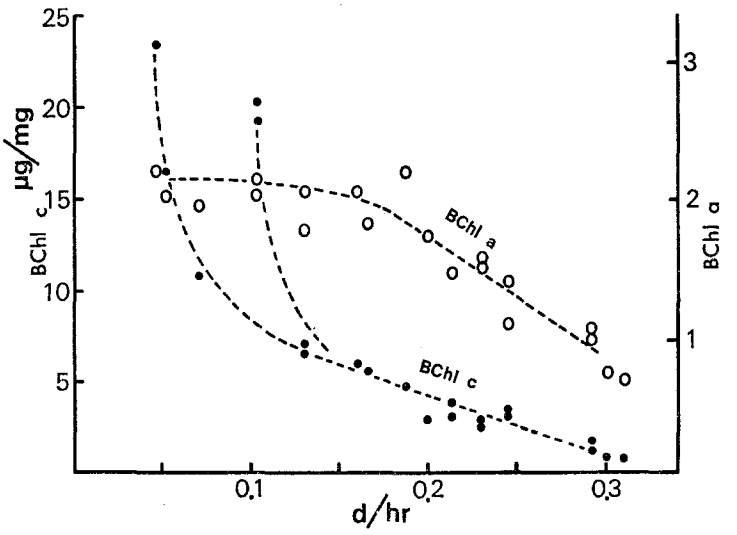

Fig. 9. The specific contents of BChl *c* (•) and BChl *a* (○) in μg/mg dry wt plotted in relation to growth rate (d/hr of second exponential phase). The curves are drawn by eye

growth rates ranging from about 0.1 to 0.3 d/hr (Cohen-Bazire and Sistrom, 1966).

In *Chloroflexus* the ratios of BChl *c*/BChl *a* changed from over 10 to about 2 as light intensity increased from about 300 to 5000 lux (Fig. 7, Table 5), or as growth rate increased over 4-fold. As intensities increased above this (to over 50000 lux), the ratios changed very little and never fell below 0.8. Although it is apparent that light intensity or growth rate affect the regulation of pigment, the two chlorophylls respond differently. The net rates of synthesis of the two chlorophylls were also examined when a highly pigmented (growing at 500 lux) anaerobic culture was suddenly shifted to an extremely high light intensity (54000 lux). Four replicate experiments were run. Since all results were similar, one experiment is represented in Fig. 10. After being exposed to the high light for 2 hrs the growth rate increased from 0.10—0.46 d/hr. This rate persisted for 4 hrs and then decreased to 0.17 d/hr. Similarly, after 2 hrs exposure to high light the rate of synthesis of BChl *c* increased from about 0.10 d/hr to 0.28 d/hr and then decreased to 0.07 d/hr. BChl *a* had an initial rate similar to that of BChl *c* and dry wt, but under high light, it shifted more rapidly to a somewhat higher rate of 0.16 d/hr. After cells had adjusted to the increased light intensity, the growth rate (dry wt) had increased 70% above the initial low light rate, while the rate of BChl *c* synthesis decreased 30% and that of BChl *a* increased 78%. Consequently, soon after high light exposure the specific content of BChl *c* decreased steadily, while the content of BChl *a* appeared nearly constant by the end of the experiment (Fig. 10). If the culture had been diluted and growth continued, the expected specific pigment contents for 54000 lux may have been attained and the growth rates adjusted accordingly.

Chlorophyll and Growth as Functions of Oxygen Tension

The amount of pigment in vigorously aerated cultures (*i.e.* pigment/ml culture) growing in darkness and in the light, did not increase over that of the inoculum, indicating that the synthesis of both bacteriochlorophylls is completely suppressed as in purple bacteria (Cohen-Bazire and Sistrom, 1966). In a few experiments a slight pigment increase occurred when the cultures became quite dense, suggesting that synthesis is possible under microaerobic conditions.

Under fully aerobic conditions the specific bacteriochlorophyll content (μg/mg dry wt) decreased with time due to dilution by growth, but total culture chlorophyll remained almost constant. In a more severe test of pigment stability under aerobic conditions, low-light grown anaerobic cultures with high specific pigment content were subjected to high light intensities (70000 lux), fully aerobic conditions, and a suboptimal growth temperature of 41°C. Even under these high stress conditions, the bac-

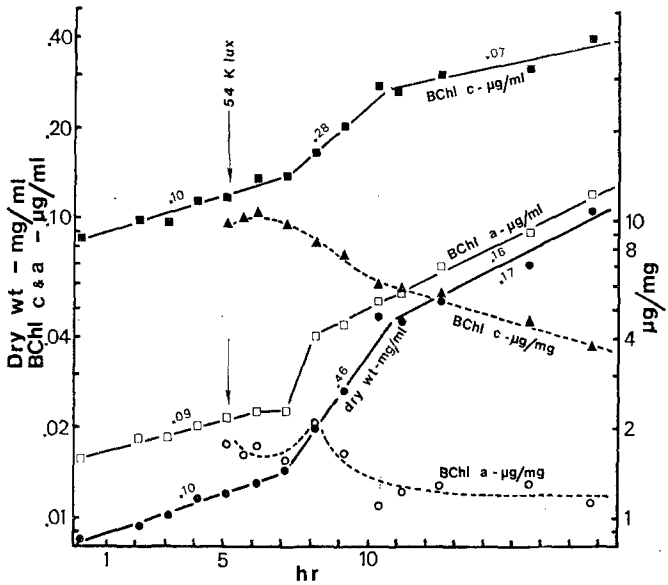

Fig. 10. The time course of dry wt increase (•) in mg/ml of medium, pigment increase (BChl c = ■, BChl a = □) in µg/ml of medium, and the specific pigment contents (BChl c = ▲, BChl a = ○) as µg/mg dry wt. At approximately 5 hrs the light intensity was changed from 500 lux to 54000 lux. Growth was under anaerobic conditions at 55°C. The rates of increase (d/hr) are indicated on the slopes

teriochlorophyll concentration per ml of culture did not decrease substantially (*ca.* 15%) during the succeeding exponential growth phase.

The 55°C growth rate of *Chloroflexus* under fully aerobic conditions was the same in the light and dark when chlorophylls were absent (0.14—0.22 d/hr). In the dark, the range of rates for highly pigmented cells was similar. With any given batch of pigmented cells, however, the aerobic growth rate was greater in the light than in the dark (*ca.* 1.6 fold). The cell yields of pigmented cells in the light were also 1.6 to 1.8 times greater than those of the dark controls.

In other experiments, the effect of bacteriochlorophyll on oxygen consumption in the light was assayed. In cultures grown aerobically over several generations (with no detectable bacteriochlorophylls), oxygen consumption rates in darkness and light (3200—22000 lux) were identical (Fig. 11). However, in anaerobically grown cultures with high chlorophyll content (BChl c = 13.3—14.5 µg/mg dry wt; BChl a = 2.4—2.5 µg/mg), light significantly reduced the oxygen consumption rate from that of the dark control. However, the degree of reduction was intensity dependent and appeared to saturate by approximately 5000 lux (Fig. 11).

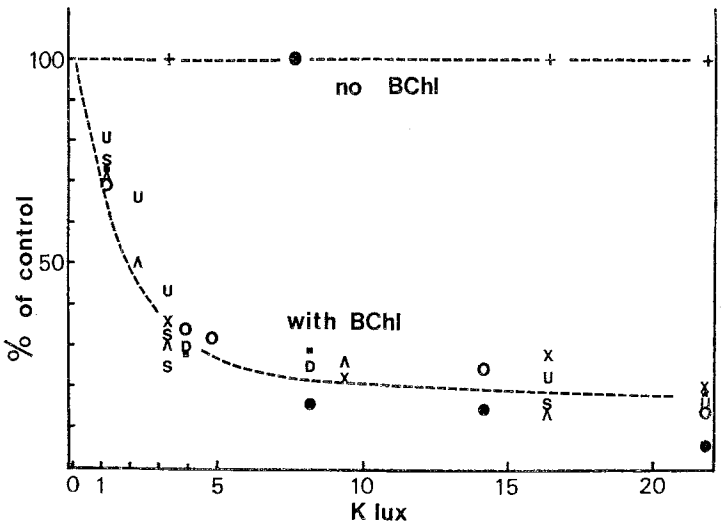

Fig. 11. The rates of O_2 consumption at 50°C as percentages of dark rate (control) *with* and *without* BChl *c* and *a* at different light intensities (kilo lux). Each type of symbol represents a separate cell batch and experiment. The contents of pigmented cells varied from 13.3 to 14.5 μg BChl *c*/mg dry wt and from 2.4 to 2.5 μg BChl *a*/mg. The actual rates of oxygen consumption in the dark ranged from 12 to 25 μg O_2/mg dry wt per min

The change in rate when switching to darkness from light, vice versa, or to different intensities was detectable within a few seconds, which may merely reflect the lower response limit for this probe and amplifier combination.

Effect of Oxygen Tension and Light on Carotenoid Pigments

To adequately study the effects of light intensity and oxygen on the synthesis of the different carotenoid pigments in *Chloroflexus*, the pigments from cells grown under different conditions would have to be separated and independently analyzed, since the carotenoid composition in *Chloroflexus* is complex (Halfen *et al.*, 1972). This was not done here, but a few observations have been made from spectra of crude methanolic extracts of cells grown under different conditions. Spectra of aerobically grown cells had a single carotenoid peak at 475 nm when grown in the dark or light at 350, 500, 3200, 5400, 16100, and 54000 lux (see Fig. 12 for a representative spectrum). The specific content (*i.e.* Abs $_{475nm}$/mg dry wt) of this pigment produced under aerobic conditions in light (3200 to 54000 lux) was about $1^1/_2$ to 2 times that of the dark controls. The amount of pigment in growing cultures (Abs_{475nm}/ml culture) increased

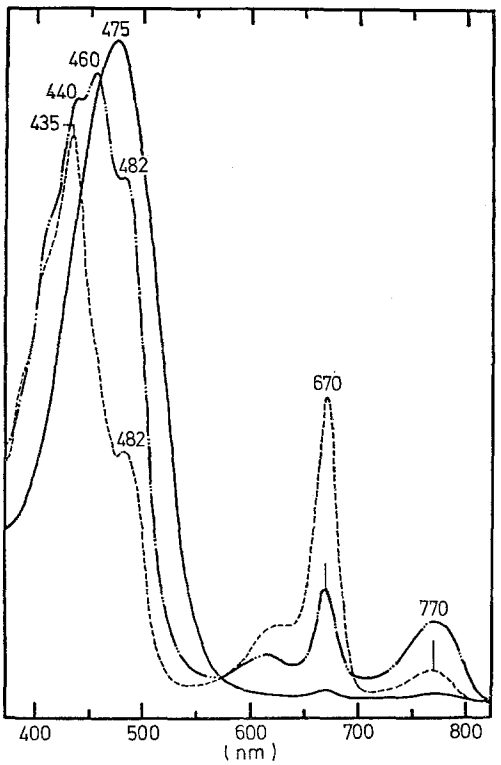

Fig. 12. Absorption spectra in methanol of pigments of *Chloroflexus* grown under different conditions. The number of peaks and the positions of the absorption by carotenoids (400—500 nm) changed with growth conditions. Aerobic cultures from 54000 lux (——); anaerobic cultures from 5400 lux (·—·—·); and anaerobic cultures from 500 lux (– – –)

significantly under all conditions (at least 5 fold), indicating that this carotenoid is synthesized in aerobically grown cells regardless of the presence or absence of light. The complex spectra of anaerobic light-grown cells showed no peak at 475 nm (Fig. 12), but this may have been obscured by the other carotenoids produced under these conditions.

All cells grown anaerobically had an absorption maximum in methanol at 482 nm (Fig. 12). This peak was not observed in aerobically grown cells. The specific content (Abs_{482}/mg dry wt) did not decrease with increasing light intensity. Two other peaks (440 nm and 455—460 nm) were observed in cells grown anaerobically at intensities greater than 5000 lux but not at 500 lux (Fig. 12).

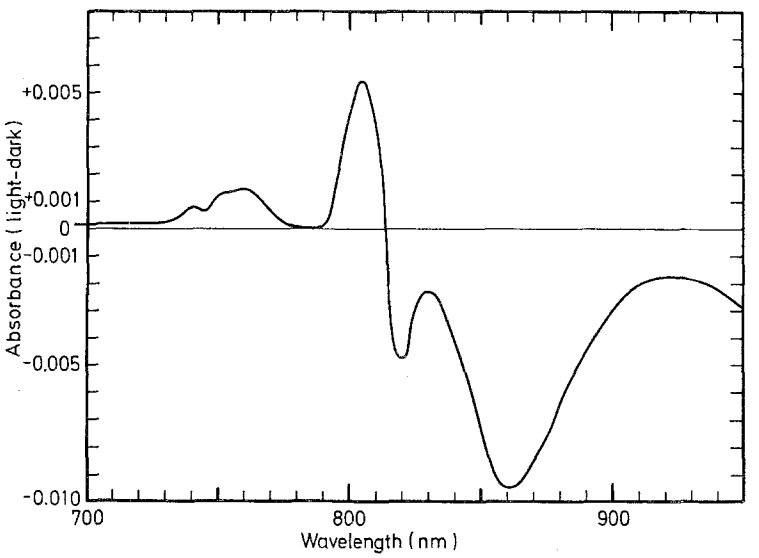

Fig. 13. Light-minus-dark difference spectrum of a cell-free preparation of *Chloroflexus* in buffer. The spectrum was determined point by point at 5 or 10 nm intervals

In vivo Absorption and Difference Spectra

The *in vivo* absorption spectrum of cell-free preparations of anaerobically grown *Chloroflexus* showed infrared maxima at 865 nm and 800 to 805 nm which correspond to those of BChl *a* (Pierson and Castenholz, 1974). There was a single maximum at 740 nm due to BChl *c* and a large peak near to 462 nm due to combined absorption by BChl *c* and carotenoids. The 2 infrared maxima of BChl *a* were approximately the same height (Pierson and Castenholz, 1971, 1974).

The light-minus-dark difference spectrum of a cell-free preparation of *Chloroflexus* in buffer with an initial Abs_{870nm} of 0.38 showed a maximum light-induced decrease in absorbance at 860 nm (Fig.13). In addition, a wavelength shift in absorbance maximum occurred at 800 to 805 nm. At saturating actinic intensity, the reversible decrease in absorbance at 860 nm represented 3.9% of the total absorbance at this wavelength.

Discussion

Pigment Identification

The shift in the 574 nm peak position in the absorption spectrum of BChl *a* when it was prepared with petroleum ether rather than ethyl ether and the anomalous phase test results are similar to the behavior described for the BChl *a* isolated by Raymond and Sistrom (1967) from

Ectothiorhodospira halophila, a phototrophic bacterium which is also moderately thermophilic. Because of these unusual characteristics, the identification of the blue pigment from *Chloroflexus* as BChl *a* is tentative. Although the pigment may not be identical to BChl *a* from *Rhodopseudomonas sphaeroides*, it is clearly very similar to it. As has recently been demonstrated (Brockman and Knobloch, 1972; Katz *et al.*, 1972), the identical chemical nature of BChl *a* in various phototrophic bacteria is questionable and the exact identity of the pigment in *Chloroflexus* needs to be verified with more precise analytical techniques.

In terms of its chromatographic behavior, spectral characteristics, and phase test results, the green pigment isolated from *Chloroflexus* appeared identical to the BChl *c* isolated from *Chlorobium*. The fine structure of the spectrum of this pigment shown in Fig.5 has not been previously reported, although published spectra of BChl *c* (Strain and Svec, 1966) showed some irregularities of the spectrum in this region but with less detail. A fine structure in the spectrum of BChl *d* in the same general region (470—625 nm) has been reported (Stanier and Smith, 1960), but it is quite different in specific shoulder locations and relative absorbancies.

Carotenoid Synthesis

The rate of synthesis of carotenoids in purple bacteria decreased with increasing light intensities and oxygen tensions (Cohen-Bazire *et al.*, 1956). This was not the case in *Chloroflexus*. At least one carotenoid was present whose synthesis was not repressed by the presence of oxygen (Abs $\max_{475\text{nm}}$). The only carotenoid analysis of *Chloroflexus* was done on anaerobic cells (Halfen *et al.*, 1972). Therefore, if molecular oxygen is in fact required for its synthesis, this carotenoid may not have been present. The carotenoids synthesized by phototrophic cultures (482 nm, 440 nm, 455—460 nm) were apparently totally repressed under aerobic conditions. In aerobic and anaerobic cultures none of the carotenoid peaks that were observed decreased with increasing light intensity. In fact, additional carotenoids (440 and 455—460 nm) appeared only at higher light intensities in anaerobic cultures. Although the response of the carotenoids in *Chloroflexus* was complex, no evidence was found for the decreased synthesis observed in purple bacteria with increasing light intensity.

Growth Rates and Pigment

Although the two chlorophylls present in *Chloroflexus* are probably identical to those of green bacteria, the relative quantities of the two chlorophylls present generally are not. In green bacteria the BChl *a* is present in small amounts compared with BChl *c*, constituting only 5—10% of the total chlorophyll present (Sybesma, 1970). In *Chloroflexus*, depending on the growth conditions, the BChl *a* may also be a minor

constituent or it may be present in equal or even somewhat greater amounts than the BChl *c* (at 54000 lux). This situation has never been observed in green bacteria which are strict anaerobes and apparently do not grow well at high light intensity. In *Chloroflexus* it appears that there is independent control of the synthesis of BChl *a* and BChl *c*. The relatively greater decrease in the specific content of BChl *c* than of BChl *a* with increasing light intensity probably does not reflect a greater sensitivity to photodestruction, since cells grown aerobically at high light intensity do not lose either pigment more rapidly than the other, even though the synthesis of both ceases. The slight decreases (*ca.* 15%) in both chlorophylls over a 6 hr growth period at suboptimal temperature probably reflected loss by cell lysis.

The growth rates and specific chlorophyll contents of *Chloroflexus* responded to increasing light intensities and oxygen tensions in a manner generally similar to that observed in the purple non-sulfur bacteria (Cohen-Bazire *et al.*, 1956; Cohen-Bazire and Sistrom, 1966). In addition, the maximum growth rates of *Chloroflexus* were comparable to the rates reported by these authors for phototrophically-grown *Rhodospirillum rubrum* and *Rhodopseudomonas sphaeroides*. The specific content of BChl *c* appears to decrease linearly with increasing light intensity (Fig.7) and with increasing growth rate (Fig.9) over a fairly wide range of values, but this linkage may uncouple at very low intensities and growth rates. The BChl *c* values become very high below 500 lux when growth rates are falling rapidly with decreasing light intensity. The specific pigment values were difficult to reproduce under these conditions, probably because it was more difficult to regulate a constant light intensity exposure. The specific content of BChl *a*, on the other hand, appears to increase much less dramatically at lower growth rates and light intensities.

Another indication of separate regulatory mechanisms for the two chlorophylls are the different rates of synthesis of BChl *c* and *a* after a shift to high light intensity (Fig.10).

These differential responses of BChl *c* and BChl *a* to light intensity or growth rate bring to our minds one principal explanation in terms of a structural basis. This is the possibility that BChl *c* is located primarily in the chlorobium vesicles (as in green bacteria), allowing its content per cell (or per mass) to vary with the number of vesicles, and that BChl *a* is principally located in the cell membrane. In the latter instance, it would be likely that the amount per cell would vary less than that of BChl *c*. In a few purple non-sulfur bacteria (*e.g. R. tenue, R. gelatinosa*) the BChl *a* may be largely restricted to the cell membrane, since only a few tubular intracytoplasmic proliferations have been seen (Pfennig, 1969). In other purple bacteria the BChl *a* content varies considerably over a wide range of light intensities and growth rates (Cohen-Bazire and

Sistrom, 1966), but these species contain a varying amount of pigment-bearing intracytoplasmic thylakoidal membranes, either lamellar or vesicular (see Oelze *et al.*, 1969), and the cell membrane itself may not contain pigment (Niederman, 1974). In the event that *Chloroflexus* has a major separation of bacteriochlorophyll sites, there is an analogy in the blue-green prokaryotes where the bulk photosystem II pigments (phycobiliproteins) are aggregated into structural units termed phycobilisomes which lie on the surfaces of the intracytoplasmic thylakoids (*e.g.*, Edwards and Gantt, 1971; Gantt and Lipschultz, 1973). Chlorophyll *a* of both photosystems and reaction center chlorophylls are located in thylakoid membranes. In blue-green algae the phycobilisomes often account for most of the light-harvesting pigments. Similarly, at lower light intensities BChl *c* greatly exceeds BChl *a* in *Chloroflexus*, and the former pigment may then be regarded as the principal light-harvesting pigment. BChl *c* may be entirely restricted to chlorobium vesicles which are closely appressed to the cell membrane (Pierson and Castenholz, 1974), much as phycobilisomes on thylakoids.

The light-induced absorbance changes at 860 nm and at 800—805 nm in *Chloroflexus* are evidence for the presence of a BChl *a*-type reaction center pigment similar to that occurring in purple phototrophic bacteria. The bulk BChl *a* of *Chloroflexus in vivo* is also similar to that of the purple bacteria in having two infra-red absorption maxima (one near to 800 nm and another between 860 and 890 nm) and is unlike the BChl *a* of green bacteria which has a single *in vivo* maximum at *ca.* 810 nm (Sybesma and Olson, 1963). The green bacteria show a maximum light-induced absorbance decrease at 840 nm (Fowler *et al.*, 1971) instead of at 860 nm or higher wavelengths as in *Chloroflexus* and purple bacteria, respectively (Frenkel, 1970). The spectral properties of BChl *a* in *Chloroflexus* may suggest a structural association with a lipo-protein membrane similar to that of purple bacteria rather than an association with the chlorobium vesicles as has been suggested for green bacteria (Fowler *et al.*, 1971). However, this suggestion of physical segregation of the two bacteriochlorophylls in *Chloroflexus* must be documented by cell-free isolations of vesicles and cell membrane.

Aerobic Metabolism and Pigment

The experiments that demonstrated a light-induced depression of oxygen consumption in pigmented cells are in agreement with numerous experiments by other authors (see Frenkel, 1970; Thore *et al.*, 1969; Oelze and Weaver, 1971).

Some have additional evidence to strongly suggest that bacteriochlorophyll-mediated electron flow in light is in direct competition with oxidative electron flow because of the sharing of several carriers (Melandri

et al., 1971; Thore *et al.*, 1969; Marrs and Gest, 1973). The most supportive evidence indicates that a portion of the photo-electron transport system of purple nonsulfur bacteria can be used alternatively with a terminal oxidase system. This route would be most used in the dark when competition for carriers would not occur. A purely respiratory chain may also be produced even during phototrophic growth (see Marrs and Gest, 1973). During the physiological adaptation of continued aerobic growth (light or dark) when bacteriochlorophyll is diluted out, the specific activity of membrane-bound respiratory enzymes (*e.g.*, succinate oxidase, NADH oxidase) increased 4—5 fold (Thore *et al.*, 1969; Oelze and Drews, 1970). However, the capability for oxygen consumption remained essentially the same on a per cell basis, since thylakoids tended to also dilute out when pigment synthesis ceased (see Lampe *et al.*, 1972).

In *Chloroflexus*, neither anaerobic light-grown cells nor pigment-less aerobic cells appear to have intracytoplasmic unit membranes except for mesosomal configurations (Pierson and Castenholz, 1974). Yet, oxygen consumption is substantial in either type of cell. In the case of the obligate anaerobe, *Chlorobium* (and other green bacteria) terminal oxidases and oxidative metabolism are unknown. *Chloroflexus*, being the only known type of facultative aerobe with chlorobium vesicles, behaves in a manner suggestive of purple non-sulfur bacteria in which the photosystem and oxidative electron transport systems share the same membranes. In *Chloroflexus*, the definite inhibition of oxygen consumption by light when bacteriochlorphylls are present would indicate the intimate contact of photosystem electron carriers and those involved in the reduction of O_2. There is no precedence for the latter being located in membranes of the type enveloping chlorobium vesicles (2—3 nm). Consequently, these results support the hypothesis that a portion of the photosystem is located in the cell membrane of *Chloroflexus*.

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