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A 4-VINYLPROTOCHLOROPHYLLIDE COMPLEX ACCUMULATED BY "PHOFIL" MUTANT OF *RHODOPSEUDOMONAS SPHEROIDES*

AN AUTHENTIC INTERMEDIATE IN THE DEVELOPMENT OF THE PHOTOSYNTHETIC APPARATUS

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SUMMARY

A photosynthetically competent mutant strain of *Rhodopseudomonas spheroides* was isolated. In addition to bacteriochlorophyll, this organism produced particle-bound precursor 4-vinylprotochlorophyllide. The spectral characteristics of the pigment complex(es) accumulated in the culture medium were very variable. The spectral form occurring within the bacteria was characterized from fluorescence data. Its particle weight, 130 000, was determined by Sephadex G200 filtration. The main components of the complex were protein, lipid and pigment (6.8 : 6 : 1, w/w). As indicated by qualitative analysis, the lipid components were characteristic constituents of the photosynthetic membrane.

Kinetics of pigments synthesis showed that the total pigment synthesis was not affected by the mutation; bacteriochlorophyll content was always lower in the mutant than in the parent strain. The repigmentation process was followed by fluorescence emission. The results indicated that the mutation affected membrane component synthesis required for the bacteriochlorophyll(ide) incorporation.

The pigment complex was concluded to be an authentic intermediate in photosynthetic apparatus morphogenesis. The reasons for its excretion are discussed.

INTRODUCTION

Differentiation of the photosynthetic apparatus in non-sulfur photosynthetic bacteria requires a coupling in the synthesis of both pigment and membrane. In the course of pigment synthesis, incorporation into the membrane system involves formation of pigment-protein complexes [1–3]. Isolation and comparative studies of such complexes provide clues to understanding membrane differentiation in these bacteria. Mutations which result in overproduction of precursor complexes yield source materials for such studies. These mutants have such a production either because their cells are unable to incorporate the normal intermediate (interesting case) or because the mutation may alter the complex (uninteresting case). Thus it is essential to ascertain the native state of the complex.

We report the composition of a 4-vinylprotochlorophyllide macromolecular compound excreted by the "Phofil" mutant of *Rhodopseudomonas spheroides* and evidence that this complex is an authentic intermediate. This mutant, the production and physiological properties of which will be described in a later paper, is a conditional mutant type; it retains the ability to synthesize bacteriochlorophyll at a reduced rate and to grow photosynthetically. Pigment synthesis is herein described.

MATERIAL AND METHODS

Organisms. The mutant strain "Phofil" was derived from the wild type *Rps. spheroides* strain Y, after exposure to ultraviolet light irradiation. The dose given, 1000 erg/mm^2, corresponded to approximately 1 % survival. After growing for three generations in dark aerobic conditions, the cells were plated in the same conditions. The mutant obtained was isolated as one green colony out of $2.5 \cdot 10^3$ colonies formed.

Culture conditions. Bacteria were grown in a synthetic medium with succinate as carbon source [4]; the medium was equally suitable for aerobic or photosynthetic growth. Photosynthetic cultures were grown, at 32 °C, in glass bottles (2 l volume) with continuous nitrogen bubbling, magnetic stirring, and illumination with incandescent bulbs (5000 lux.) Partial or total bleaching resulted from aerobic growth in the light in volumes of 10–15 ml; the degree of bleaching depended on the number of generations in such conditions. Semi-aerobic conditions under constant oxygen partial pressure were obtained by gassing with a 5 % oxygen/95 % nitrogen mixture (Air Liquide).

Inocula were prepared, in all cases, from a single colony grown aerobically on solid medium.

The cellular population was estimated from the absorbance at 1200 nm, with the correspondance of $4 \cdot 10^9$ cells per ml for $A_{1200\,nm} = 1$.

Isolation of pigment complex. The washed pellet of a photosynthetic preculture was grown in photosynthetic conditions for the time of about one generation. The extracellular pigment complex was first separated from the bacteria by centrifugation (10 min, 12 000 $\times g$), then centrifuged at high speed (90 min, 100 000 $\times g$). The pellet was resuspended in 10^{-2} M phosphate buffer (pH 6.6), eluted on a Sephadex G 50 column and again centrifuged (90 min, 100 000 $\times g$).

Analytical procedures. Bacteriochlorophyll was determined in the acetone/methanol (7 : 2, v/v) extract using the molar extinction coefficient given by Clayton [5].

The molar extinction coefficient of 4-vinylprotochlorophyllide is the same as that for 4-vinylprotochlorophyll, $22.1 \cdot 10^3$ M$^{-1} \cdot$ cm^{-1} at 623 nm in ethyl ether [6]. From this value we deduce the extinction coefficients of the pigment inside the complex after detergent action: $\varepsilon_M = 27.8 \cdot 10^3$ M$^{-1} \cdot$ cm^{-1} and $\varepsilon_p = 45$ lg$^{-1} \cdot$ cm^{-1} at 631 nm. Protein was estimated by the method of Lowry et al. [7].

Total lipid was extracted with chloroform/methanol (2 : 1, v/v) by the Bligh and Dyer modified method [8]. Total phosphorus was determined by the method of Barlett [9]. Lipid compounds were examined by thin layer chromatography (silica gel G plates from Merck) with solvent systems A: chloroform/methanol/water, 65 : 25 : 4 v/v and B: chloroform/acetone/methanol/acetic acid/water, 6 : 8 : 2 : 2 : 1 v/v. The dried chromatograms were stained with iodine vapors or rhodamine 6 G for

total lipid detection, with phosphomolybdate for phospholipid staining, ninhydrin for aminolipids, "chlorox" reagent for -NH_2 and -NH groups, periodate/Schiff's reagent for terminal -OH, Dragendorf's reagent for choline and α-naphtol for glycolipids [10].

Pigments were examined by thin-layer chromatography with the solvent system: benzene/ethyl acetate/ethanol (8 : 2 : 1, v/v).

Spectrometry. Absorbance measurements were recorded on a Cary 14 R spectrophotometer. Bacteriochlorophyll(ide) fluorescence spectra were obtained by using a Dumont 6911 photomultiplier tube and an analysis monochromator (Baush & Lomb – band pass 5 nm – grating blazed at 750 nm) together with a scanning drive, amplifier (Lemouzy E. R. I. C.)and potentiometric recorder (Sefram). Excitation was effected with a Xenon Lamp (75 W) and a monochromator (Baush & Lomb "high intensity" band pass 5 nm). A set of two complementary filters had to be used for good stray light rejection: VW_2 Barr-Stroud filter cutting off the infrared light of the excitation lamp in front of the sample and RG 10 Schott filter cutting off the visible light in front of the analysis monochromator. For 4-vinylprotochlorophyllide fluorescence, an EMI (9558) photomultiplier tube and a single cut-off filter (Corning 9787) in front of the sample were used. The spectra, recorded at room temperature, have not been corrected.

Radioactivity measurements were made in glass vials by liquid scintillation counting. 10 cm^3 of a toluene scintillation cocktail mixture (each l contains 6 g of 2.5 diphenyloxalole, PPO, and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP)) were added. The samples were counted in a Packard 3320 liquid scintillator.

RESULTS AND DISCUSSION

Spectral characteristics

The absorption spectrum of the complex varies with the characteristics of the culture medium. As will be shown in a later paper, the various forms obtained do not differ in their biochemical composition but correspond to different macromolecular conformations of the same excreted complex, depending upon the physicochemical properties of the medium. Fig. 1 shows four absorption forms. The complex with its red peak located at x nm is denoted Px. This peak varies from 636 nm to 654 nm. The Soret absorbance consists of one single band for the extreme forms (P 636 on one hand, and P 651–P 654 on the other hand), but becomes asymmetric for the intermediate ones. P 631, similar to the complex accumulated by the 2/73 mutant strain of *Rps. spheroides* [11] is obtained after detergent (Triton X-100, 0.05 %) has acted on any other form. The "one Soret band" forms have a definite fluorescence emission maximum, respectively located at 641, 658 and 682 nm for P 636, P 651 and P 654, whereas the emission spectra of the other forms depend on the exciting light: P 648 fluoresces at 641 nm when excited with a 445 nm light and at 658 nm with a 475 nm excitation light.

The absorption spectrum of chromatophores, prepared as in ref. 12, do not show any contribution of any form of the complex. Thus unlike the A_1 a^+ r mutant strain of *Rhodopseudomonas capsulata* [13], the precursor complex is not (or is insufficiently) bound to the membrane. To recognize the intracellular spectral form, we followed the fluorescence data in repigmenting bacteria, which had been previously

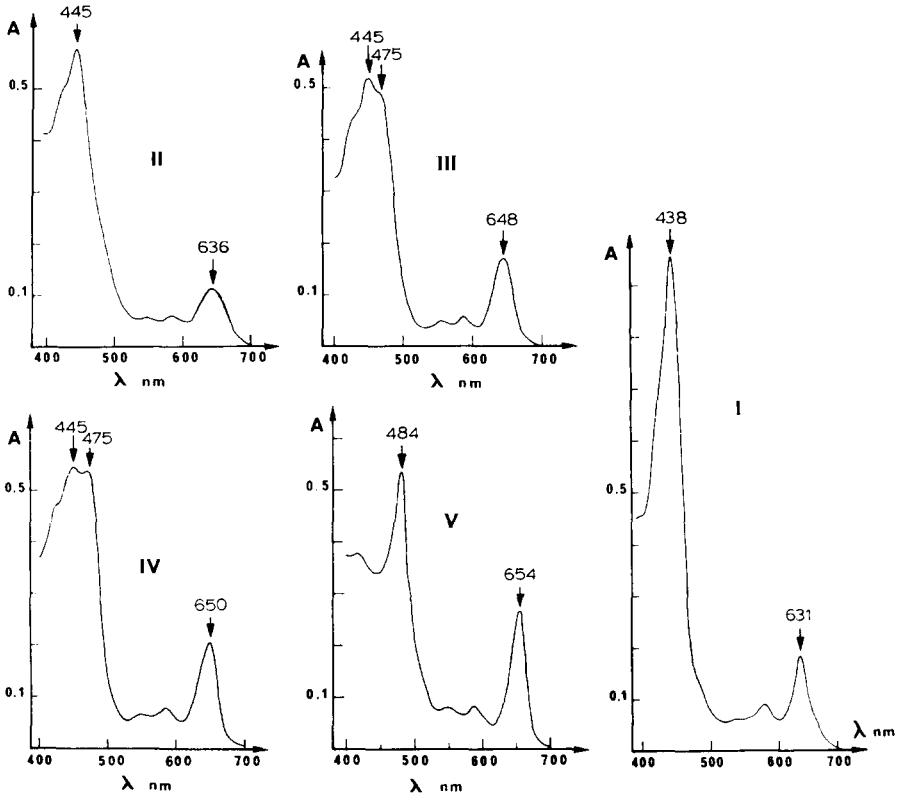

Fig. 1. Absorption spectra of the pigment complex from different culture supernatants. Spectra II and V represented extreme forms, III and IV were those of intermediate forms and spectrum I derived from any other by Triton X-100 (0.05 % final) action. The spectra were not adjusted to the same pigment content.

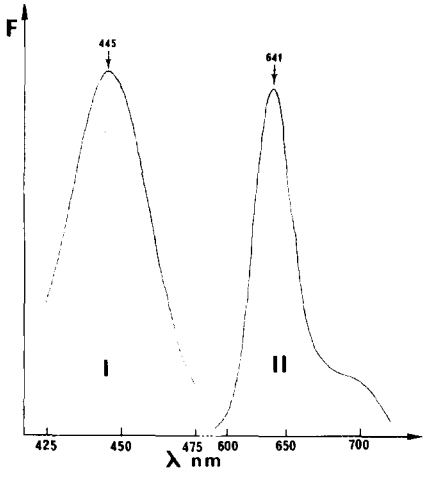

Fig. 2. Fluorescence excitation and emission spectra of mutant cells 100 min after transfer of bleached cells (aerobically grown in the light) to 5 % oxygen/95 % nitrogen in the dark. I. Excitation spectrum for fluorescence emission at 641 nm. II. Emission spectrum due to exciting light of 445 nm. F: Fluorescence intensity in arbitrary units. The maximum wavelength of the emission spectrum did not change with the exciting light.

bleached. Emission and excitation fluorescence spectra were characteristic of P 636 (Fig. 2).

Biochemical composition

The main components of the pigment complex were protein, lipid and pigment in addition to traces of unidentified sugar(s).

The pigment was a normal precursor of bacteriochlorophyll [14], identified as 4-vinylprotochlorophyllide by its absorbance spectrum in ether and that of its phaeophytin derivative. Pigments, extracted from the complex by chloroform/methanol/water partition, were separated by thin-layer chromatography into two bands (R_f 0.23, R_f 0.37). After scraping off and extracting from the silica by ethyl ether, the pigments of the two bands revealed the same absorbance spectrum. Iodine coloration showed an unsaturated lipid associated with the R_f 0.23 band. After polar lipid precipitation in cold acetone [10], the pigment migrated in one band with an R_f value of 0.37. Pigment was not phytylated as shown by comparing the migration pattern with literature data [15] and by the evaluation of the "HCl number" of about 15 % which is in agreement with the value attributed to 4-vinylprotochlorophyllide from *Cucurbita pepo* by Jones [16]. It is noteworthy that we never found phaeophytin or oxidized derivative which stresses the remarkable stability of the pigment within the complex. This suggests efficient protection of the pigment and rules out the possibility that the pigment was excreted alone before binding to the lipoproteic moiety.

Qualitative analysis of lipids has shown five components. The lipids were identified by their reactions with specific spray reagents and by comparison of their R_f values with those given in the literature (Table I). The major lipids were phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylcholine (PC). A ninhydrin-positive component, probably the ornithine-lipid (OL) described by Gorchein [17] and a phospholipid similar in R_f values to diphosphatidylglycerol

TABLE I

STAINING BEHAVIOUR AND R_f VALUES ON THIN LAYER CHROMATOGRAPHY OF THE LIPIDS FROM THE PIGMENT COMPLEX PRODUCED BY "PHOFIL" MUTANT OF *RPS. SPHEROIDES*

The solvent systems A and B were described in Materials and Methods. Literature data were from Kates [10] and Gorchein [17]. Specific staining for sugar was negative.

Spot	Staining behaviour for					R_f values $\times 10^2$		$R_f \times 10^2$ in literature		Lipid*
	Phosphate	NH_2	NH	OH	Choline	A	B	A	B	
I	+++	−	−	++	−	44	77	48	75	PG
II	+++	+++	+	−	−	60	61	62	65	PE
III	±	±	+	−	−	57	72	60		OL**
IV	++	−	−	−	+	38	24	33	40	PC
V	+	−	−	−	−	42	87	71	85	DPG

* Abbreviations: PG, phosphatidylglycerol; PE, phosphatidylethanolamine; OL: Ornithine-lipid; PC: phosphatidylcholine; DPG: diphosphatidylglycerol.

** OL spot was slightly stained by phosphate reagent. Gorchein pointed out this staining as an artefact because OL migrated as PE in solvent A and as PG in solvent B.

[DPG) were present in small amounts. As all the lipids, OL excepted, were phosphorylated, the total weight lipid content was derived from lipid phosphorus determination, by multiplying by a factor of 25 [4].

Quantitative determination gave the following weight partition: protein 6.8/phospholipid 6/pigment 1. The biochemical study presented here was performed simultaneously on two distinct spectral forms (P 636 and P 654). No differences were noticed in the quantitative determinations or in the qualitative lipid analysis. Moreover the composition of the complex proved to be quite reproducible. This indicates that the isolated material is the native complex itself or at least a stoichiometric association of the native complex with another material excreted by the mutant.

Filtration of the various forms of the complex on Sephadex G200 showed that the native form P 636 was also the lighter one, with a particle weight of 130 000 (Fig. 3). Taking the ratio 1/13.6 for the weight contribution of the pigment in the complex, the number of pigment molecules per P 636 is given by: 130 000/13.8 · 615 = 16 (where 615 is the molecular weight of 4-vinylprotochlorophyllide).

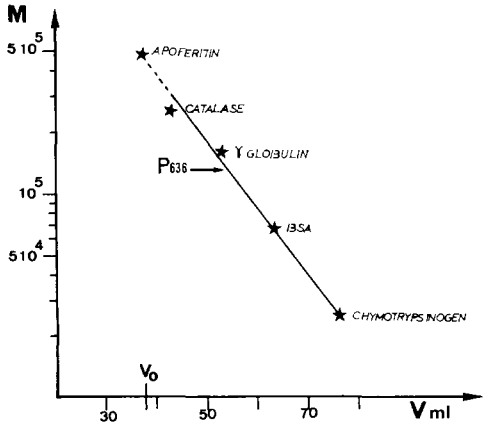

Fig. 3. Particle weight estimation of P 636 on G 200 Sephadex gel. The standard proteins used and their molecular weights were: horse apoferitin (480 000), catalase (250 000), γ globulin (160 000) bovine serum albumin 67 000) and chymotrypsinogen A (25 000) · V_0 was the outer volume determined as the blue dextran elution volume.

Pigment synthesis

In order to get further information as to the origin of the complex excretion, comparison of pigment synthesis in the mutant and in the wild type was undertaken.

Non-lysis verification

One first had to discard the trivial possibility that the pigment might be liberated by a lysis of part of the cells during growth. Fig. 4 shows the radioactivity distribution between the pellet and the trichloracetic acid precipitable supernatant material during the growth of an inoculum initially labelled with [^{3}H]Trp. Trichloracetic acid will precipitate the previously labelled soluble proteins liberated by a possible lysis. Thus a lysis will be shown by a diminution of the pellet activity and an increase in the activity of the trichloracetic acid precipitable supernatant material. No such behaviour was observed (Fig. 4).

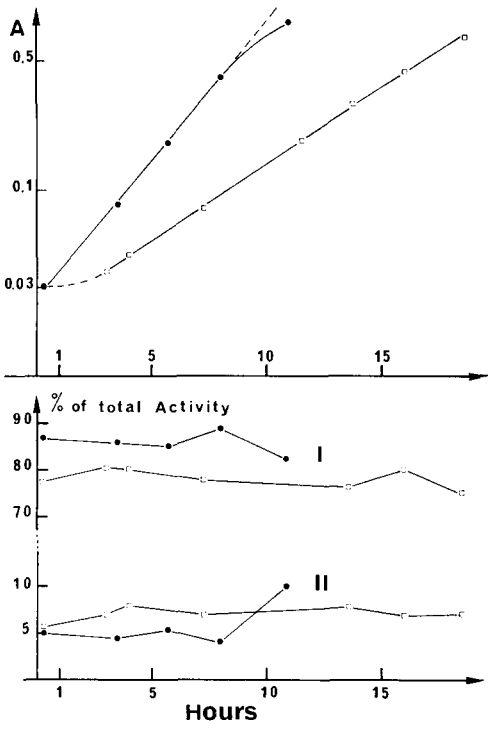

Fig. 4. Bottom: radioactivity distribution during the growth of [^{3}H]Trp-labelled inocula of wild type (●) and mutant (□) strains. The cells were precultivated for 3 generations in 30 ml volume of a medium containing 3 μg of [^{3}H]Trp (specific activity 1 Ci/mmol) and 10 μg of unlabelled Trp. Bacteria were centrifuged, washed, and grown in unlabelled medium. Samples were centrifuged and activity was measured on the washed pellet (I) and the trichloracetic acid (5 %) precipitable fraction of the supernatant (II). Top: Growth curves. Ordinate: Absorbance at 1200 nm.

Pigment synthesis capacity

The step affected by the mutation is not necessarily the one immediately before or after the formation of the accumulated complex. The excretion may arise from an inability of the membrane to accept a nevertheless normal intermediate. In a more general way it may reveal a saturation of the membrane caused by a decrease in its ability to effect pigment intermediate transformations up to bacteriochlorophyll. In such a case, one expects a choking up of the membrane up to the deficient step. This would result in the excretion of the less tightly bound intermediate or intermediates above that step.

Experiments on repigmentation kinetics show that the mutation does not affect the actual pigment synthesis. Fig. 5 shows the kinetics of pigment synthesis in photosynthetically growing cultures of both strains. In order to follow the repigmentation process, initial depigmentation was not achieved so that the photosynthetic growth began without too large a lag. Bacteriochlorophyll synthesis in the parent strain (Fig. 5 WT) occurs rapidly during the initial phase up to the steady state phase [18] where rates of growth and pigment synthesis become parallel. Bacteriochlorophyll synthesis in the mutant (Fig. 5 M) occurs also in two phases; the main difference is

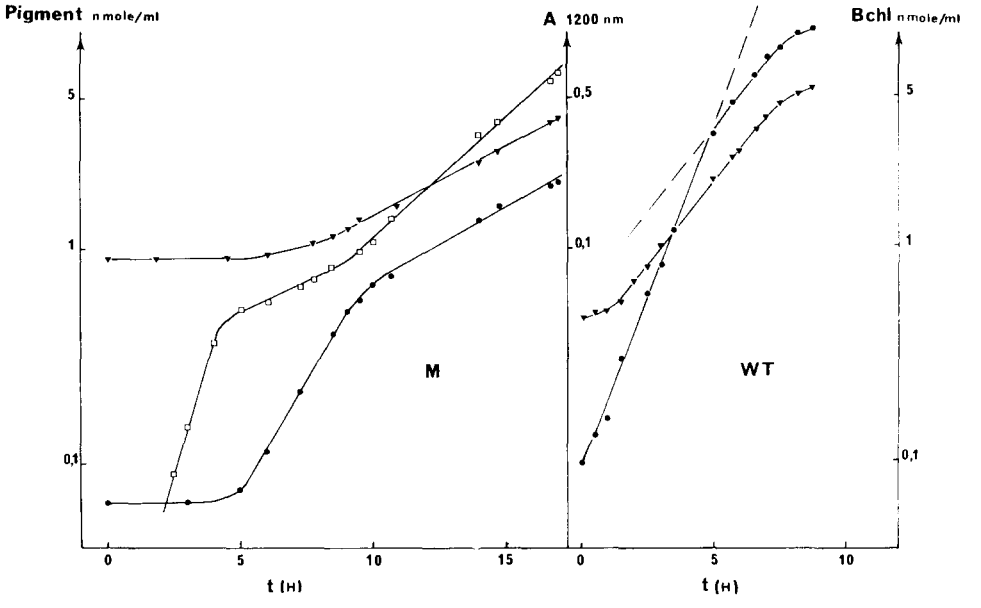

Fig. 5. Growth and pigment synthesis in photosynthetic cultures of mutant (M) and parent (WT) strains. Aerobic inoculum preparation was described in the text. (▼) $A_{1200\,nm}$; (●) Bacteriochlorophyll; (□) 4-vinylprotochlorophyllide.

that the bacteriochlorophyll synthesis and the growth start only after a considerable lag. Production of 4-vinylprotochlorophyllide occurs throughout the culture, its rate is maximal during the latency of bacteriochlorophyll synthesis, diminishes during the rapid phase and increases again in the steady state conditions.

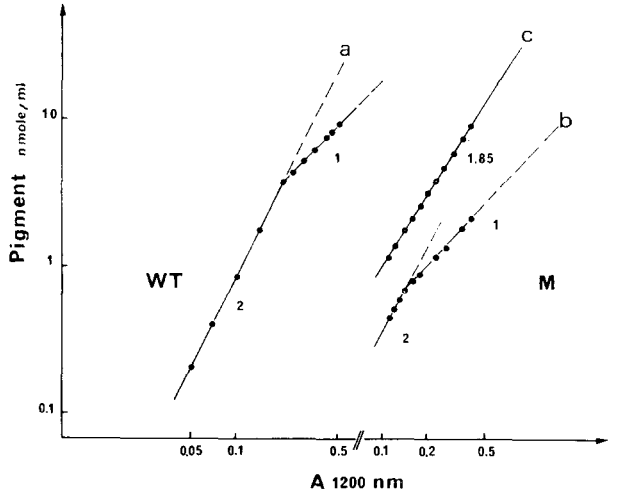

Fig. 6. Dependence of the logarithm of pigment content in terms of the logarithm of $A_{1200\,nm}$. The slopes of the curves give the differential rates of pigment synthesis. The values observed during the lag phase were not plotted. a: Bacteriochlorophyll of parent strain; b: Bacteriochlorophyll, and c: total pigment (Bacteriochlorophyll+4-vinylprotochlorophyllide) of mutant strain.

As the growth and the pigment synthesis are exponential functions of time, the logarithm of the pigment content varies linearly with the logarithm of $A_{1200\,nm}$. The slope of the curve is the differential rate of pigment synthesis. Fig. 6 shows the differential rate of bacteriochlorophyll to be 2 for the rapid phase and 1 for the slow phase (steady state) in both parent and mutant strains. To determine the true differential rate of chlorophyllous pigments synthesis in the mutant strain we have to take into account the bacteriochlorophyll and its precursor. Fig. 6 shows that chlorophyllous pigments synthesis occurred throughout the growth at a constant rate of 1.85, close to the maximum rate of 2, for the wild type.

In Fig. 7 the experimental data have been replotted to present the changes in the pigment content per cell during growth. The bacteriochlorophyll content is always lower in the mutant than in the parent strain; in steady state conditions, the bacteriochlorophyll values are, respectively, 5 nmol and 17 nmol per $4 \cdot 10^9$ cells. The total chlorophyllous pigment contents of both strains are close (the two curves are superposable) during the rapid phase of bacteriochlorophyll synthesis in the wild type. Whereas the parent reaches the steady state, however, the mutant cells keep synthetizing chlorophyllous pigments at the same rate (1.85).

Total pigment synthesis is not affected: this indicates a normal process at least up to the 4-vinylprotochlorophyllide step. The observed kinetics are not consistent with the hypothesis of a mutation, of the leaky type, affecting the transformation of 4-vinylprotochlorophyllide or of another intermediate before bacteriochlorophyll. For in this case the maximum synthesis rate of bacteriochlorophyll in the mutant should be inferior to that of the wild type, and excretion should be in a constant ratio to bacteriochlorophyll synthesis. That the fast phase of bacteriochlorophyll integration stops in the mutant at a cell pigmentation level markedly inferior to that of the wild type (5 nmol/ml instead of 17 nmol/ml) is, on the other hand, an argument in favour of the hypothesis of a deficient membrane, which can only integrate a part of the synthesized pigments.

In order to attempt a localization of that deficiency, we tried to see whether any intermediate after 4-vinylprotochlorophyllide was being accumulated in the cell. Cellarius and Peters [19] have shown that beside the 885 nm fluorescent form,

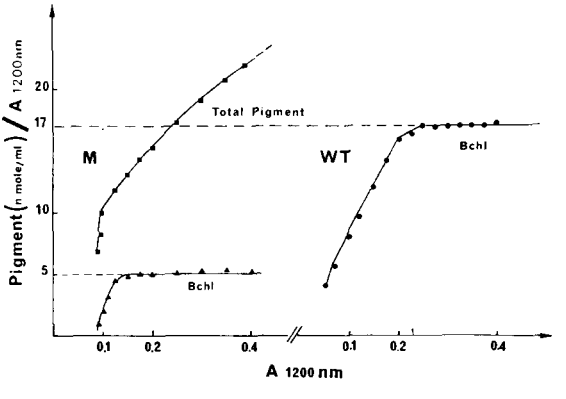

Fig. 7. Cellular level of pigment content during the repigmentation of mutant and parent cells. Bchl, bacteriochlorophyll.

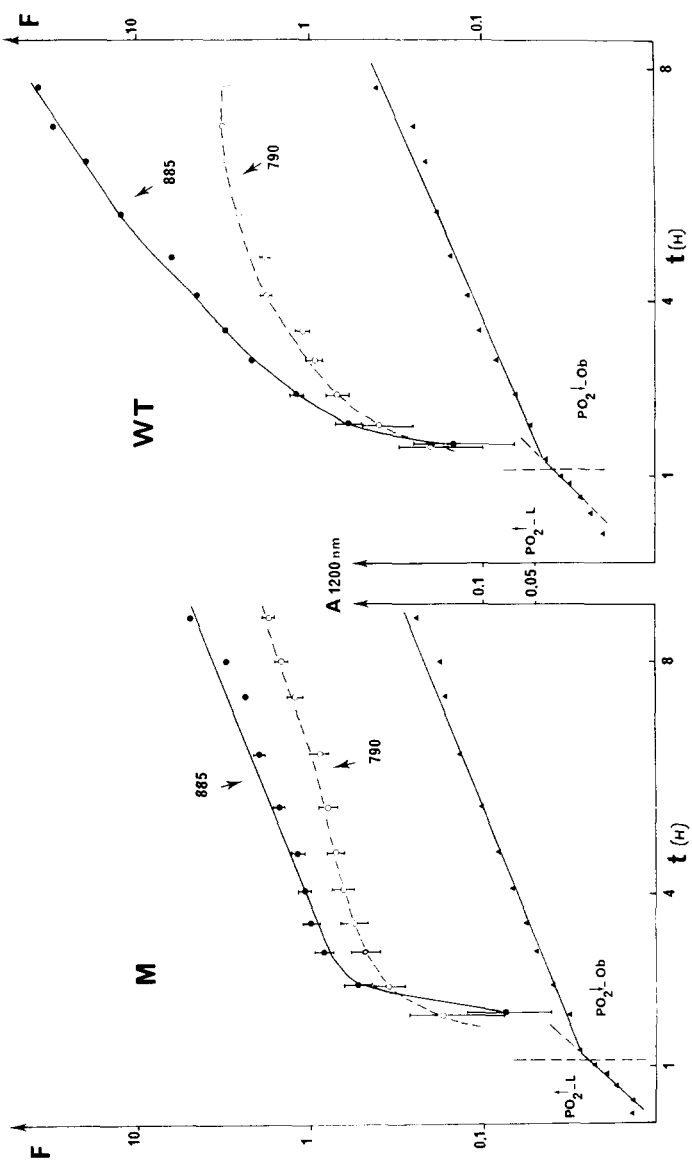

Fig. 8. Growth and Bacteriochlorophyll production in repigmenting cells of both mutant and parent strains. 885 and 790: intensity of fluorescence emission peaks in vivo, at 885 nm and 790 nm respectively, with an exciting light of 590 nm. $PO_2 \uparrow L$: *aerobic growth in the light*; $PO_2 \downarrow ob$: *Semi aerobic* (5 % oxygen/95 % nitrogen) growth in the complete obscurity. Vertical bars indicate the uncertainty in the values, as determined from the noise level of the photomultiplier tube. F = fluorescence intensity in arbitrary units.

another form of bacteriochlorophyll(ide), F 790, appears at the beginning of the repigmentation process. This form, which arises from a shift between pigment and membrane synthesis, was characterized as a pool from which pigment is integrated into the photosynthetic apparatus. The ratio F 790/F885 may be considered as an indicator for the delay of the membrane components synthesis with regard to the pigment. Fig. 8 shows the growth and bacteriochlorophyll(ide), F 885 and F 790, formation in a culture after total depigmentation by aerobic growth in the light (20 generations) and transfer to darkness in a 5 % oxygen/95 % nitrogen atmosphere. F 790 bacteriochlorophyll(ide) exists in both mutant and parent strains. In the wild type, the amount of F 790 increases with a diminishing slope, indicating that, after an initial delay, the membrane comes up with the pigment causing progressive depletion of the pool. The mutant behaves quite differently, with a nearly constant ratio F 790/ F 885. Thus the F 790 pool remains high with regard to the bacteriochlorophyll. This indicates the very last step F 790 bacteriochlorophyll(ide) → F 885 bacteriochlorophyll to be the deficient one, causing accumulation above it.

CONCLUSION

The mutation affects neither the total pigment capacity nor the enzymatic pathway of bacteriochlorophyll(ide) synthesis, but rather the synthesis of membrane components required for the incorporation of bacteriochlorophyll(ide). In the absence, seemingly, of feed-back regulation, the bacteriochlorophyll(ide) content increases in a direction parallel with growth (Fig. 8). The same behaviour may exist with the other precursors between 4-vinylprotochlorophyllide and bacteriochlorophyll(ide); nevertheless their intracellular concentration has to be low because typical fluorescence emission of such compounds was not observed in repigmenting cells, as it was for bacteriochlorophyll(ide) and 4-vinylprotochlorophyllide. In such conditions, the 4-vinylprotochlorophyllide excretion is the result of excess accumulation of chlorophyllous pigments inside the cells. The high number of photosynthetic bacteria mutants accumulating 4-vinylprotochlorophyllide [20], indicates the 4-vinylprotochlorophyllide-protochlorophyll(ide) transformation as a trouble spot in the bacteriochlorophyll pathway.

We assume the accumulated pigment complex to contain the authentic 4-vinylprotochlorophyllide intermediate in the differentiation process of cytoplasmic into intracytoplasmic membrane. The biochemical analysis is compatible with this conclusion. We cannot, however, eliminate the possibility of a stoichiometric binding between the authentic intermediate and other material excreted with it. Indeed we did not find typical components of the cell wall as it was for the pigment complex produced by the mutant strains A_1a^- and A_1a^+r of *Rps. capsulata* [21]. In the same way all the lipid components are characteristic of the intracytoplasmic membrane (chromatophores) of *Rps. spheroides* [22].

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