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ELECTRON PARAMAGNETIC RESONANCE STUDIES ON PHOTOSYNTHETIC BACTERIA

I. PROPERTIES OF PHOTO-INDUCED EPR-SIGNALS OF *CHROMATIUM* D

HEINZ SCHLEYER

Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. (U.S.A.)

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SUMMARY

(1) Photo-induced EPR-signals of the free radical type were investigated in a variety of preparations derived from the photosynthetic bacterium *Chromatium* D. Materials ranged from intact, actively photosynthesizing organisms to cell-free "simplified systems" such as chromatophore-type preparations to synthetic photochemical systems containing bacteriochlorophyll in solution. Closely similar signals were observed in terms of the general properties such as *g*-value, linewidth and line shape, microwave saturation behaviour, and lack of detectable hyperfine structure.

(2) Improvements of the time response capabilities of the instrumentation made fast measurements possible—present time limit a few msec—and allowed detailed kinetic studies on all these materials. Characteristic time courses of both formation and decay of photo-induced EPR-signals were observed. They are seen to fall into two classes of patterns which are described in detail for intact photosynthesizing bacteria and for the classical chromatophore-type preparation. These patterns are termed the "cell pattern" and the "chromatophore-type pattern", respectively.

(3) The "cell pattern" is characterized by a relatively slow and complex time course of formation with a fast initial rise at the onset of irradiation and a much faster, comparatively simple time course of decay. It is only observed when intact bacteria are studied under physiological conditions and is strongly dependent on such metabolic parameters as age of the cultures, substrate levels, and preillumination history. Changes introduced by altering the metabolic situation are reversible. No evidence for the existence of lag periods in either formation or decay has been obtained.

(4) The "chromatophore-type pattern" shows a fast monotonic rise with no complexities and a much slower and typically biphasic decay. It is a quite stable phenomenon, strictly repetitive, and relatively independent of the quality and intactness of the preparation used. This pattern is observed with the classical chromatophore-type preparations and also with a large number of further simplified systems.

(5) The transition from the cell pattern to the chromatophore-type pattern is readily achieved by unspecific treatments and is found to be irreversible. It occurs at a level of organization very close to that of the intact organism and is accompanied by a gradual loss of the overall photosynthetic capacities of the materials.

Abbreviation: DPPH•, 1,1-diphenyl-2-picryl-hydrazyl.

(6) A comparison of these results with other measurable parameters of bacterial photosynthesis shows that existence and demonstratability of these photo-induced EPR-signals parallel strictly the photochemical activities of the bacteriochlorophyll molecule, including its participation in bacterial photosynthesis. On the other hand, the existence of the two characteristic time course patterns parallels the overall photosynthetic capacities of the materials, but does not correlate with the known capacities of the materials to form ATP in the process of photophosphorylation. The results demonstrate clearly that the paramagnetic species detected by EPR-spectroscopy are closely linked to the overall processes of photosynthesis and are participating in the electron transfer system of these bacteria.

INTRODUCTION

Since the first observations of electron paramagnetic resonance (EPR-) signals from photosynthetic materials in 1954 by COMMONER, HEISE AND TOWNSEND[1] and shortly thereafter by the Berkeley group[2] these signals have evoked considerable interest due to their postulated role in what is called "primary events of photosynthesis". Photo-induced EPR-signals have since been found in a wide range of materials under grossly varying conditions reaching from the level of fully functioning organisms to extracts of pigments. A number of laboratories have participated in these studies and a bulk of publications covering this controversial subject exists (see for example literature quoted in refs. 3, 4, and 5). In spite of all these efforts neither the chemical or physical nature of the species responsible for the observed signals nor their relationship to the overall processes of photosynthesis have been established beyond the stage of hypothesis and speculation.

In the past four years we have carried out an extensive investigation of photo-induced EPR-signals in photosynthetic bacteria together with light-induced absorbance changes and other measurable parameters of bacterial photosynthesis. These studies are based on our earlier work on the electron transfer systems of these bacteria[6] using suitable spectrophotometric techniques developed in this laboratory.

The specific aims of these studies are: (1) to establish the relationships between the observed photo-induced EPR-signals and the processes of bacterial photosynthesis; (2) to investigate in more detail the mechanisms in which formation and decay of the detected paramagnetic species are related to other known or even as yet unknown (*e.g.*, the "natural" electron acceptor) components of bacterial photosynthesis, and (3) to identify the chemical nature of the species observed.

Here we describe results obtained with the photosynthetic bacterium *Chromatium* D. This organism, rather than one of the more commonly used Athiorhodaceae, has been chosen for its character as obligate anaerobe. This eliminates some of the difficulties and ambiguities involved in separating "oxidative" and "photosynthetic" electron carriers, especially in spectrophotometric studies, and helps to define more readily a general "redox state" of the cells. The occurrence of a photo-induced EPR-signal in *Chromatium* has been reported previously by CALVIN and co-workers[7]; they state that both rise and decay are fast for cells and chromatophores (less than one sec).

MATERIALS AND METHODS

Bacteria

Chromatium strain D, isolated from shake cultures, is grown in an inorganic medium (modification of ref. 8) containing 3 % NaCl, and Na_2S, $Na_2S_2O_3$, and $NaHCO_3$ as substrates. The bacteria are cultured anaerobically in 500-ml flat bottles in an illuminated waterbath at 30 ± 2°. Relatively uniform illumination during growth is provided by two lines of reflector type flood lamps (75 W) on each side of the bath.

The bacteria are harvested by mild centrifugation at room temperature in different states of their growth, most often after 24–36 h in the late log-phase or the early stationary phase. Some of the inorganic precipitate from the medium is first removed by filtration through coarse grade filter paper.

Unless otherwise noted, the bacteria are washed twice with deoxygenated solutions containing 3 % NaCl (w/v) and phosphate (20 mM, pH 7.5), isotonic with the growth medium. Adjustment to appropriate concentrations is made by a final centrifugation step using the same solution. Where desired, substrates or other agents are added at this time. All operations including transfer of the material into the sample cell are performed as much as possible in darkness and in an inert atmosphere of helium gas so as not to upset the "natural redox state" of the materials to be studied.

Starvation is achieved by suspension of the bacteria in carefully deoxygenated isotonic growth medium from which reducing sulphur compounds are omitted. The degree of starvation is controlled by the length of exposure to illumination at 30°.

"Chromatophore-type preparations" are obtained in the usual fashion using either sonic treatment or cell breakage in a French pressure cell. The suspending medium is NaCl–phosphate solution as above or a 10 mM glycylglycine buffer (pH 7.5)[9].

Bacteriochlorophyll content of the cells or of derived preparations is routinely determined in aliquots of the final suspensions using either methanol or a methanol–acetone mixture (2:7, v/v)[10] as solvent. The following molar absorption coefficients are applied: $4.2 \cdot 10^4$ M^{-1} cm^{-1} at 7720 Å for methanol and $7.5 \cdot 10^4$ M^{-1} cm^{-1} at 7680 Å for the methanol–acetone mixture, as independently determined with pure preparations of bacteriochlorophyll. In general the bacteriochlorophyll content serves as a convenient measure of the concentration of the material. No correlation to other quantities such as dry weight, wet weight, number of cells or "protein" is attempted here, but is being pursued as required in a different context[11].

The "photosynthetic capacity" of the bacteria is estimated by measuring the rate of light-dependent CO_2 fixation using $^{14}CO_2$ uptake (ref. 12) or a Radiometer pCO_2 electrode Type E 5036 (ref. 11).

Light-dependent oxidation and reduction of *c*-type cytochromes are followed with a dual wavelength spectrophotometer[13]. All chemicals used are of analytical grade.

EPR-spectroscopy

The EPR-studies are performed with a Varian X-band spectrometer, Model V 4500-10A, equipped with 100 kHz field modulation. For experiments at room temperature the standard "Aqueous sample cell" (Varian V 4548) is used. The dimensions of the "active volume" under these conditions are approximately 23 mm × 8 mm ×

0.25 mm. The latter figure corresponds to the pathlength for the actinic irradiation. Studies at low temperatures are carried out in 3-mm bore, 4-mm outer diameter quartz sample tubes immersed in liquid nitrogen or in a stream of cold gas at various temperatures using the Varian V 4557 accessory. In certain special applications 0.8-mm quartz capillaries are used.

EPR-spectra (approximately first derivative presentations of the resonance absorption) as well as the time courses are displayed on potentiometric recorders (Leeds and Northrup Speedomax and Esterline Angus Speedservo with 10–90 % response times of 1 sec and 1/8 sec, respectively). Most often the EPR-spectra are recorded directly as a function of the applied magnetic field, H_0, on an X, Y-recorder (Moseley, Model 2D-2AM). The field is monitored by a partially temperature compensated Hall effect sensor (Siemens, Model SV 131 I). For kinetic studies the EPR-spectrometer is adjusted to the resonance conditions. The peak of the first derivative curve is then monitored as a function of time.

For fast events a suitable wide band recording system has been developed[14]. The essentially unfiltered output of the phase sensitive detector of the EPR-spectrometer is fed either into a single stage passive *RC* network or into an active filter providing an overall d.c. gain of approximately 10. The output of these external filters is fed into a multichannel recording system—Schwarzer Oscilloscript (manufactured by Fritz Schwarzer GmbH, München-Pasing, Germany)—which has a 3-db bandwidth of approximately 160 Hz and a recording width of 40 mm per channel, linear to within ± 1 % of full scale. The actinic light is simultaneously monitored in one of the channels. The shortest filter time constants routinely employed in our investigations are $RC = 1$ or 3 msec. This has proved to be a reasonable choice for most of our experiments with relatively low actinic intensities. Recording chart speeds of up to 200 mm sec^{-1} are used.

Illumination equipment

A multifilament projection lamp (Westinghouse, Type DMX, 115 V, 500 W) was chosen as the actinic light source optimal for our purposes. This lamp is mounted in a water cooled housing. The operating voltage is controlled by means of a "Variac" autotransformer.

A relatively uniform collimated beam is generated and made only slightly convergent before entering the slotted entrance plate of the TE 102 microwave cavity containing the sample. A standard photographic shutter is used in a focal plane of the illumination system to allow for sufficiently fast shutter speeds. These are routinely monitored *via* a beam splitter and a vacuum photocell (RCA 929) built into the lamp housing. Shutter speeds are calibrated with a photovoltaic detector in place of the sample inside the cavity. Risetimes of 10–90 % determined in such a manner are found to be less than 1.5 msec.

Suitable filters and neutral attenuators can be accommodated in the collimated beam. Most of the studies are carried out with a Kodak Wratten filter No. 88 A inserted. This limits the spectral region for actinic irradiation to a broad band ranging from approximately 7300 Å to longer wavelengths. The long wavelength cut-off is then determined solely by the absorption characteristics of the bacteria themselves rather than by the instrumentation. This spectral region has been chosen to make the EPR-studies compatible with absorption spectrophotometric studies in the blue

and green region of the visible spectrum. The activation is hereby limited to the longest wavelength absorption bands of bacteriochlorophyll *in vivo* excluding other spectral regions where accessory pigments would cooperate *via* energy transfer. For operations at very high actinic intensities a water filter of 25 mm path length is employed to prevent excessive and unnecessary heating of both sample and microwave cavity.

Actinic irradiation intensities are determined with a Silicon cell (Hofmann No. 110 C) mounted inside the microwave cavity exactly at the position normally occupied by the sample. The detector is operated under virtually short circuit conditions to achieve linear response over a wide range of actinic intensities and to reduce the temperature sensitivity of the detector. Calibration of the detector system is obtained by comparison against a thermopile or a Kahlbaum bolometer which in turn are calibrated by a comparison with a secondary standard lamp of the National Bureau of Standards.

g-Values and field calibration

g-Values and H_0-field calibrations are obtained with the aid of a number of accurately known free radical standards such as 1,1-diphenyl-2-picryl-hydrazyl (DPPH$^\bullet$), 1,4-benzosemiquinone, or peroxylamine-disulfonate free radical in connection with the Hall effect sensor described. The Hall effect system is calibrated in this field range by using known hyperfine splitting constants of free radicals containing ^{14}N.

Determination of spin concentrations

For an approximate determination of the concentration of unpaired spins, dilute solutions of peroxylamine-disulfonate free radical in 0.1 M $KHCO_3$ are used. This material is prepared after the modified method of MURIB[15] or commercially obtained (K and K Laboratories, Jamaica, New York). The purity of the starting material is determined spectrophotometrically (ε_M 21.6 M^{-1} cm^{-1} at 5450 Å) or by chemical titration with potassium ferrocyanide. Aqueous solutions of $CuSO_4 \cdot 5\ H_2O$ or quinhydrone[16] and solutions of recrystallized DPPH$^\bullet$ in benzene (ε_M $11.9 \cdot 10^3$ M^{-1} cm^{-1} at 5250 Å) are employed as additional standards for these calibration procedures.

Spin concentrations are determined using either a double integration technique or the "first moment method" where applicable. The data determined in this manner are to be considered approximate; they are accurate to within a factor of two or three. The reproducibility of the data is much higher, however, generally around 5 %. For a detailed discussion of some of the problems involved in determining spin concentrations see ref. 17.

EXPERIMENTAL RESULTS

General properties of the photo-induced EPR-signals of Chromatium D

Intact bacteria. Upon irradiation of a suspension of intact cells of *Chromatium* D with near infrared radiation, or with visible light, a photo-induced free radical type EPR-signal is readily obtained (Fig. 1). The signal, which decays rapidly in the dark has a *g*-value of about 2.0025 $\pm$ 0.0004 close to the free electron value and is very slightly asymmetric. The width of the line, expressed as ΔH between the extreme values in the first derivative presentation corresponding to inflection points of the

resonance absorption curve, is found to be 10–12 Oersted. Even with low microwave power and with modulation amplitudes as low as 200 mOersted, no hyperfine structure can be detected in these signals.

A more detailed analysis under conditions of low modulation amplitudes and non-saturating microwave power levels shows the observed line shape to fall between the simple theoretical cases of a Lorentzian and a Gaussian curve, but much closer to the latter (Fig. 1). The relationship between EPR-signal intensity, expressed as peak to peak amplitudes, and applied microwave power is given in Fig. 2 for intact cells of *Chromatium* D and for a chromatophore-type preparation. This in combination with the line-shape data, suggests an inhomogeneously broadened spin system.

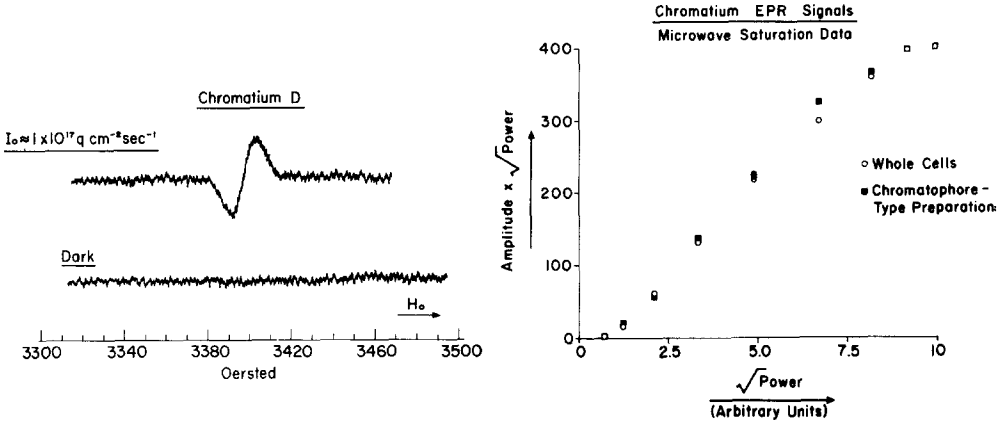


Fig. 1. Photo-induced EPR-signal of intact cells of *Chromatium* D. 36-h culture, suspended in 3% NaCl–phosphate solution. Final bacteriochlorophyll concentration, 2.2 mM. Incident actinic intensity $1 \cdot 10^{17}$ quanta cm^{-2} sec^{-1}; wavelength > 7300 Å; optical pathlength, 0.25 mm. Microwave frequency, 9514.5 MHz; modulation amplitude, 2.4 Oersted. Scan rate, 30 Oersted min^{-1}; filter time constant, 1.0 sec.

Fig. 2. Microwave saturation characteristics. Magnetic resonance absorption intensity is expressed as peak-to-peak amplitude of the signal in the first derivative presentation. Microwave power is expressed in arbitrary units. Attenuation data are obtained by calibration with a non-saturable standard.

"Simplified preparations". In addition we have investigated a large number of "simplified systems"* (*cf.* ref. 18) prepared from *Chromatium* D in order to detect any possible differences. Essentially indistinguishable photo-induced EPR-signals are found in intact cells under the conditions of actual bacterial photosynthesis and in cell-free particulate preparations such as chromatophore-type preparations with respect to the general properties, *i.e.*, *g*-values, line shapes, linewidths, and microwave saturation (relaxation) behavior (for an example, see Fig. 2).

The same statement can be made for heat-treated cells (10 min, +57.5°; see Fig. 12), cells suspended in non-physiological media such as glycerol–water or ethanediol–water mixtures, and both whole cells and cell-free preparations studied under non-physiological conditions—for example in the frozen state at temperatures as

* We define a "simplified system" as any preparation derived from intact cells which has lost part, but not all of the capabilities presumed to exist in the intact overall system of bacterial photosynthesis.

low as −196° (ref. 19). This has also been found true for crude extracts of the pigments, in good agreement with very early observations of CALVIN and collaborators on methanol extracts from *Rhodospirillum rubrum*[3]. More recently, very similar photo-induced EPR-signals have been obtained from pure bacteriochlorophyll in solution[20] and from a crystallized bacteriochlorophyll protein complex isolated from a different organism (H. SCHLEYER AND J. M. OLSON, unpublished observations).

All the general properties of the photo-induced EPR-signals observed in the intact and simplified systems from *Chromatium* D are found to be very similar to those in Athiorhodaceae such as *R. rubrum* and *Rhodopseudomonas spheroides* (both wild-type and mutant strains) as studied by us as well as by many others[5,7,30] and in Chlorobacteriaceae[21]. This suggests that at least very similar if not the same paramagnetic species are produced in all photosynthetic bacteria studied so far.

The photo-induced EPR-signals from the bacterial systems in turn show some marked similarities to the so-called "fast narrow signals" observed in several algae[22] and in chloroplasts and quantosome preparations[18] from higher plants where they are generally assumed to be related to "System I" of photosynthesis.

Steady state studies. The observed photo-induced EPR-signals saturate readily with higher actinic intensities. An example for intact cells of *Chromatium* D under conditions optimal for bacterial photosynthesis (*e.g.*, $NaHCO_3$ and $Na_2S_2O_3$ added) is given in Fig. 3. The actinic intensity at half saturation corresponds to approximately $1.4 \cdot 10^{16}$ quanta cm^{-2} sec^{-1}. This is close to the values of half saturation found for the oxidation of cytochrome *c*-555 ($\Delta A_{4220\,Å} - \Delta A_{4600\,Å}$) and for light-dependent CO_2 fixation[12]. Similar saturation phenomena are observed with most types of simplified systems studied so far. No simple, theoretically predictable, relationship has been found to fit the experimental observations such as the one shown in Fig. 3. This is true even with very dilute suspensions and therefore greatly reduced errors due to the unavoidable inhomogeneities of actinic irradiation. For any specific kind of material, however (*e.g.*, intact cells in a certain metabolic state or a cell-free preparation), the results are accurately reproducible.

No accurate determination of the maximal steady state spin concentration has been attempted at the present stage due to the principal systematic uncertainties associated with such a procedure[17]. Our preliminary data indicate that the ratio of

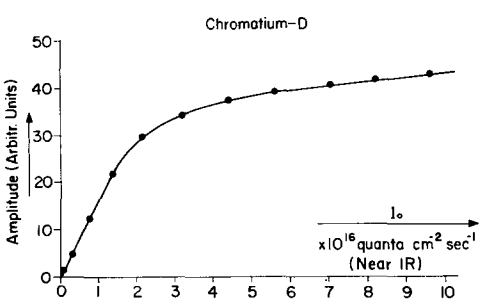


Fig. 3. Photo-induced EPR-signal of intact cells of *Chromatium* D. Light saturation characteristics. 36-h culture, starved for 24 h. Bacteriochlorophyll concentration is 2.3 mM. Actinic intensity as shown, wavelength > 7300 Å. Signal amplitude (peak-to-peak) is measured in preilluminated state after 60 sec of irradiation and expressed in arbitrary units. Half maximal saturation is found at approximately $1.4 \cdot 10^{16}$ quanta cm^{-2} sec^{-1}.

the steady state concentration of "unpaired spins" to the concentration of bacteriochlorophyll under saturating actinic intensities is of the order of $3 \cdot 10^{-3}$. Only upon chemical treatment with potassium ferricyanide or 1,4-benzoquinone can much higher ratios be obtained (ref. 20, see also ref. 17). A detailed quantitative study of the ratio of "unpaired spins"/bacteriochlorophyll as a function of metabolic and growth conditions is currently in progress.

Attempts to correlate the photo-induced EPR-signals directly with spectrophotometrically observable absorbance changes have either failed so far or yielded inconclusive results[23,24]. As reported earlier no simple kinetic correlation exists between photo-induced EPR-signals in intact cells of *Chromatium* D and the oxidation and reduction of the various *c*-type cytochromes under these conditions[23]. This situation is further complicated by the fact that the specific role of these cytochromes in the various processes of photosynthesis is poorly understood at the present time[6,25].

The correlation of absorbance changes at 4350 Å (corresponding to an unknown chemical event, but presumably linked to bacteriochlorophyll) with the EPR-signal formation in *R. rubrum* chromatophores by CALVIN and coworkers[24] is not applicable to intact cells of *Chromatium* D under conditions of actual photosynthesis, nor is it valid for any other intact photosynthetic bacterium investigated so far. As long as the bacteria are studied under physiological conditions in the presence of substrate and are not subjected to damaging procedures, absorbance changes at 4350 Å are not observed. A similar situation has been reported by CHANCE AND SCHÖNER[26] for near infrared absorbance changes in whole cells of *Chromatium* D. Special treatments of the intact bacteria are required[27,28] to bring absorbance changes in these wavelength regions into the detection range of even the most sensitive spectrophotometers available. No significant changes in steady state levels of photo-induced EPR-signals are caused by these treatments. This fact and some discrepancies in the kinetic findings wich have emerged from recent studies on *R. rubrum* chromatophores (see for example refs. 29, 30, 31 and 52) make a translation of the absorbance changes into chemical terms and a possible correlation of some of the optical changes—especially those related to the reaction center bacteriochlorophyll[28,46]—with our EPR-results on bacteria under conditions of actual photosynthesis extremely difficult.

Likewise, no direct correlation of the "fast narrow signal" of higher plant and algal photosynthesis with any spectrophotometrically observable absorbance change has been found as yet, although a relationship between the species detected by EPR-techniques and the so-called P 700 reaction center chlorophyll of "System I" has frequently been postulated.

Time course of formation and decay in intact cells

As described above, no significant differences in the general properties of the photo-induced ERP-signals from a variety of intact and simplified preparations of *Chromatium* D have been found. If one follows, however, the time courses of formation and decay of these signals with sufficiently fast detection systems, very characteristic differences are observed in comparing intact cells under conditions of actual photosynthesis with various types of simplified systems.

This paper will be limited to a detailed discussion of the characteristic diagnostic patterns observed with intact cells of *Chromatium* D under conditions of actual photosynthesis and with the classical chromatophore-type preparation as an example

of a simplified system. Paper II and III of this series[32,33] will be concerned with a number of aspects particular for intact cells under more or less physiological conditions.

Experimental results. A 24-h-old culture of *Chromatium* D, grown and harvested as described, is suspended in NaCl–phosphate buffer and transferred into the EPR sample cell in darkness in an atmosphere of helium gas. Harvested at this stage of their growth, the bacteria contain a considerable amount of endogenous reducing substrates but are low in HCO_3^- as shown by adding various substrates and checking spectrophotometrically for substrate effects[6]. The suspension of the bacteria is thermally equilibrated with its environment in the microwave cavity for a period of 15 min in darkness before actinic irradiation is applied.

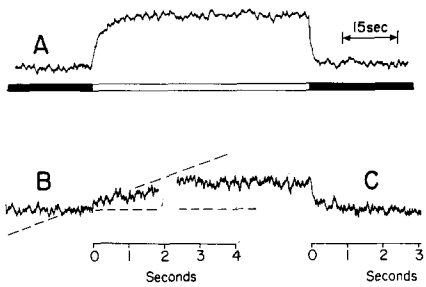


Fig. 4. Time course of formation and decay of photo-induced EPR-signal in intact cells of *Chromatium* D. 24-h culture, suspended in NaCl–phosphate solution. Bacteriochlorophyll concentration is 0.99 mM. Incident actinic intensity $1.0 \cdot 10^{17}$ quanta cm^{-2} sec^{-1}; wavelength > 7300 Å; optical pathlength, 0.25 mm. Time scales as indicated. A. "Slow" recording. Esterline-Angus Speedservo recorder (1/8 sec response time). Filter time constant 0.3 sec. B and C. Excerpts of simultaneous fast recordings. Schwarzer Oscilloscript; sensitivity $2 \cdot 10^{-2}$ V mm^{-1}. Time constant of active filter 30 msec.

The time course of formation and decay of photo-induced EPR-signals observed when this anaerobic sample is irradiated is shown in the recordings reproduced in Fig. 4. Part A of this figure shows a "slow" recording with a 0.3-sec time constant and the time scale as indicated. Parts B and C are excerpts from simultaneous fast recordings with time constants of 30 msec for the initial portion of the "on"-process and the decay process, respectively.

Prior to the irradiation in this recording, the bacteria had seen two periods of irradiation with the same actinic intensity, each 60 sec long, followed by 60 sec of darkness. At this moment the bacteria are therefore in a preilluminated state[14]. Details on the effects of the preillumination history on the kinetic patterns for intact cells of *Chromatium* D are being discussed in ref. 32.

The incident actinic intensity in this experiment corresponds to about 95 % of the saturation value. The maximal loss of actinic intensity for a single pass through the sample cell at $\lambda = 8100$ Å, the highest *in vivo* absorption band for cells grown under these conditions, can be estimated from the bacteriochlorophyll concentration (0.9 mg/ml) to be of the order of $A = 1.75$ based on an assumed *in vivo* absorption coefficient of $7.0 \cdot 10^4$ M^{-1} cm^{-1} (ref. 51). This figure corresponds to a "worst case consideration"; for any other wavelength applied the loss of actinic intensity can be expected to be smaller and the irradiation to be more homogeneous.

The time course of the formation of the signal is quite complex, as can be seen in Figs. 4A and 4B in spite of the rather high actinic intensity chosen. Lower actinic intensities tend to bring out the complexities much more clearly; this is readily seen in Fig. 5 and in Fig. 12, where intact cells and heat treated cells are compared for several actinic intensities.

In the experiment shown in Fig. 4 the time needed to reach 95 % of the final steady state signal intensity is 8.4 sec. Neglecting the complex rise pattern an "approximate half-time of formation" can be estimated to be 1.6 sec. There is a clearly discernible, very fast initial component immediately after the onset of irradiation which is instrument limited even in the fast recording (Fig. 4B) and completely lost in the slow recording of Fig. 4A. A much slower, but still somewhat complex rise to the final steady state amplitude follows. This situation is characteristic for intact cells and is fully discussed in ref. 33; see also ref. 34.

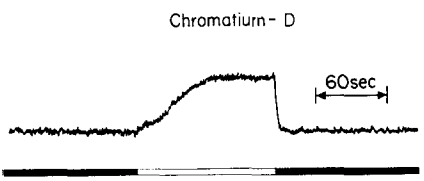


Fig. 5. Time course of formation and decay of photo-induced EPR-signal in intact cells of *Chromatium* D. 36-h culture, starved for 24 h, suspended in NaCl–phosphate solution. Dark adapted cells in helium atmosphere; irradiation period, 120 sec. Incident actinic intensity, $1.4 \cdot 10^{16}$ quanta cm^{-2} sec^{-1}. Wavelength > 7300 Å.

Within a series of consecutive irradiations of these cells (60 sec irradiation, 60 sec darkness) both the height of the first initial "jump" immediately after onset of irradiation and the final steady state amplitude, reached after about 10 sec in this experiment, do not depend on preillumination history. However, prolonged preillumination tends to shorten the over-all time it takes to reach the final steady state and thus to mask the discontinuity in the time course pattern.

The final steady state amplitude is also not affected by the prevailing levels of endogenous or added substrate, but does somewhat depend on the conditions under which the bacteria are grown (for example, "high light" *vs.* "low light") with corresponding variations in the pigment composition of the cells.

In contrast to the time course of formation the decay process upon termination of the actinic irradiation is remarkably fast. In the experiment shown in Fig. 4 the value of the "half-time of decay" is estimated to be 50 ± 5 msec or less—thus still somewhat limited by the response time of the active filter chosen (*i.e.*, 30 msec). Even under more favourable conditions—with higher cell concentration and faster instrumental time constants—no evidence for the existence of faster decay components has been found within the present limits of the instrumentation (about 1.5 msec). Therefore, a directly determined half-time of decay can be considered an appropriate parameter to describe the decay process.

A detailed analysis of the decay mechanism is made difficult by the low steady state concentrations of the paramagnetic species under these conditions. An attempt to fit simple kinetic characteristics to the decay process is shown in Fig. 6. Here the actual data from a fast recording (10 msec time constant) are replotted (crosses,

solid line) and compared with a first order (full squares, dashed line) and a second order (open circles) time course calculated from the experimentally determined half-time of the reaction.

The time courses of formation and decay shown in Figs. 4 and 5 are characteristic of bacteria harvested in this particular stage of their growth and treated as described. For the sake of brevity in the discussion, this pattern will be termed the "cell pattern". It is characterized by a relatively slow and complex time course of formation—with a fast initial rise at the onset of irradiation—and a very much faster, comparatively simple time course of decay.

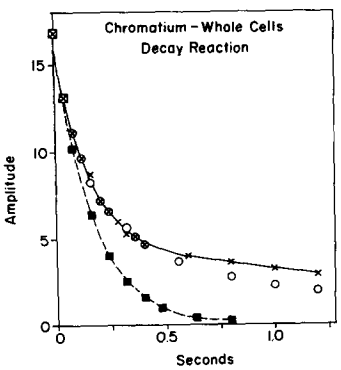


Fig. 6. Decay of photo-induced EPR-signal in intact cells of *Chromatium* D. 48-h culture (low in endogeneous substrate), suspended in NaCl–phosphate solution. Incident actinic intensity, $3.3 \cdot 10^{16}$ quanta cm^{-2} sec^{-1}; wavelength > 7300 Å. Schwarzer Oscilloscript; time constant of active filter 10 msec. A first order decay process indicated by squares and a second order decay process (assuming 1:1 stochiometry of reactants), indicated by open circles, are calculated from the experimentally determined half-time (average of four recordings). Experimental data replotted from an individual actual recording are shown as crosses connected by a solid line.

The "cell pattern" is only seen when the bacteria are studied under physiological conditions where active photosynthesis is carried out as demonstrated by parallel studies with light-dependent CO_2-fixation[12]. The "cell pattern" depends strongly on such metabolic parameters as age of the culture, endogenous substrate levels (of S^{2-}, $S_2O_3^{2-}$, HCO_3^-), concentration of added substrates, and on the preillumination history of the bacteria. Changes in the pattern induced by alteration of the metabolic situation—for example, by adding substrates or by starvation—are reversible within a limited range of conditions. For a detailed discussion of these effects see refs. 32 and 33. The "cell pattern" is, on the other hand, remarkably insensitive to other chemicals which are not substrates of bacterial photosynthesis[19].

If attempts are made to control these metabolic factors closely, exactly reproducible patterns of time courses can be consistently obtained, in contrast to a number of reports in the literature (see for example ref. 18). In fact, these patterns are so characteristic that deviations from normality can be used to diagnose difficulties with the culture long before more conventional microbiological techniques are able to do so.

Due to the complex effects of preillumination history this "cell pattern", while reproducible, cannot be considered a strictly repetitive phenomenon[14]. This precludes for all practical purposes the use of conventional time averaging techniques to en-

hance the signal to noise ratio. Time averaging would tend to mask some of the discontinuities seen in the formation of photo-induced EPR-signals with intact cells. It would also wipe out the small but characteristic changes in the decay process (see ref. 32). With prolonged dark periods of the order of 10 min or more between irradiations the time course patterns become strictly reproducible for a given material. But even for modest improvements in the sensitivity (for example averaging over 10 irradiation periods) total dark time between irradiations would have to be of the order of 2 h—a time period too long for work on physiologically intact materials.

We have found no evidence for the occurrence of any "lag period" in either formation or decay of these signals in intact cells of *Chromatium* D, or in any other photosynthetic bacterium, under any of the experimental conditions encountered so far, but see ref. 35.

The "cell pattern" can also be influenced by a number of processes of an unspecific nature such as variations in the osmotic environment or heat treatment. The bacteria are remarkably sensitive to these unspecific treatments and the changes in the time course patterns caused by these treatments are irreversible. In no way has it been possible to restore the original "cell pattern"; all the changes proceed in the direction towards the "chromatophore-type pattern" (for details see below).

Time course of formation and decay in a chromatophore-type preparation

A quite different pattern of time courses of formation and decay is found with chromatophore-type preparations obtained from intact cells of *Chromatium* D. Fig. 7 shows a characteristic example of such an experiment. The particles are prepared by sonic treatment and, after high speed centrifugation, suspended in NaCl–phosphate solution. The time course is recorded under conditions similar to the ones used for intact cells in Fig. 4A. An experiment with faster recording (10 msec time constant) and a somewhat more concentrated sample is illustrated in Fig. 8. In this experiment, the irradiation lasts only for about 5 sec.

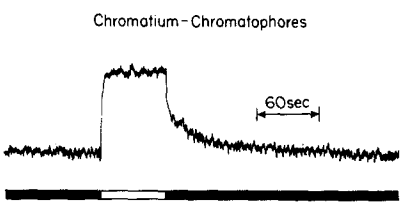


Fig. 7. Time course of formation and decay of photo-induced EPR-signal in chromatophore-type preparation from *Chromatium* D. Bacteriochlorophyll concentration, 1.3 mM. Incident actinic intensity, $1.5 \cdot 16^{16}$ quanta cm^{-2} sec^{-1}; wavelength > 7300 Å. Esterline Angus Speedservo recorder; filter time constant, 0.3 sec.

With these preparations the amplitude of the photo-induced EPR-signal rises very rapidly in a monotonic fashion to its maximal and final height, much faster and much more simply than in the case of actively photosynthesizing bacteria (*cf.* Fig. 4). On turning off the actinic irradiation the signal decays slowly in a characteristic biphasic fashion with clearly discernible fast and slow components. This pattern of time courses, with its fast rise to a final steady state value and its much slower biphasic decay process, will be termed the "chromatophore-type pattern".

The steady state amplitudes observed are quite independent of the methods used to prepare the material and relatively insensitive to various unspecific treatments (*e.g.*, changes in the osmotic environment) which affect the "cell pattern" drastically.

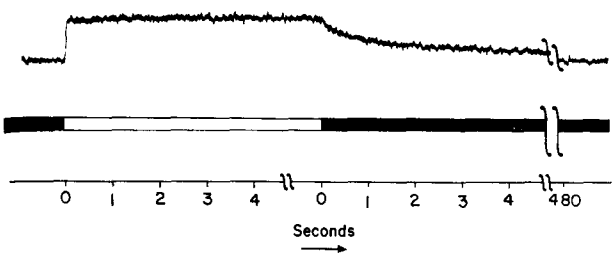


Fig. 8. Time course of formation and decay of photo-induced EPR-signal in chromatophore-type preparation from *Chromatium* D. Bacteriochlorophyll concentration 1.5 mM. Incident actinic intensity, $3 \cdot 10^{16}$ quanta $cm^{-2} sec^{-1}$; wavelength > 7300 Å; optical pathlength, 0.25 mm. Schwarzer Oscilloscript; time constant of active filter, 10 msec.

No further increases in amplitude are observed even after prolonged irradiation for up to 30 min at room temperature. It is also interesting to note that in spite of the much slower decay processes found with the chromatophore-type preparations (see below) the ratios "unpaired spins"/bacteriochlorophyll obtained with saturating actinic intensities are of the same order of magnitude as those obtained with intact bacteria[11].

The "on-rate", measured with the fast recording system, is found to be proportional to the actinic intensities over a range of about 100:1. Under no conditions have deviations from a simple monotonic time course been observed. All the discontinuities seen with intact bacteria[33,34] are absent under all conditions applied so far within the time resolution and sensitivity limits of our instrumentation. In addition, no effects of preillumination have been detected.

Preliminary estimates of apparent quantum yields of "unpaired spin" formation, using the observed initial rates of formation, result in values between 0.5 and 1.0, in agreement with an earlier value reported by CALVIN and collaborators for *R. rubrum* chromatophores[5].

As shown in Figs. 7 and 8 the decay of the photo-induced EPR-signals in a chromatophore-type preparation is relatively slow and biphasic with at least two readily discernible components. The time course can in a satisfactory manner be described by an expression containing the sum of two exponential terms (Fig. 9). This empirical curve fitting, however, does neither prove nor even imply that two different chemical processes with individual time constants are involved. In fact, although biphasic patterns are widely seen in systems related to photosynthesis[24,36,37], no reasonable explanation for its validity on chemical grounds can be given at the present stage of our knowledge. A plausible mechanistic explanation for a treatment by use of the "Elovich" equation, as COPE[38] suggested, is presently not available either.

A set of data with various actinic intensities is shown in Fig. 10. The time constants k_{fast} and k_{slow} have been obtained by fitting two exponential terms to the data as outlined in Fig. 9. For comparison a first order rate constant k_{obs} has been calculated directly from the observed half-times of decay. From these results it seems

that k_{fast} depends more strongly than k_{slow} on the actinic intensity and, therefore, on the steady state concentration of unpaired spins, while irradiated. Since the steady state amplitudes, when studied as a function of actinic intensity, follow roughly a square root law the faster component of the decay reaction thus seems to depend on the steady state amplitude in an approximately linear fashion. A similar relationship has been reported for bacteriochlorophyll in solution[20].

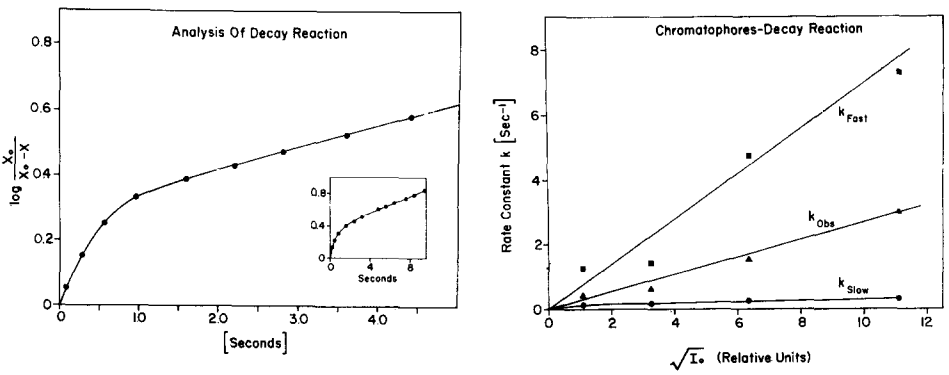


Fig. 9. Chromatophore-type preparation from *Chromatium* D. Analysis of decay reaction. Experimental conditions same as in Fig. 8.

Fig. 10. Chromatophore-type preparation from *Chromatium* D. Decay of photo-induced EPR-signal as function of actinic intensity. Recording and analysis of experimental data as in Fig. 9. Abscissa: square root of incident actinic intensity in arbitrary units. The value 10 on the abscissa corresponds to approximately $8.1 \cdot 10^{16}$ quanta cm^{-2} sec^{-1}.

In contrast to the situation encountered with intact bacteria under conditions of actual photosynthesis, the time course patterns observed with "chromatophore-type preparations" are readily reproducible and found to be strictly repetitive. They are quite stable phenomena and do not strongly depend upon the "quality and intactness" of the preparation used. Practically identical patterns are found with preparations obtained by a variety of procedures. Duration of the sonic treatment, aging of the material at $+4°$, storage at room temperature for several hours, changes in the osmotic environment, excessive irradiation with near infrared radiation or with visible light do not affect the time courses of either formation or decay to any larger extent.

Only comparatively small but definite kinetic differences are found when chromatophore-type preparations possessing high photophosphorylation activity are compared with those where photophosphorylation is inhibited by various means. This relationship, which is much more subtle in nature than the effects described here, is currently under detailed investigation in our laboratory[39].

Time course patterns similar or identical to the ones reported here for chromatophore-type preparations (*cf.* Figs. 7 and 8) can also be obtained from whole cells after exposure to a variety of unspecific treatments (see below). The changes in the direction towards the chromatophore-type pattern are seen, however, long before a general breakage of the bacteria or an acute leaching out of the pigments into the suspending medium can be detected.

Time course patterns of formation and decay seen with even more simplified (*i.e.*, less intact) systems, on the other hand, are virtually identical to the ones

described here for the classical chromatophore-type preparations. The main differences are being found in the generally much slower decay rates; examples are crude extracts of pigments from the bacteria[3] or isolated bacteriochlorophyll in solution[20]. In all these cases the characteristic features of the chromatophore-type pattern are clearly preserved even to such details as the order of magnitude of the ratios of k_{fast}/k_{slow} (see Fig. 10) and dependence on actinic intensities.

Transitions between the time course patterns

The time courses of formation and decay of photo-induced EPR-signals from *Chromatium* D have been seen to fall into two categories which we have termed "cell pattern" and "chromatophore-type pattern".

It is of considerable interest to learn at what level of intactness and organization of the materials the transition in time course pattern occurs and, in addition, by what technical means this change can be produced.

One convenient way to achieve the transition is found in the exposure of intact bacteria to hypotonic conditions for a limited period of time. Dilute buffer solutions (for example 10 mM phosphate, pH 7.5) or simply distilled water are generally used. The degree of perturbation is controlled by the duration of the exposure. For the EPR measurements the perturbed material is then brought back to the original tonicity by centrifugation and resuspension in the usual NaCl–phosphate solution. Control samples are handled in exactly the same manner using NaCl–phosphate solution throughout. All treatments are again carried out with strictly deoxygenated solutions under a helium atmosphere in darkness.

Results obtained in such an experiment are illustrated in Fig. 11. During the exposure to hypotonic conditions—here to distilled water for approximately 5 min—the photosynthetic capacity of the cells expressed in terms of light-dependent CO_2-fixation drops to about 15 % of its original value. Viability tests performed in collaboration with Dr. JANE GIBSON show that about 85 % of the untreated sample, but less than 20 % of the osmotically perturbed sample give rise to visible colonies in an anaerobic culture[12]. Simultaneous measurements of the characteristic cyto-

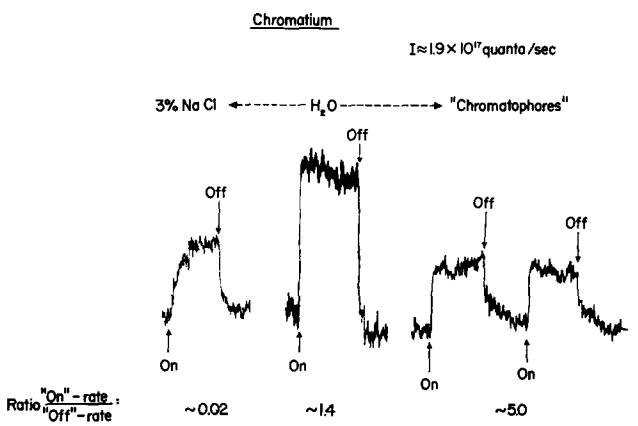


Fig. 11. Effect of osmotic treatment on intact cells of *Chromatium* D. Experimental conditions similar to Fig. 4A; for details of the treatment, see text.

chrome responses using the dual wavelength spectrophotometer yield essentially indistinguishable results for the perturbed and the control samples.

A quite similar transition can readily be achieved by heat treatment. Results of such an experiment are illustrated in Fig. 12 where the time course patterns are compared for intact cells of *Chromatium* D and heat treated cells at three different actinic intensities.

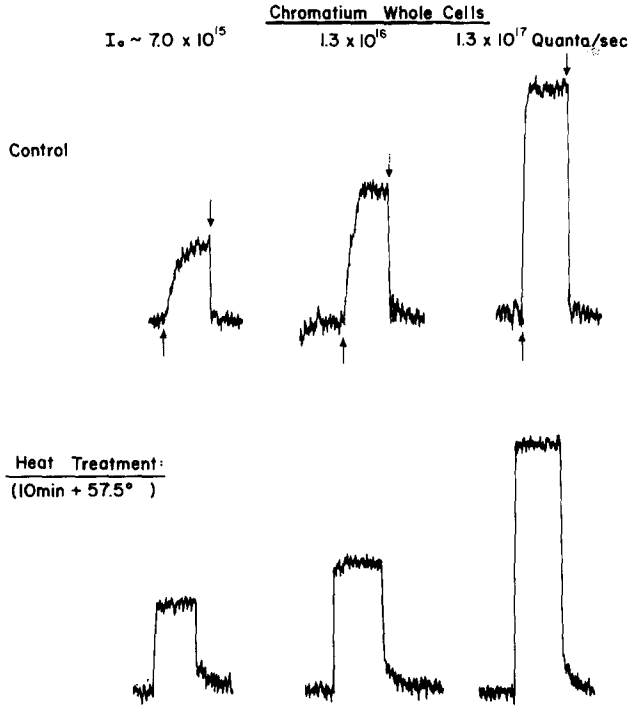


Fig. 12. Effect of heat treatment on intact cells of *Chromatium* D. Experimental conditions similar to those of Fig. 4A. Actinic intensities quoted are calculated for a total receiving area of 1.84 cm^2.

These results demonstrate clearly that the kinetic patterns associated with formation and decay of photo-induced EPR-signals, but not the existence of the signals, are affected by these perturbations, as are the more complex physiological functions (growth, CO_2-fixation, *etc*). The common result of all these treatments is the acceleration of the formation of the signals accompanied by a gradual loss of the complexities seen in this time course (*cf.* Figs. 4 and 5). For the decay process the common result of these perturbations is a general slowing down of the process and the appearance of the biphasic character (*cf.* Figs. 7 and 8).

In contrast the light-induced absorbance changes attributable to oxidation and reduction of *c*-type cytochromes are virtually unaffected by these treatments applied to intact cells of *Chromatium* D[12,34]. This is in line with the findings that when carefully prepared, closely similar photo-induced cytochrome responses can still be obtained from chromatophore-type preparations of *Chromatium* D—both with steady actinic light[41] and, more recently, also with pulsed excitation[29].

CONCLUSIONS AND DISCUSSION

In this paper we report on the properties of photo-induced EPR-signals of *Chromatium* D. The investigations cover a wide span of materials, from intact cells under conditions of actual photosynthesis to more and more simplified systems and finally to synthetic, purely photochemical systems.

Our studies have revealed no significant differences in the general properties of the signals from all these materials. The most characteristic parameters in this respect are the apparent g-value at resonance (very close to the free electron value), the width and the shape of the absorption line, the lack of any detectable hyperfine structure, the relaxation behaviour, and the temperature dependence of all these parameters. The only exceptions known so far are a slightly smaller linewidth observed with bacteriochlorophyll in solution[20] and a somewhat broader line in the case of a bacteriochlorophyll protein complex isolated from a different source (H. SCHLEYER AND J. M. OLSON, unpublished results). The fact that these signals from the various preparations have virtually identical properties suggests that the same or a very similar species is detected by the magnetic resonance technique. Our recent findings of closely similar photo-induced EPR-signals from pure bacteriochlorophyll in solution[20] suggest even more strongly a central role and a direct involvement of the bacteriochlorophyll molecule in the formation of these signals. Further, independent support is provided by the fact that identical biphasic kinetic patterns (except for smaller values of rates and rate constants) are observed with photochemical systems containing bacteriochlorophyll in solution[20] and all simplified systems derived from *Chromatium* D, but not with the photosynthesizing bacteria.

In contrast, the situation with chlorophyll *a* in higher plant and algal photosynthesis is not as well defined; for more recent work see WEAVER[22] and FUJIMORI[42]. That chlorophyll *a* can participate in one-electron transfer processes, even at low temperatures and with high quantum yields, has been demonstrated by TOLLIN and co-workers[43].

Spectroscopic techniques applied to bacterial photosynthesis do not readily lead to unambiguous chemical identification of the species involved but they can be used to eliminate a number of chemical reactants. Our experiments show that the presence and function of cytochromes and cytochromoids is not essential and that neither quinones nor non-heme iron proteins are absolutely required for the formation of the signals. This does of course not rule out that in the intact systems of bacterial photosynthesis some of these species will participate in the occurring reactions.

As to the possible assignment of a chemical structure to the paramagnetic species, our results point to an inhomogeneously broadened spin system with unresolved hyperfine structure. This is in full agreement with what one would predict for a molecular radical species having its unpaired spin density in a highly delocalized π-electron system as it exists in porphyrins. This view is experimentally supported by the recent findings of FEHER AND MAUZERALL[44] of a somewhat analogous free radical species photoproduced from uroporphyrin III under appropriate conditions.

The current lack of suitable theories for these "chromophores" does not allow prediction of detailed features of both EPR- and optical absorption spectra for the free radical species. The situation is further complicated by the fact that the chemistry of bacteriochlorophyll is still poorly understood. Recent developments of refined

theoretical treatments by GOUTERMAN and associates[45] have opened possibilities that might help to overcome the difficulties. Experimental work along these lines is well under way in this laboratory (*cf.* also ref. 20).

The relation to bacterial photosynthesis

On the basis of spectroscopic observations alone one cannot rule out the possibility that the observed species are resulting from a derailment rather than participating in the natural processes of photosynthesis. This argument applies to the paramagnetic species and to all photo-induced absorbance changes. On these grounds the interpretation of some spectroscopic findings has occasionally been critisized in the past (*cf.* for example ref. 26). Such a complication is only partially relieved by the fact that the photo-induced EPR-signals, with the kinetic relationships described here, are readily demonstrated in intact bacteria under physiological conditions, where *c*-type cytochromes are the only other reactive species spectrophotometrically detected (refs. 6, 25, 36, 37). A definitive solution can only be obtained from detailed correlation experiments linking the spectroscopically observed phenomena with some of the more physiological expressions of bacterial photosynthesis. Results using this approach for the photo-induced EPR-signals are reported here. A similar, clarifying investigation of the absorbance changes related to the bacteriochlorophyll absorption *in vivo* has not yet been performed.

The kinetic data, obtained in a time range not previously accessible, enabled us to study the correlation of photo-induced EPR-signals with a number of other measurable parameters of bacterial photosynthesis. For the purpose of this discussion a qualitative comparison of several of these parameters with the EPR-data is made for a selected group of materials (Fig. 13).

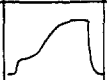
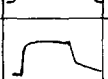
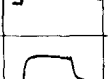
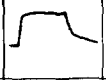
Level of Organization	Material	Photo-chemical Activities	Cytochrome Responses	ATP Synthesis	CO_2 Fixation	EPR-Time course pattern	Photo-induced EPR signals
Intact System	Intact Bacteria	+	+	+	+ (100 %)		+
	Perturbed Cells	+	+	+	(+) (~15 %)		+
"Simplified Systems"	Chromato-phore type preparation	+	+	+			+
	Perturbed Chromatophores	+	(+)				+
	Crude Extract of Pigments	+					+
Molecular Level	Bacterio-chlorophyll in Solution	+					+

Fig. 13. A comparison of parameters of bacterial photosynthesis as observed in intact cells and preparations of *Chromatium* D.

It is seen from the information presented that existence and demonstratability of photo-induced EPR-signals parallel the photochemical activities of the bacteriochlorophyll molecule including its participation in bacterial photosynthesis. Examples of such photochemical activities in systems ranging from chromatophore-type preparations of various qualities to more and more simplified systems are numerous (see for example ref. 47). In contrast, the existence of the two characteristic time course patterns of formation and decay—the cell pattern and the chromatophore-type pattern—parallels the overall photosynthetic capacities of the materials.

It should be emphasized that the existence of the two characteristic kinetic patterns does not correlate with the capacity of the materials to form ATP in the process of photophosphorylation. Some preliminary data have been obtained with intact cells of *Chromatium* D in our laboratory[48] and SMITH AND RAMIREZ[49] have reported relevant data for intact cells of *R. rubrum*. But, unfortunately, a detailed study of the formation of ATP in intact photosynthetic bacteria is still not available. On the other hand, a regulating influence of the ATP level on some electron transfer processes in these bacteria has been postulated[50].

As shown, the transition between the two time course patterns takes place between the levels of organization of the intact bacteria and those of a classical chromatophore-type preparation, but very much closer to the intact organism. The only other parameters for which we can demonstrate distinct changes of behaviour in correlation with these transitions are the more complex physiological expressions of bacterial photosynthesis such as growth, viability, and light-dependent CO_2-fixation.

Photo-induced absorbance changes attributable to oxidation and reduction of *c*-type cytochromes do not exhibit changes in behaviour at this level. These findings are in line with the observation of virtually unchanged cytochrome responses in chromatophore-type preparations from *Chromatium* D at least at room temperature[29,41]. It should be stressed, however, that this is typical only for *Chromatium* D but is not normally found with other photosynthetic bacteria, for reasons which are not fully understood. In contrast, the photo-induced EPR-signals in other photosynthetic bacteria behave in a fashion quite analogous to that reported for *Chromatium* D in both general properties and time courses (*cf.* also refs. 19 and 40).

The kinetic results compared with the other parameters provide the strongest evidence available at present that the free radical type species investigated by EPR-spectroscopy are participants in the overall process of bacterial photosynthesis. The paramagnetic species have all the properties required for a functional component within the "photosynthetic unit" which is mediating the light-driven flow of electrons (or reducing equivalents) from the natural substrates to the ultimate electron acceptor. This overall system constitutes the basic and fundamental part of photosynthesis, *i.e.* the synthesis of organic matter by the organisms under illumination. So far definitely established constituents of the "photosynthetic unit" are bacteriochlorophyll[28,29,47]—with the detailed reaction mechanisms as yet unknown—and *c*-type cytochromes[6,25,36,37]. In the case of *Chromatium* D under our experimental conditions reducing substrate and ultimate electron acceptor can be represented by S^{2-} and HCO_3^-, respectively.

By measuring light-dependent reduction of CO_2 or growth as a parameter of bacterial photosynthesis not all of the electrons driven by light are accounted for; instead an absolute minimal requirement of photosynthesis is established. Substantial

evidence exists for an additional cyclic flow of electrons in various photosynthetic systems. While a detailed account of the contribution from a cyclic flow is not available for intact cells of *Chromatium* D (but see refs. 6, 25, and 50 for attempts) such a cyclic pathway has to be considered in relation to the functional role of the various cytochromes, the photo-induced EPR-signals, the reaction center bacteriochlorophyll, the electron acceptor, and the process of photophosphorylation. Preliminary results indicate that kinetic EPR-studies are valuable investigating tools in this respect as well.

Finally, the lack of a correlation of EPR-signals with some directly observable spectrophotometric events deserves some comment. Spectrophotometric studies have revealed a variety of light-dependent absorbance changes attributable to the absorption of bacteriochlorophyll *in vivo*[27,28,46]. In spite of the generally recognized specificity of spectrophotometric techniques the nature of these changes is still very little understood and at present diffcult to interpret in terms of chemical events. The paramagnetic resonance technique is selective for a certain magnetic property rather than specific for a certain chemical species. It responds in a very sensitive fashion to a class of chemical entities (*e.g.*, the free radical type species) which is absent in the dark as demonstrated by the lack of paramagnetic resonance absorption.

The limit of detectability of "unpaired spins" in photosynthetic systems is of the order of $3 \cdot 10^{-11}$ moles. With bacteriochlorophyll concentrations typically of the order of 1 mM, the active part of the EPR sample cell contains roughly $3 \cdot 10^{16}$ molecules and approximately $8 \cdot 10^{13}$ unpaired spins are formed under light-saturated conditions. If we assume a maximal value ε_M approx. $1 \cdot 10^5$ M^{-1} cm^{-1} for an absorbance change associated with the photo-induced EPR-signals we can calculate that in comparison studies an absorbance change of approximately $7.5 \cdot 10^{-3}$ units would have to be detected at some, in principle, unknown wavelength if the same samples and sample geometry are used. It is not unreasonable to expect that an absorbance change of this magnitude, with the time course characteristics as described in the RESULTS section, might be overlooked among some of the other absorbance changes known and observed.

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