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ELECTRON SPIN RESONANCE IN ZERO MAGNETIC FIELD OF THE REACTION CENTER TRIPLET OF PHOTOSYNTHETIC BACTERIA

A.J. HOFF and H. GORTER DE VRIES

Biophysics Department and Centre for the Study of the Excited States of Molecules, Huygens Laboratorium, State University of Leiden, Leiden (The Netherlands)

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Summary

The decay rates k_x, k_y, k_z of the individual spin levels of the light-induced triplet state have been accurately measured by the zero-field resonance technique under conditions of very low light intensity and a microwave sweep rate of 2.5 MHz/μs, which is in excess of that commonly used in optical detection magnetic resonance experiments. The rates k_u found correspond well with those previously determined under somewhat different conditions (Hoff, A.J. (1976) Biochim. Biophys. Acta 440, 765—771) and with those inferred from the deacy at 4.2° K of the triplet-triplet absorption after picosecond excitation (Parson, W.W. and Monger, T.G. (1977) Brookhaven Symp. Biology 28, 195—212). Thus there seems no reason to doubt that P^R corresponds to the triplet state detected by ESR. In a recent publication Clarke and Connors (Clarke, R.H. and Conners, R.E. (1976) Chem. Phys. Lett. 42, 69—72) published values of the rates k_u which differ substantially from ours and which lead to a mean lifetime in excess of that of P^R. We show that erroneous rates are obtained when the microwave sweep rate is not made fast relative to the decay of the individual spin levels.

Zero-field splitting parameters for a member of photosynthetic bacteria have been measured with an accuracy of better than 0.4% for $|D|$ and 1% for $|E|$. The enhanced precision as compared to conventional ESR allows one to discriminate between species of one family. Deuteration reduces the k_u values by a factor of about 2, with little spin selectivity. This effect is much larger than previously observed for chlorophyll *a*.

The present results explain the decrease in fluorescence intensity observed on microwave saturation in zero-field optical detection magnetic resonance experiments, and they also show that the simple exciton model is inadequate to derive the geometry of the reaction center dimer from the observed zero-field splitting and decay rates.

Introduction

In 1972 Dutton et al. [1] discovered a triplet state in photosynthetic bacteria using low temperature electron spin resonance (ESR). This triplet state is observed only under reducing conditions, it is strongly spin polarized [2] and it can be seen in reaction centers which are devoid of antenna pigments.

By fast optical absorption spectroscopy Parson et al. [3] and Cogdell et al. [4] observed a state called P^R in reaction centers of *Rhodopseudomonas spaeroides* in which the primary acceptor had been reduced. This state attributed to a bacteriochlorophyll triplet on the basis of its absorption difference spectrum, has a rise time of about 15 ns and decays with a halftime of about 6 μs at room temperature, and 110 μs at 77°K [5]. At 4.2°K its decay is biphasic, the two components having halftimes of 86 and 540 μs, respectively, and relative amplitudes of 2 to 1 [6].

The two kinds of observation are commonly regarded as different spectral manifestations of the same triplet state, but a definite proof is lacking. One of the few ways to prove this contention is the comparison of rise and decay kinetics as measured with both techniques. Clarke et al. [7,8] and Hoff [9] carried out ESR experiments in zero-field at 2°K. They observed two lines from which zero-field splitting parameters, $|D|$ and $|E|$, were deduced that corresponded very well to those extracted from the conventional ESR signals. Both Clarke et al. [7,8] and Hoff [9] measured the decay kinetics of the three components of the triplet state, but they arrived at rather divergent results, i.e. $k_x = 2660$, $k_y = 3183$, $k_z = 1596\ s^{-1}$ [8] and $k_x = 9300$, $k_y = 8500$, $k_z = 2100\ s^{-1}$ [9], where k_x, k_y and k_z are the decay rates of the x, y and z components, respectively. The average decay halftime derived from the latter values, 104 μs, agrees well with the average halftime from the optical work at 4.2°K (117 μs) [6] and at 77°K (110 μs) [5]. The decay rates obtained by Clarke and Connors [8], however, give an average halftime of 282 μs, the difference being due mainly to their different values of k_x and k_y. The results of Hoff have been criticized by Clarke et al. [10], who suggested that the fast decay rates observed by Hoff were caused by high levels of illumination.

The determination of accurate values of the decay rates for the three components of the triplet state yet has another aspect, since Clarke recently suggested that from these decay rates together with the zero-field splitting parameters one may derive information about the geometrical structure of the reaction center dimer via the model of Sternlicht and McConnell [11].

In order to clear up the existing discrepancy between the results of Clarke et al. [7,8] and those of Hoff [9] and Parson and Monger [6] we have carefully examined the dependence of the three triplet decay rates on the rate of excitation and also on the characteristics of the microwave perturbation at resonance. We conclude that the values previously found by Hoff [9] were essentially correct. The results of our investigations leave little doubt that the state P^R in fact is the same triplet state as seen by magnetic resonance techniques.

The bacterial triplet state is generally assumed to result from a back reaction between the photo-oxidized primary donor and the photo-reduced intermediate bacteriopheophytin [12—16]. One might expect that in zero magnetic field this back reaction would equally populate the x, y and z triplet compo-

nents. The populating probabilities p_u ($u = x, y, z$) found previously [9] deviated considerably from the value 1/3. The analyses of the zero-field resonance results in ref. 9 was carried out following the procedure of van Dorp et al. [17]. In this procedure no account is taken of the possibility of energy transfer between the reaction center and the antenna pigments. Since the zero-field resonance measurements were carried out with whole cells, such energy transfer has to be accounted for. We have been able to do so by applying the Vredenburg-Duysens relation [18,19] (see Appendix). The p_u values now obtained are much closer to 1/3 and to the values inferred by Parson and Monger [6].

Since the zero-field resonance technique lends itself so well for the precise determination of values of $|D|$ and $|E|$ (the accuracy of conventional ESR is of the order of 5%, whereas zero-field resonance yields values of $|D|$ and $|E|$ with a precision better than 0.4 and 1%, respectively) and since these parameters are one of the few handles we have to gain information on the detailed structure of the primary donor, we have determined $|D|$ and $|E|$ for a member of photosynthetic bacteria. The differences are small (most fall within the accuracy of ESR determinations [20]), with the exception of *Chromatium vinosum*, a thiorhodaceae.

Complete deuteration does not affect the zero-field splitting parameters, but it slows the decay rates by a factor of about 2. This effect is much larger than for monomeric chlorophyll *a*, where it is only 20% [21].

Materials and Methods

The bacteria *Rhodospirillum rubrum* and *Rps. sphaeroides* (wild type) were grown as described [22]. Deuterated *R. rubrum* was a kind gift of Dr. J.J. Katz. After centrifugation the cells were taken up in growth medium and 10 mM morpholinopropane sulfonic acid (pH 8) to an absorbance varying from 1 to 0.05 in 1 mm at 870 nm. Reduction was carried out under N_2 atmosphere by adding excess solid dithionite. After 2-fold dilution with glycerol, the samples were quickly frozen at 77° K.

Zero-field resonance was detected via the Microwave-Induced Fluorescence technique developed by van Dorp et al. [17,23]. The samples were excited by broad banded light from an appropriately filtered mercury arc lamp, centered at 423 nm, or 542 nm, with bandwidths of 91 and 73 nm, respectively. In some experiments narrow banded excitation via appropriate interference filters (Balzer) at 553 or 590 nm (bandwidth 10 nm) was used. Incident power ranged from 440 mW/cm^2 to levels as low as 0.8 mW/cm^2. A few experiments were carried out using a He-Ne laser (λ = 628.4 nm). Detection took place via narrow band interference filters or an Ebert monochromator. Spectra were recorded in a single scan using amplitude modulation of the microwaves and lock-in detection. The microwaves were generated by a Hewlett-Packard HP 8690 B sweep oscillator, of which the output was amplified by a Varian solid state amplifier VSP-7435-KL-496 to a level of 1—2 W.

The kinetic experiments were carried out following the procedure as described in detail by van Dorp et al. [17]. Care was taken to perturb the triplet system with resonant microwaves in a time much shorter than the fastest

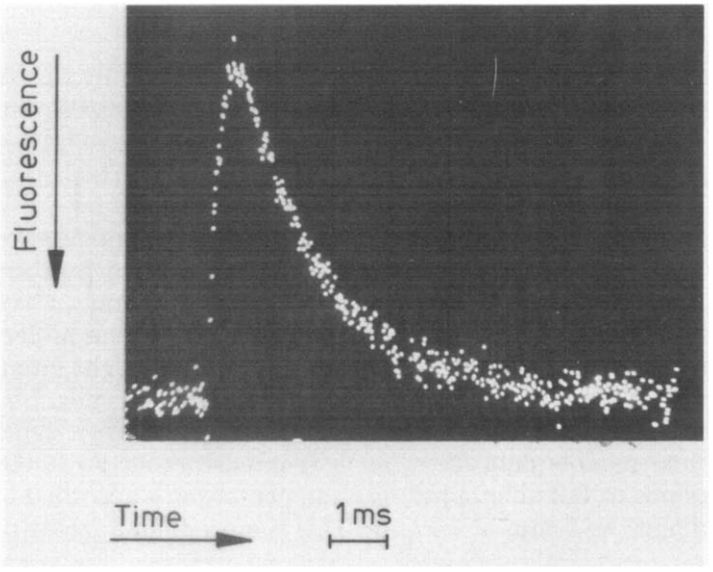


Fig. 1. Typical low light response of the fluorescence intensity of deuterated *R. rubrum* at 906 nm to a 10 μs microwave pulse at 461 MHz. Light intensity 5.5 mW/cm^2 (about 50 quanta/reaction center per s) at 542 cm (10 cm $NiSO_4$ 50 g/l, HA3, Calflex c, Schott OG 515 and neutral density filters). Number of scans 2^{17}, temperature 1.4 K.

decay times encountered, either by applying square pulses of width 10 μs at the resonance frequency, or by sweeping through the transition in about 6 μs. The response as detected by changes in fluorescence was monitored by a Hewlett-Packard 5480 B signal averager (Fig. 1). For the lowest light levels up to 2^{19} response curves were accumulated. Following the procedure of van Dorp et al. [17] the populating probabilities p_u ($u = x, y, z$) can be extracted from the changes in steady-state level of fluorescence on connecting the triplet sublevels in different ways with resonant microwaves. We have employed the Vredenberg-Duysens expression [18], which relates the level of fluorescence to the number of closed reaction centers, to calculate calues for p_u (see Appendix for details). The k_u values were found by extrapolating to zero light intensity curves of the apparent decay rate r_u versus light intensity (see Eqn. 7 of ref. 17). Light intensities, expressed in mW/cm^2, were measured by a Yellow Springs YS1 radiometer, placing the probe at the sample location with the optics adjusted to optimum intensity. Where necessary the light level was reduced by calibrated neutral density filters (combinations of Schott NG 11, 5 and 4). The number of quanta absorbed in the sample was calculated using a molar extinction coefficient of 95 $mM^{-1} \cdot cm^{-1}$ at 850 nm (*Rps. sphaeroides*) or 890 nm (*R. rubrum*) [24], taking the absorption spectrum of a dilute suspension of bacteria as measured with a Cary 14 spectrophotometer equipped with a scattered light attachment to compute the relative extinction coefficient at the wavelengths of excitation.

Results

The discrepancy between the values of the k_u previoulsy reported by Clarke et al. [7,8] and Hoff [9] could be caused by (i) different light intensities; (ii) different absorbtivity of the sample; (iii) different wavelength of excitation, and (iv) other reasons, as sample preparation, the duration of the perturbation by resonant microwaves, etc.

The first three causes are interdependent as they all influence the number of quanta observed. This is an important parameter, as it can be shown that the rate of depopulation r_u of the triplet component T_u is a function of the number of quanta absorbed (ref. 17, Eqn. 7). In order to evaluate the molecular decay rates k_u, which are obtained by extrapolating r_u to zero light intensity, we have therefore taken pains to set the lower light limit as low as possible. By increasing the microwave power 50-fold, and by employing extensive signal averaging, we could obtain reliable data for r_u at ligth intensities as low as 0.8 mW/cm^2, which corresponds to 3.2 quanta per reaction center per s absorbed in a sample of 0.02 absorbance in 1 mm at 540 nm. This is more than a factor of

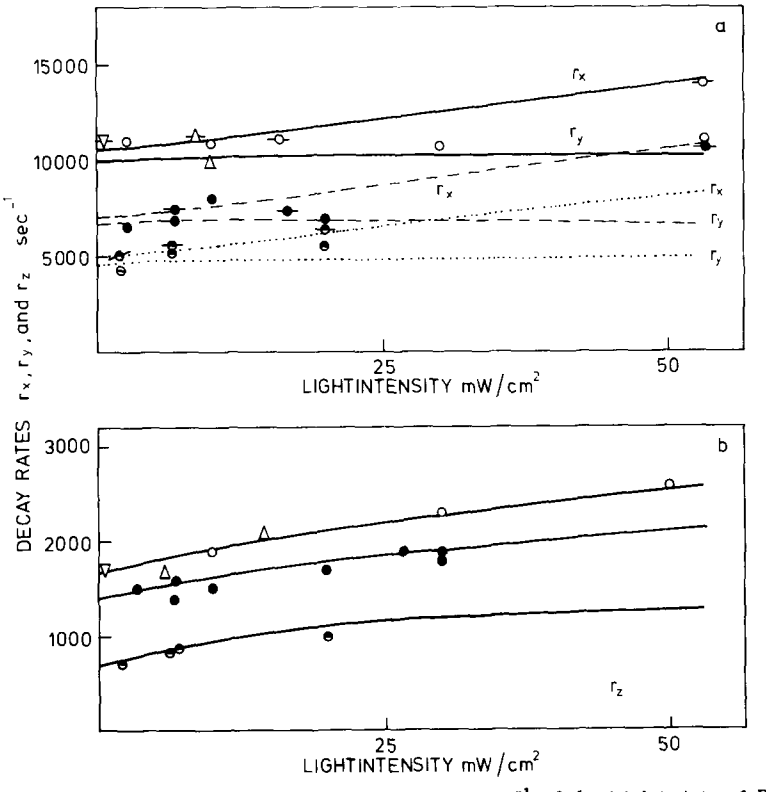


Fig. 2. (a) Characteristic decay rates r_x and r_y in s^{-1} of the triplet state of *Rps. sphaeroides* wild type (———), *R. rubrum* (- - - - - -) and *R. rubrum* deuterated (· · · · · ·) at 1.4°K. Excitation: ▽ and △; Balzer 590 and 553 nm interference filters, bandwidth 10 nm, respectively; ○, ● and ⊖. broad band centered at 542 nm, bandwidth 73 nm (filters as in Fig. 1). The ligth flux was varied with Schott NG neutral density filters. Drawn curves represent computer simulated curves. (b) Decay rate r_z of *Rps. sphaeroides* (▽, △, ○), *R. rubrum* (●) and *R. rubrum* deuterated (⊖) at 1.4°K. Excitation as in a.

TABLE I

DECAY RATES OF THE TRIPLET COMPONENTS AT 1.4°K

	k_x	k_y	k_z
Rps. sphaeroides wild type	10 500 ± 1000	10 000 ± 1000	1700 ± 100
R. rubrum	7 000 ± 500	6 500 ± 500	1300 ± 100
R. rubrum deuterated	4 500 ± 500	5 000 ± 500	700 ± 100

25 lower than the lowest level attained by Clarke et al. (illumination by a laser beam of 20 mW lowest output power at 590 nm (Clarke, R., private communication)). At our lowest light flux less than 0.5% of the reaction centers are in the triplet state. In Fig. 2 data obtained under various experimental conditions are collected. Table I shows the extrapolated values k_u which are seen to be similar to those previously obtained in this laboratory [9]. However, k_x and k_y still deviate appreciably from those reported by Clarke et al. [7,8] *. He-Ne laser illumination (λ = 628.4 nm, incident power 1 mW) gave essentially the same results as those obtained with broadband excitation at shorter wavelengths. Omitting glycerol from the sample medium did not change the k_u values to any significant extent. Our results were reproducible, and were independent of the wavelength of excitation (taking into account the difference in absorbance of the sample). In order to rule out selective excitation effects due to the spectral narrowness of the excitation microwave pulse (50 kHz) we have carried out kinetic experiments by sweeping the entire microwave transition. For fast sweeps (0.4 μs/MHz) no difference was found with the k_u values as determined by our pulse method. For slower sweeps, however, important deviations resulted. Using a 40 μs/MHz sweep for instance (this corresponds to the fastest built-in sweep of the HP oscillator), values of k_x, k_y and k_z of 2010, 2470 and 1350 s^{-1}, respectively, were found (at a light intensity of 7 mW/cm^2). We note that these values are close to the ones found by Clarke et al. [7,8], suggesting that their decay rates were slowed down by a period of microwave irradiation that was too long compared to the sublevel lifetimes.

Also in Fig. 2 are displayed the values for r_u obtained for deuterated *R. rubrum*. It is seen that the rates of depopulating are about a factor of 1.5—2 lower than in protonated *R. rubrum*, which are similar to the rates obtained for protonated *Rps. sphaeroides*. We observe only little spin selectivity for the isotope effect.

The probability of populating the three components of the triplet state were determined by the method detailed in the Appendix. Briefly, the triplet components x, y and z were connected for about 2 ms with saturating resonant microwaves in three different ways, viz. $x \sim z$, $y \sim z$ and $x \sim y \sim z$. The differences between the fluorescence intensity under those three conditions and the intensity without microwaves was measured by averaging the fluorescence response for 2^8—2^{15} repetitive scans, under various intensities of illumination.

* The belief that the values of r_u become independent of the light intensity in the lower light limit [8] is erroneous as borne out by the theoretical analysis of van Dorp et al. [17] and by our present experimental results. In fact, precisely at low light fluxes, k_y and especially k_z become markedly dependent on light intensity.

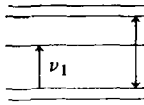
The excitation probability K which enters as a parameter in the formalism of the Appendix was calculated from the measured incident energy and the known absorbance of the sample. For 300 mW/cm^2 at 590 nm K is 1300 s^{-1}. The values of p_u calculated from the experimental data are only weakly dependent on K. Although the relative changes in fluorescence induced by the saturating microwave field were small (typical values ranging from 0.5 to 0.03%) they could be accurately measured with our repetitive pulse procedure. To a good approximation these relative changes proved independent of light intensity.

Our method of analysis of the fluorescence changes differs from that given by van Dorp et al. [17] in two respects: (i) we have explicitly taken into account energy transfer from the reaction center complex on which the triplet state is located to other (antenna) pigments by using the Vredenberg-Duysens relation [18,19]; (ii) we have estimated the fraction of reaction center molecules that are affected by the microwave field. The accuracy with which p_u may be determined depends critically on a proper assessment of this fraction. In an effort to excite the whole zero-field resonance line we originally frequency modulated the microwave source, but this procedure was found to be unsatisfactory. Instead we estimated the fraction of the line that is saturated by performing "hole-burning" experiments [9]. Taking estimates of this fraction from 5 to 10% of the total linewidth, we arrive at values of p_u that are rather similar for the three organisms studied, viz. $p_x \sim 45$, $p_y \sim 35$ and $p_z \sim 20$%. The estimated error is about 20% of these values, p_z tending to lower values, and p_x and p_y becoming more equal with proportionally, less "background" fluorescence. Apparently p_z is now about a factor of 2 higher than the value of p_z obtained with the direct formalism of van Dorp et al. [17]. Its value, however, is considerably below the value 0.33 arising from triplet generation via an isotropic mechanism.

Table II gives the $|D|$ and $|E|$ values of a number of photosynthetic bacteria.

TABLE II

FREQUENCIES, LINEWIDTHS AND ZERO-FIELD SPLITTING PARAMETERS

	MHz (±0.8)		MHz (±0.5)		10^{-4} cm^{-1}	
	ν_1	ν_2	$\Delta\nu_1$	$\Delta\nu_2$	$D \pm 0.6$	$E \pm 0.3$
R. sphaeroides (wild type)	460.6	654.9	13.3	13.5	185.9	32.4
R. rubrum (wild type)	460.6	666.3	12.6	15.8	187.8	34.3
R. rubrum (wild type) (no glycerol)	459.8	666.0	13.1	16.1	187.6	34.3
R. rubrum (deuterated) (no glycerol)	461.0	669.7	12.5	16.7	188.4	34.8
R. rubrum FR 1 *	460.8	666.5	12.0	14.7	187.9	34.3
R. rubrum FR 1 * mutant VI	462.5	670.0	13.1	17.9	188.8	34.6
R. gelatinosa	459.5	653.7	12.1	11.7	185.5	32.3
R. capsulata	461.4	643.5	13.2	13.5	184.2	30.3
R. palustris	458 ± 2	657 ± 2	14 ± 1	17 ± 1	186 ± 2	33 ± 1
C. vinosum	431.0	633.5	11.8	11.6	177.4	33.7

* *R. rubrum* FR 1 and its carotenoidless mutant VI were a kind gift of Dr. G. Drews.

These values concord with those found by conventional ESR [29]. Note that the increased accuracy of zero-field resonance permits to discriminate between species that yield the same $|D|$ and $|E|$ when studied by conventional ESR, where the experimental uncertainties are far greater.

Discussion

The results of our investigations leave little doubt that the decay rates of the three triplet sublevels correspond to the biphasic decay found by Parson and Monger [6] for the state P^R at 4.2°K following picosecond excitation. This strenghtens the notion that P^R and the ESR-detectable triplet state are one and the same.

Clarke et al. [10] have derived formulae relating the zero-field splitting parameters and the decay rates of a dimer to those of the constituent monomers under the assumption of exciton interaction between the monomers. We have not been able to fit our data in a consistent manner with this model. It seems clear that the theory of Sternlicht and McConnell [11] on which the calculations of Clarke et al. [10] are based is not an adequate formalism to describe the bacterial triplet. This is perhaps not suprising, since in view of the delocalized charge in the cationic dimer it is probable that the dimer triplet state has appreciable charge-transfer character. Also, it is know from work on paracyclophanes with polar substituents that their triplet state shows reduced zero-field splittings and enhanced decay rates compared to the monomers, which has been attributed to charge-transfer effects [25,26]. This suggests that it is not sufficient to take into account only the excition character of the bacterial triplet, but that also ionized states should be considered before one can arrive at pertinent conclusions about the dimer structure. We note that the negative sign of the change of fluorescence upon the application of resonant microwaves now finds a natural explanation. Regardless of the exact nature of the radical pair mechanism, it will always give probabilities of population for the x, y and z components that do not differ much from each other. This means that the z component has a far greater equilibrium population than the x and y components, since this population is proportional to p_u/k_u, and $k_z << k_x, k_y$. Application of resonant microwaves then transfers population from the z to the x or y levels, i.e. the total triplet concentration will decrease, and the S_0 populations accordingly increase because the excess population in the x or y level decays rapidly to S_0. An increase in $[S_0]$ would mean an increase in fluorescence, if one neglects the presence of antenna pigments, contrary to observation. However, the application of the Vredenberg-Duysens equation leads to a decrease of fluorescence (the concentration of open traps increases), conform the experimental results. Deuterium substitution reduces the k_u values by about a factor of 2. This is surprising, since in monomeric chlorophyll *a* the effect is only 20% [21]. In free base porphyrins a similar doubling of the lifetime of the triplet state on deuteration has been reported, but there it is almost completely due to the inner N-H hydrogens [27,28]. Apparently, dimerization of the chlorophylls leads to an increased deuterium effect, perhaps owing to the involvement of protons in the ligands that keep the chlorophylls together. It would be of interest to carry out isotopic substitution experiments on in vitro

aggregated chlorophyll species, such as studied by Kooyman et al. [29].

The discrepancy between the values of the populating probabilities in zero field found in this work and the value of 1/3 that might be expected from the radical pair theory in its simplest form can be understood by looking somewhat closer into the details of this mechanism.

The conversion of the singlet state of the radical pair into its triplet state, $(P^+I^-)^S \rightarrow (P^+I^-)^T$, is governed by the product of the inverse of the square of the energy difference between the interacting states and a matrix element of a perturbing hamiltonian H_1 between the singlet and triplet eigenstates of the radical pair.

The energy difference between the triplet and singlet eigenstates in zero laboratory magnetic field depends on the exchange interaction J between the unpaired electron of the whole radicals, on their dipole-dipole coupling energy, and on hyperfine interactions. From the magneto-dependence of the yield of the triplet P^TI it was concluded [14,15] that even at higher laboratory magnetic fields one does not have pure $S\text{-}T_{m=0}$ mixing, and consequently it was assumed that the exchange interaction J plays an important role in the singlet-triplet conversion.

The hyperfine interaction presumably is of the same order of magnitude as the dipole-dipole interaction between the two unpaired electrons of the radical pair. This means that for $(P^+I^-)^T$ in zero laboratory field the triplet electron spin is no longer quantized in the symmetry planes of the molecule, and consequently T_x, T_y and T_z are no longer the correct zero field eigenstates. When the two unpaired electrons recombine to form the state P^TI, the electron dipole-dipole coupling becomes much stronger and T_x, T_y and T_z do become the zero field eigenstates of P^TI. Without detailed knowledge of the geometry of P^+I^- and of the anisotropic hyperfine interactions one cannot tell how the eigenstates of (P^+I^-) project out into the eigenfunctions T_x, T_y and T_z. We must conclude that because of our ignorance of sign and magnitude of J, and of the detailed structure of P^+I^-, it is at present impossible to predict the relative populations of T_x, T_y and T_z after the recombination process. Our determination of p_u indicates that there is a slight preponderance for populating the $T_{x,y}$ levels above that of the T_z level.

In summary we conclude that our present results on the populating probabilities of T_x, T_y and T_z are much closer to those expected from the radical pair theory in its simplest form than our previous values of p_u [9]. There is, however, a need for extension of this theory. We are currently performing electron spin resonance in low fields (less than 100 G) in an attempt to learn more on the conversion between singlet and triplet state of the radical pair.

Appendix

The determination of the populating probabilities p_u ($u = x, y, z$) from zero field resonance kinetic experiments on photosynthetic bacteria

The analysis of zero-field resonance kinetic experiments in dilute crystals has been described in detail by van Dorp et al. [17]. Application of this analysis to the present case is hampered by (i) the absence of the third resonance $x \sim y$, corresponding to the transition $2E/h$; (ii) the impossibility to directly measure

the populating of the triplet components under conditions that all levels are connected by resonant microwaves [19]; (iii) the fact that the fluorescence monitored is not solely due to the reaction center but for more than 90% to the antenna chlorophyll molecules, and (iv) the large influence on the antenna chlorophyll fluorescence yield of the state of the reaction center primary donor [19]. In this Appendix we will show, that by modifying the treatment of van Dorp et al. [17], the first two limitations can be circumvented, whereas incorporation of the Vredenberg-Duysens relation between the state of the reaction center and the fluorescence yield allows extraction of meaningful values for the populating probabilities p_u ($u = x, y, z$).

We start our analysis with the differential equations set up by van Dorp et al. to describe the populating and depopulating of the triplet system:

$$\frac{d[S_0(t)]}{dt} = -K[S_0(t)] + \sum_u k_u[T_u(t)]$$

$$\frac{d[T_u(t)]}{dt} = -k_u[T_u(t)] + p_u K[S_0(t)] \tag{1}$$

with $u = x, y, z$ and $\Sigma p_u = 1$, where $[S_0(t)]$ and $[T_u(t)]$ are the fractional populations at time t of the singlet ground state and the triplet components, respectively, and k_u and p_u are the depopulating rates and the populating probabilities. K is the excitation rate. The solution is given by $[S_0(t)] = a + \Sigma_u b_u \exp(-r_u t)$. a and b are simple functions of K, k_u and p_u, r_u is a complex function of these parameters. For very small light intensities r_u approaches k_u, the true depopulating rate. Since the population of the first excited singlet state S_1 is neglected we can write $\Sigma_u[T_u(t)] = 1 - [S_0(t)]$. Abbreviating $\Sigma_u[T_u(t)]$ to $[T(t)]$, we obtain for steady-state conditions under any microwave configuration

$$[T(\infty)]^\dagger = 1 - [S_0(\infty)]^\dagger \tag{2}$$

were the dagger sign stands for $x \sim z$, y; $y \sim z$, x; $x \sim y \sim z$ or x, y, z. The notation $\sim$ means: connected by saturating microwaves.

The Vredenburg-Duysens relation [18] states that

$$\frac{1}{\phi} = A + B[\text{fraction open traps}]. \tag{3}$$

Here, ϕ is the fluorescence quantum yield of antenna chlorophyll, A and B are experimental constants. It has been shown that this relation holds well for the bacterial system [19]. The bacterial reaction center has been brought in the state PIX^- by chemical reduction. This state in itself does not constitute a closed trap since the reaction $PIX^- \xrightarrow{h\nu} P^+I^-X^- \rightarrow P^TIX^-$ is still possible, and is at low temperatures 100% effective [6,19]. Following Holmes et al. [19] we therefore equate the fraction of closed traps to the fraction that is in the triplet state, $[T(\infty)]$. We can then write

$$\frac{[T(\infty)]^\dagger}{[S(\infty)]^\dagger} = \frac{1/\phi_0 - 1/\phi}{1/\phi - 1/\phi_m} \tag{4}$$

with $1/\phi_0 = A + B$ and $1/\phi_m = A$; ϕ_0 and ϕ_m are the fluorescence yield when all the traps are open, or closed, respectively. Introducing the fluorescence intensity $F = I\phi$, one obtains

$$\frac{[T(\infty)]^{\dagger}}{[S(\infty)]^{\dagger}} = \gamma \frac{F^{\dagger} - F_0}{\gamma F_0 - F^{\dagger}} \tag{5}$$

with $\gamma = F_m/F_0$.

In Fig. 3 we have schematically depicted the fluorescence level under various microwave conditions, after a steady-state situation under continuous illumination has been reached. The increase in fluorescence yield upon switching on the light has actually been observed [19]. When all triplet levels are connected by microwaves, i.e. in the $x \sim y \sim z$ configuration, it can easily be shown [17] that

$$\left(\frac{[T(\infty)]}{[S(\infty)]}\right)_{x \sim y \sim z} = \frac{K}{k}$$

where K is the rate of excitation and k is the average rate of depopulation: $k = \frac{1}{3} \Sigma_u k_u$. The quantity c defined in the figure can now be expressed as

$$c = F^{x \sim y \sim z} - F_0 = \frac{\gamma - 1}{\gamma(k/K + 1)} \cdot F^{x \sim y \sim z} \tag{6}$$

This is a useful result, since the fluorescence intensity F_0 cannot be measured directly. It is now an easy matter to derive expressions for

$$\frac{[T(\infty)]^{\dagger}}{[S(\infty)]^{\dagger}} \quad (\dagger = x \sim z,\ y;\ y \sim z,\ x \text{ and } x,\ y,\ z)$$

in terms of p_u, K and k_u on the one hand [17], and in terms of the experimentally measurable quantities c, u, v and w (see Fig. 3) on the other hand. Taking the k_u values as the extrapolated values of r_u for $I \to 0$ (from Fig. 1),

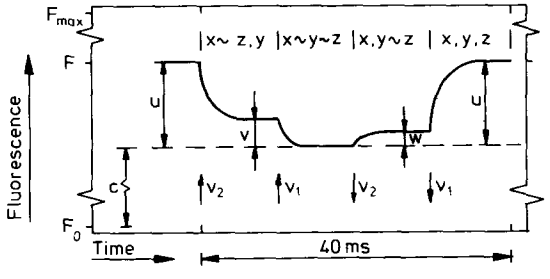


Fig. 3. Schematic representation of the changes in fluorescence intensity following the application of resonant microwaves corresponding to the $y \sim z$ (ν_1) and $x \sim z$ (ν_2) transition between the components of the triplet state. Upward and downward arrows indicate the switching on and off, respectively, of microwave power of saturating intensity. The response time of the system was made longer compared to that for kinetic experiments to improve the signal to noise ratio.

the p_u values can now be solved from the resulting set of three linear equations. The value of the quantities γ is $\gamma = 2$ for *Rps. sphaeroides* R-26 and $\gamma = 4$ for *Rps. sphaeroides* wild type and for *R. rubrum* (van Grondelle, R., personal communication).

In this analysis we have not used the relation $\Sigma p_u = 1$, i.e. the deviation of the sum of the calculated p_u values from the value 1 is a useful check for their accuracy. The values of p_u determined in this way should be independent of the parameter K, i.e. of the light intensity, when appropriate values of c, u, v and w are used. This proved to be the case. It is noted that the changes in fluorescence yield, i.e. the values of u, v and w are measured sequentially and repetitively (2^8—2^{15} repetitions being averaged, depending on the light intensity). Values of u, v and w are therefore to a high degree insensitive to lamp fluctuations etc.

When determining values of p_u with the above formalism one should be aware of the fact that for certain values of p_u (determined solely by the relative values of k_u), the relative population of the three triplet components is equal, so that no change in fluorescence is detected when connecting the sublevels with resonant microwaves [17]. Conversely, very small relative changes in fluorescence yield values of p_u that are very close to the above solution. It is therefore important to estimate the level of background fluorescence (i.e. fluorescence unreactive to microwaves) [30]. In our data reduction we have taken into account this effect by careful analysis of the bandwidth of detection, fluorescence bandwidth, zero-field resonance linewidth and the part of this line excited by the resonant microwaves [9].

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