AGE AND FLUORESCENCE CHARACTERISTICS IN SOME SPECIES OF ATHIORHODACEAE

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Abstract.—Nonsulfur photosynthetic bacteria (Athiorhodaceae) exhibit a time-variable fluorescence in addition to a constant fluorescence. All species examined show upon aging a remarkable gain in the variable component at the expense of the constant component while the total fluorescence remains essentially invariant. This result can be rationalized by supposing a change in distribution of bacteriochlorophyll in photosynthetic units as cells age. Alternatively, one may assume operation of two photochemical systems—one cyclic and predominant in young cells, the other noncyclic and predominant in old cells. It is also noted that a hitherto unreported minor fluorescence with maximum emission at $\sim$ 860 nm exists in addition to the well-known main fluorescence band at $\sim$890 nm. The rise in variable fluorescence is associated with the main band, a result in accord with the notion that the bacteriochlorophyll component responsible and absorbing at 870 nm is directly in contact with the energy trap.

Introduction.—Fluorescence intensity in green plants under continuous illumination, after a period of dark incubation, varies with time in a characteristic way.[1–5] Typically, the fluorescence transient of the green alga *Chlorella pyrenoidosa*,[5] excited by blue light, rises from base level 0 (constant fluorescence) through a series of variations in intensity to reach a maximum level P (variable fluoresence). This increase in fluorescence from 0 to P is believed[6, 7] to follow the reduction of Q (primary electron acceptor of pigment system II; quencher of fluorescence) and A (the intersystem electron transport pool of intermediates). An analogous phenomenon was discovered in bacteria by Vredenberg and Duysens in 1963.[8] They showed that the yield of bacteriochlorophyll (BChl) fluorescence rose in *Rhodospirillum rubrum* during continuous illumination much as in green plants and that this increase in fluorescence was proportional to the concentration of the bleached energy trap (P890). Clayton[9] confirmed these findings in chromatophores of *Rhodospirillum rubrum*, *Rhodopseudomonas spheroides*, and *Chromatium*, and also found an increase in fluorescence during illumination, when there was no bleaching of energy traps; this effect was correlated with the accumulation of reducing power.

In this report, we show that the older the cells the greater the percentage of variable fluorescence. For example, *Rhodopseudomonas spheroides*, when excited with medium light intensity, shows 2 per cent variable fluorescence when "young" (exponential cells) and 66 per cent when "old" (stationary cells). In general, purple bacteria have three infrared bands in their absorption spectra (at about 800, 850, and 880 nm) and only one fluorescence band at about 900

nm (see review by Olson and Stanton[10]). Our data indicate clearly that, in a variety of species, there are usually two fluorescence bands (at about 860 and 890 nm) and that most of the variable fluorescence is found in the long-wave component.

Experimental.—In most of our work we used three purple bacteria: *Rps. spheroides*, *R. rubrum*, and *R. molischianum*. However, the generality of the phenomena under investigation was established by examination of several other purple bacteria, e.g., *Rps. capsulata*, *Rps. gelatinosa*, *R. fulvum*, and an unidentified species provisionally assigned to the *Rhodopseudomonas* group and labeled FRNY. All the bacteria were cultivated as described by de Klerk.[11] Bacteria were suspended in their own culture media, and their concentration was adjusted to give the same optical density at 850 nm.

Fluorescence measurements were made with a spectrofluorometer described elsewhere.[12] The exciting light was obtained from a transmission-grating monochromator equipped with a Xenon light source. When emission spectra were made, the exciting light was either 480, 515, or 520 nm with a half bandwidth of 6.84 nm. A blue Corning filter (C.S 4–97) was used to remove the second-order overlap. Fluorescence in the characteristic range, 800–950 nm, was observed at right angles to the exciting beam. It passed through a red Corning filter (C.S. 2–62), then through a Bausch and Lomb monochromator (half bandwidth, 6.6 nm), and finally to a Dumont 6917 photomultiplier. The signal was amplified and recorded on a Sefram recorder. The excitation wavelengths and intensities were chosen to obtain maximal differential responses for the two types of fluorescence.

For recording variable and constant fluorescence levels, we used a stopped-flow method described previously.[12] In this procedure, the constant fluorescence is measured when the test system flows through a capillary, and the variable fluorescence is measured when the flow is halted. By running the time-course curve, i.e., fluorescence intensity as a function of time of illumination, we found conditions for maximum variable fluorescence.

The emission spectra of the variable and constant fluorescence were determined with the use of an automatically driven wavelength scan. Spectra were not corrected for the photomultiplier sensitivity. The photomultiplier, however, had an almost flat response in the region of observation; some distortion was possible because of the spectral dependence of the transmission efficiency of the observation monochromator.

In all experiments, the concentration of BChl and the intensity of the exciting light were kept constant, unless otherwise noted.

We did not attempt to exclude air except in one case in which we passed argon through the test system for about 15 min. No changes were observed in fluorescence characteristics.

Results and Discussion.—(1) *Growth curve of bacteria:* To define our system, we first present (in Fig. 1) a growth curve of *Rps. spheroides* (*open circles*) as obtained by batch culture in a photochemostat.[13] There are three distinct growth phases: *A*, the exponential (or logarithmic) phase in which the cells are dividing at maximum rates; *B*, the intermediate phase in which a portion of the cell population is still growing at maximum levels; and *C*, the stationary phase. The amount of bacteriochlorophyll synthesized during growth, as determined by extraction,[14] is also shown (*open triangles*). During growth phase *A*, the amount of BChl per unit cell density remains constant, but as the culture enters phase *B*, the biosynthesis of BChl continues at an almost unchanged rate even though increase in cell mass is lessening and gradually decreases into phase *C*. The ratio of BChl to unit cell mass changes from approximately 0.3 to 3.0 from phase *A* to *C*. Similar results are obtained for other bacteria.

Cohen-Bazire and Kunisawa[15] showed that BChl per cell increased when the

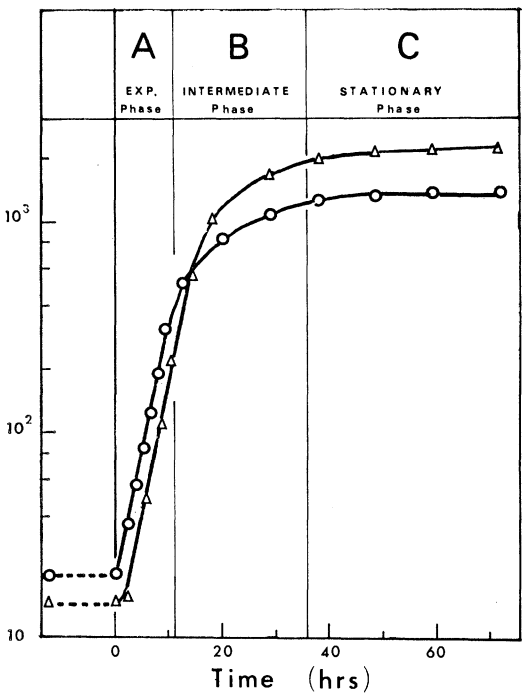

FIG. 1.—Growth curve for *Rps. spheroides*. ○—○—○, Cell population as measured by turbidity at 1200 nm (arbitrary units). ▲—▲—▲, BChl concentration measured by extraction (arbitrary units).

light intensity decreased. This finding was in accord with our observations, because as the cells were grown under constant light conditions, it could be expected that as the culture became more dense the quantity of light per unit time reaching a given cell decreased.

(2) *Fluorescence as a function of intensity of exciting light:* It is well known that the steady-state fluorescence yield increases at high light intensities in chlorophyll *a*–containing plants.[16–20]

Lavorel[21] and later Govindjee *et al.*[22] have shown that the yield of constant fluorescence (0) is not dependent on light intensity, whereas that of the variable fluorescence increases with light intensity to reach a maximum (P) which may be three times greater than the level of constant fluorescence. We obtain similar results with bacterial fluorescence.

Figure 2 shows the plot of the relative yield (ϕ/I) as a function of 480-nm light intensity (I) (100 units on the abscissa equals approximately 40,000 ergs $cm^{-2}sec^{-1}$). The yields, presented above, are directly related to the state or concentration of photochemical centers. Percentage of variable fluorescence to total (variable + constant) fluorescence increases by a factor of about 2–3 when light intensity is increased from a low value (4,000–20,000 ergs $cm^{-2}sec^{-1}$) to a high value (40,000 ergs $cm^{-2}sec^{-1}$). In one case (*R. molischianum*), this ratio increased from 27 to 66 per cent. These results can be rationalized on the basis of the model proposed by Vredenberg and Duysens[8] in which excess fluorescence is associated with increased bleaching of the energy trap at high light intensities.

(3) *Variable to constant fluorescence as a function of age:* Figure 3 shows fluorescence spectra obtained using the stopped-flow method for cell suspensions of *Rps. spheroides* harvested in phase A and in phase C. The BChl concentrations were adjusted in both cases to be the same (A at 852nm = 0.375). The cell mass in phase A was approximately 2.5 times that in C. It can be seen that, although the total fluorescence per unit of BChl varied only slightly for both types of cells, there was a striking increase in the ratio of variable to constant

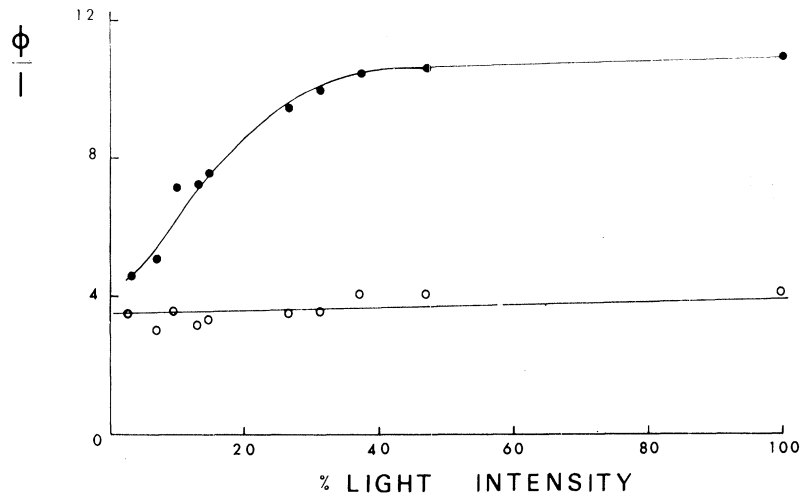

FIG. 2.—Variable (●—●—) and constant (○—○—) fluorescence yields as function of light intensity. *Coordinate:* ratio of fluorescence intensity to light intensity (arbitrary units). *Abscissa:* per cent of total excitation light intensity (see text). Organism: *Rps. spheroides.*

fluorescence in phase *C* cells. An increase from approximately 3 per cent for phase *A* cells to 35 per cent for phase *C* cells was found, whereas the level of constant fluorescence decreased in phase *C* cells.

Table 1 shows the results of over 40 determinations on several species of the nonpurple sulfur bacteria in all phases of growth. In all cases, the percentage of variable fluorescence was found to increase with physiological age.

Spectra: Absorption spectra of phase *A* and phase *C* cells have been included in Figure 3. Two features of these data should be noted: (*a*) Two fluorescence bands at 860 nm and 890 nm (F860 and F890) from bacteriochlorophyll B850 and B870 were found in both phase *A* and *C* cells. Thus, the efficiency of energy transfer from B850 to B880 was not 100 per cent even in young cells of *Rps. spheroides.* Earlier work on *R. rubrum, Rhodomicrobium vannielii, Chromatium D,* and *Rhodopseudomonas sp. NHTC 133* (see review in ref. 10) suggested that only one BChl complex (890) fluoresced. This was not the case in *Rps. spheroides,* as shown in our research. Measurements on *Rhodopseudomonas*

TABLE 1. *Percentage of variable fluorescence as a function of the physiological age of cells.**

Species	Percentage of Variable to Total (Constant + Variable) Fluorescence Phase *A* (exponential cells)	Phase *B* (intermediate cells)	Phase *C* (stationary cells)
Rps. spheroides	2	28	66
Rps. FRNY	2	13	60
R. rubrum	0	18	56
R. molischianum	2	20	†
Rps. capsulatus	4	30	†

* Cells were grown under identical culture conditions. Fluorescence was measured in each case with identical BChl concentrations (±5%) with the same light intensity (±7%).

† Not determined.

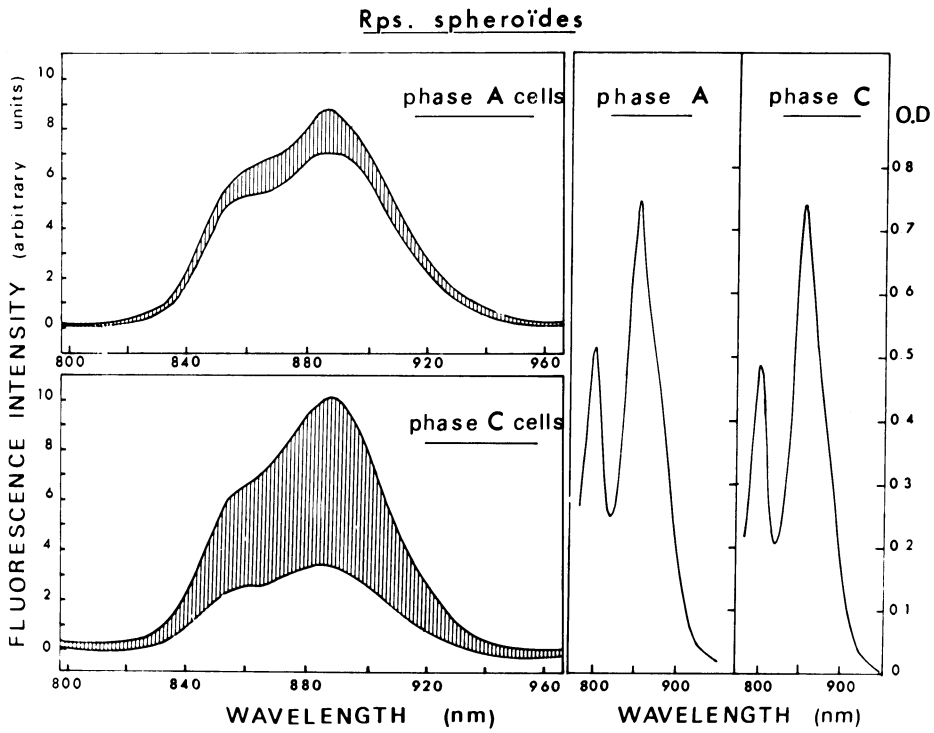

FIG. 3.—Figures on left show fluorescence emission spectra (arbitrary units) for cells in phase *A* and phase *C*. Vertically hatched areas (upper envelopes) indicate variable fluorescence and bottom envelopes indicate constant fluorescence.

Figures on right exhibit optical absorption spectra of phase *A* and phase *C* cells with equal concentrations of chlorophyll.

FRNY showed a small shoulder at 860 nm in addition to the peak at 890 nm. Two-banded emission spectra have been independently reported by Karapetyan[23] for *Rhodotheca sp.* (*b*) The fluorescence from B870 increased more than that from B850; this could be seen by plotting the ratio of variable to constant fluorescence ($P - 0/0$) in phase *C* cells. For instance, this ratio was ~1.6 at 860 nm and ~2.0 at 890 nm.

Clayton[24] has shown that in green bacteria (*Chloropseudomonas ethylicum*), variable fluorescence arises from bacteriochlorophyll rather than from chlorobium chlorophyll (Chlb Chl), whereas both BChl and Chlb Chl contribute to constant fluorescence.‡ Our results on *Rps. spheroides* (confirmed in *Rhodopseudomonas* FRNY) suggest that it is preferentially the fluorescence from B870 that changes during fluorescence induction. This is consistent with the idea that the BChl-complex which transfers energy directly to the energy trap should show the greatest variation in fluorescence yield.

Interpretations.—To explain the increase in the variable and the decrease in the constant fluorescence as the bacterial cells age, two alternative hypotheses may be proposed: one is concerned with changes in the size of the photo-

synthetic units (PSU) and the other with the relative importance of the "noncyclic" and the "cyclic" light reactions.

The increase in BChl content per cell with age may be attended by structural changes in the pigment apparatus. One may assume that as more BChl is deposited on the available surface, the ratio of active center BChl to bulk BChl is not modified; i.e., in old cells, the photosynthetic units exhibit closer packing and their effective size is smaller. If energy transfer occurs by exciton migration, channeling of energy from bulk BChl to the traps will be more efficient for the more densely packed photosynthetic units. This should decrease the fluorescence yield in the 0 state when all traps are available;[25] however, the maximum level in the *P* state where no traps are available should be independent of the size of the photosynthetic units. The increase in photon collection efficiency could be an adaptive response to decrease in light intensity usually prevalent in old cultures. This does not necessarily lead to the results that over-all photosynthesis is more efficient in old cells, because dark processes could become limiting.

That bacterial photosynthesis involves two pigment systems and two light reactions—one "cyclic" and the other "noncyclic"—has recently been suggested.[26–28] A model for the noncyclic system could be based on the flow of electrons from H_2A (the external electron donor), through ZH (the primary electron donor), BChl (the energy trap for the noncyclic process), X (the electron acceptor), and thence to NAD^+. For the cyclic system, coupled to photophosphorylation, electrons would flow from B′Chl (the energy trap in the cyclic process) to X and thence back to B′Chl. The noncyclic reaction is suggested here to be the more efficient one in old cells. Low constant and high variable fluorescence would result if either ZH or X were the quencher of fluorescence. Constant fluorescence would be low because ZH would predominate in dark. As the photochemical reaction proceeded, ZH would be oxidized to Z and variable fluorescence would rise. To account for the low photosynthetic activity of old cells, the cyclic reaction is postulated to be inefficient, thus limiting the amount of ATP old cells can generate. (Further, one may assume that the "cyclic" process does not compete with fluorescence; it might involve instead the triplet state of BChl.) On the contrary, in young cells the cyclic reaction predominates and phosphorylation proceeds efficiently. With the help of ATP, H_2A reduces NAD^+ (see refs. 29 and 30). To explain the high constant and low variable fluorescence in young cells, the concentration of ZH is postulated to be low and the noncyclic light reaction marginal. A decision between the models proposed should be possible when more data on the comparative biochemistry of young and old cells become available.

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‡ A recent paper by K. L. Zankel, D. W. Reed, and R. K. Clayton on fluorescence and photochemical quenching in photosynthetic reaction center preparations obtained from a blue-green mutant of *Rps. spheroides* (these PROCEEDINGS, **61**, 1243 (1968)) came to our attention after submission of this report. Of special interest in relation to our findings is their elegant demonstration that the variable fluorescence originates from the energy trap P-870.

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