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Rose Bengal Radicals and their Reactivity

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The one-electron oxidised (radical cation) and one-electron reduced (radical anion) forms of the photosensitiser rose bengal have absorption maxima at 470 nm (molar absorption coefficient 21 100 $dm^3 mol^{-1} cm^{-1}$) and 420 nm (molar absorption coefficient 37 600 $dm^3 mol^{-1} cm^{-1}$), respectively. The radical anion ($RB^{\cdot -}$) undergoes electron-transfer reactions both with oxygen ($k = 1.5 \times 10^8$ $dm^3 mol^{-1} s^{-1}$) and iron(III) complexed with ethylenediaminetetraacetic acid (EDTA) ($k = 4.5 \times 10^8$ $dm^3 mol^{-1} s^{-1}$) and diethylenetriaminepentaacetic acid (DTPA) ($k = 8.6 \times 10^8$ $dm^3 mol^{-1} s^{-1}$). Rose bengal reacts both with the solvated electron and the semireduced nicotinamide adenine dinucleotide radical ($NAD^{\cdot}$) to give $RB^{\cdot -}$ with second-order rate constants of 3.1×10^{10} and 9.6×10^8 $dm^3 mol^{-1} s^{-1}$. The one electron-reduction potential E_1^7 for rose bengal, measured *via* pulse radiolysis, was found to be −511 mV.

Rose bengal (RB) is a well known and much studied water-soluble photosensitiser with a very strong visible absorption band around 550 nm.[1,2] The triplet quantum yield (ϕ_T) is believed to be unity[3] and the corresponding singlet oxygen yield (ϕ_Δ) is also very high.[4] However, the interaction of oxygen with the RB triplet is not quantitative and *ca.* 25% of the triplets do not yield singlet oxygen. Thus other processes such as electron-transfer reactions involving the RB triplet and oxygen may also occur:[5]

$$RB(T) + O_2 \rightarrow RB^{\cdot +} + O_2^{\cdot -}$$

In the biological situation electron-donor molecules can also play a role in such processes, that is

$$RB(T) + D \rightarrow RB^{\cdot -} + D^{\cdot +}$$

and, in the presence of oxygen, the superoxide radical ($O_2^{\cdot -}$) and H_2O_2 can also arise. In the presence of redox active metal ions (*e.g.* iron and copper), a classical Fenton reaction will arise, yielding highly reactive and biologically damaging hydroxyl radicals ($OH^{\cdot}$). Previous (*e.g.* ref. 2, 5) photo-studies of RB have been somewhat confused due to spectral overlap of triplet and radical spectra. In this paper we have used the Paterson Institute nano-second pulse radiolysis facility to characterise the $RB^{\cdot +}$ and $RB^{\cdot -}$ radicals unambiguously. That is, we report the molar absorption coefficients and absorption spectra of both radicals, the one-electron reduction potential of $RB^{\cdot -}$ and rates of reaction of several molecules of biological interest with $RB^{\cdot -}$. The $RB^{\cdot -}$ can also arise from RB without direct light excitation and, as an example of such a process, we have studied the interaction of semi-reduced nicotinamide adenine dinucleotide ($NAD^{\cdot}$) with RB

$$NAD^{\cdot} + RB \rightarrow RB^{\cdot -} + NAD^+$$

Experimental

RB was obtained from Aldrich as a high-purity product and used as supplied. All solutions were made up in 0.1 mol dm^{-3} phosphate buffer, pH 7, and saturated with either nitrous oxide or argon, as appropriate. The iron(III) complexes with EDTA and DTPA were prepared by dissolving measured amounts of $Fe_2(SO_4)_3$ (BDH) in acidified distilled water (pH 2) to which the corresponding chelator (Sigma) was added. The solution was adjusted to pH 7 with concentrated sodium hydroxide. The solution contained up to 2.6 times excess of the chelator compound to the molar concentration of Fe^{3+}. Paraquat was also obtained from Sigma.

The pulse radiolysis technique has been described previously,[6] and for this study we used either almost exclusively oxidising environments (N_2O–10^{-1} mol dm^{-3} azide ion) or exclusively reducing environments (10^{-1} mol dm^{-3} formate). Under these conditions we unambiguously form $RB^{\cdot +}$

$$e_{aq}^- + N_2O + H_2O \rightarrow N_2 + OH^- + OH^{\cdot}$$

$$OH^{\cdot} + N_3^- \rightarrow N_3^{\cdot} + OH^-$$

$$N_3^{\cdot} + RB \rightarrow RB^{\cdot +} + N_3^-$$

or $RB^{\cdot -}$

$$RB + e_{aq}^- \rightarrow RB^{\cdot -}$$

$$OH^{\cdot} + HCO_2^- \rightarrow CO_2^{\cdot -} + H_2O$$

$$RB + CO_2^{\cdot -} \rightarrow RB^{\cdot -} + CO_2$$

The assumption that these two systems react cleanly to give the respective one-electron oxidised and reduced radicals is reasonable as many examples of such reactions are known both from this laboratory and others.[7–10]

Involvement of $H^{\cdot}$ radicals in these reactions can effectively be ignored. In the presence of azide $H^{\cdot}$ is rapidly scavenged to give $HN_3^{\cdot}$ which is eventually converted to $N_3^{\cdot}$.[9,11] In the presence of formate ions $H^{\cdot}$ reacts with formate ions to give more $CO_2^{\cdot -}$ radical.[12]

The radical molar absorption coefficients for $RB^{\cdot -}$ and $RB^{\cdot +}$ were obtained using the thiocyanate radical $(CNS)_2^{\cdot -}$ produced from N_2O-saturated solutions of KCNS in water as a dosimeter.[13] The molar absorption coefficient at 500 nm used for $(CNS)_2^{\cdot -}$ was 6550 $dm^3 mol^{-1} cm^{-1}$ with a *G* value of 632 nmol J^{-1} (calculated from the yield/extinction product reported by Schuler *et al.*[14] of 46 400 molecules per 100 eV $dm^3 mol^{-1} cm^{-1}$ at 472 nm. Both molar absorption coefficients are the result of at least five different concentrations of RB, and a correction has been made for the decay of $N_3^{\cdot}$ and $CO_2^{\cdot -}$ by routes other than electron transfer. Overall the error limits on these measurements are in the range ±15%. Pulse radiolysis can also be used to determine the one-electron reduction potentials[15] and we have obtained this for RB using paraquat (PQ) as a redox indicator at three different paraquat–RB concentrations. The $PQ^{\bullet +}/RB^{\bullet -}$ ratio was determined at 605 nm.

Results and Discussion

Fig. 1(*a*) and (*b*) show the absorption spectra, at several times after the pulse, for $RB^{\bullet -}$ and $RB^{\bullet +}$, respectively. From the spectral and kinetic data obtained we can be fairly certain that only one species is produced in each case.

The spectrum of $RB^{\bullet -}$ was obtained using formate solutions saturated with argon. Under these conditions the spectrum obtained shortly after the pulse is very similar to that produced after 200 μs. This would indicate that the species formed from the e_{aq}^- reaction with RB is the same as that from $CO_2^{\bullet -}$, *i.e.* the reduced radical $RB^{\bullet -}$. As can be seen, $RB^{\bullet -}$ exhibits a peak at 420 nm with $\varepsilon(RB^{\bullet -}) = 37600$ dm^3 mol^{-1} cm^{-1} (using a *G* value of 673 nmol J^{-1} for the total reducing species in N_2O-saturated solution as calculated using the method of Schuler *et al.*;[16] a value of 3.2×10^9 dm^3 mol^{-1} s^{-1} was used for the rate constant for the reaction of $HCOO^-$ with $OH^\bullet$ [17]). For $RB^{\bullet +}$ a peak at 470 nm with a somewhat lower molar absorption coefficient of 21100 dm^3 mol^{-1} cm^{-1} is obtained (from a *G* value for $N_3^\bullet$ of 622 nmol J^{-1} based on the discussion of Ye *et al.* in ref. 9). These radicals show different decay rates, under the conditions chosen, so that $RB^{\bullet -}$ decays to *ca.* half its initial concentration in 150 ms, whereas $RB^{\bullet +}$ decays by *ca.* two orders of magnitude faster.

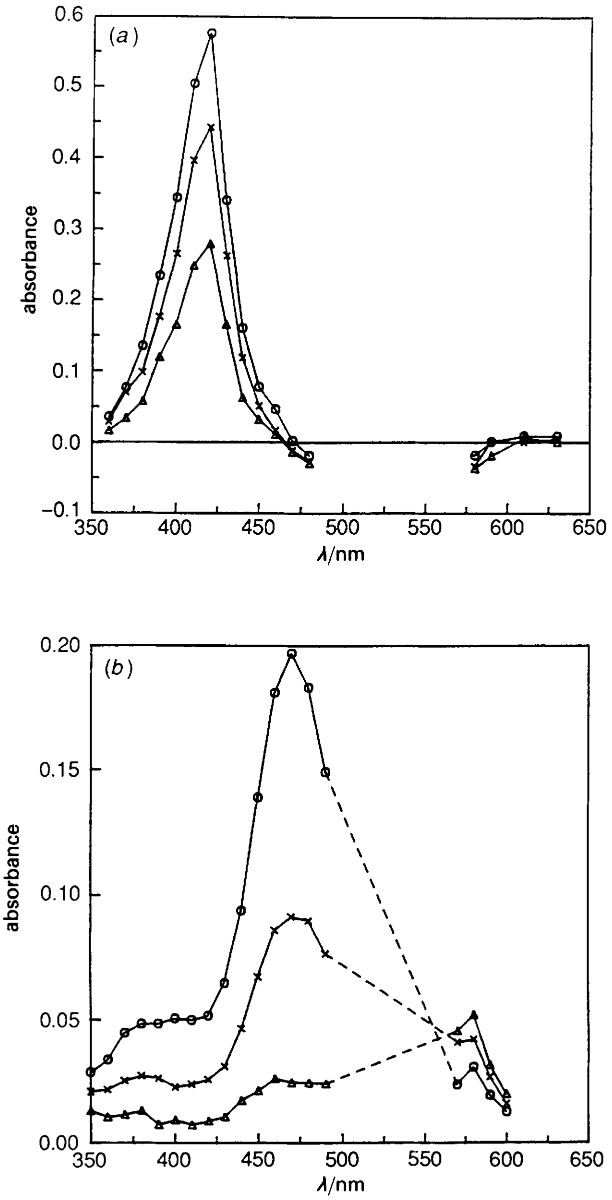

Fig. 1 Transient absorption spectra of rose bengal (10^{-4} mol dm^{-3}) in argon-saturated solution of 0.1 mol dm^{-3} formate (dose ≈ 1.9 Gy) (*a*) and in N_2O-saturated solution of 0.1 mol dm^{-3} azide (dose ≈ 8.1 Gy) (*b*). Times after pulse were (*a*) ○, 200 μs; ×, 4.1 ms; △, 43 ms; and (*b*) ○, 60 μs; ×, 336 μs; △, 2.32 ms. Spectra are normalised to a dose of 10 Gy

The semioxidised radical, derived from RB, decays by second-order kinetics with a second-order rate constant of 1.6×10^9 dm^3 mol^{-1} s^{-1}. The decay of the semireduced radical is much slower, as can be appreciated from the intercept of fig. 3(*d*) (later). The lifetime observed is almost certainly controlled by trace amounts of oxygen remaining in the solution (see below). Independent EPR measurements suggest that the true rate constant for $RB^{\bullet -}$ decay is 2×10^4 dm^3 mol^{-1} s^{-1}.

Fig. 2 shows the structure of $RB^{\bullet +}$, RB, $RB^{\bullet -}$ and the dihydro fully reduced form of RB in their ionised forms at pH 7.[18] As can be seen, $RB^{\bullet -}$(**3**) has normally three negative charges whereas $RB^{\bullet +}$(**1**) is formally a mono-negative species. Thus, it is quite reasonable to expect the second-order decays of $RB^{\bullet -}$ to be slower than those of $RB^{\bullet +}$. Interestingly, while the $RB^{\bullet -}$ decays, possibly *via* disproportionation (to RB and RB^{2-}), and this leads to no residual absorption in the spectral range studied, $RB^{\bullet +}$ decays to a product that absorbs at 580 nm. We are not aware of any data on the absorption spectra of $RB^{\bullet +}$ dimers to compare with our observations. The pulse radiolysis experiments also allow us to obtain the second-order rate constants for the formation of $RB^{\bullet -}$, *i.e.* $k(RB + CO_2^{\bullet -})$ as 3.2×10^8 dm^3 mol^{-1} s^{-1} and for the for-

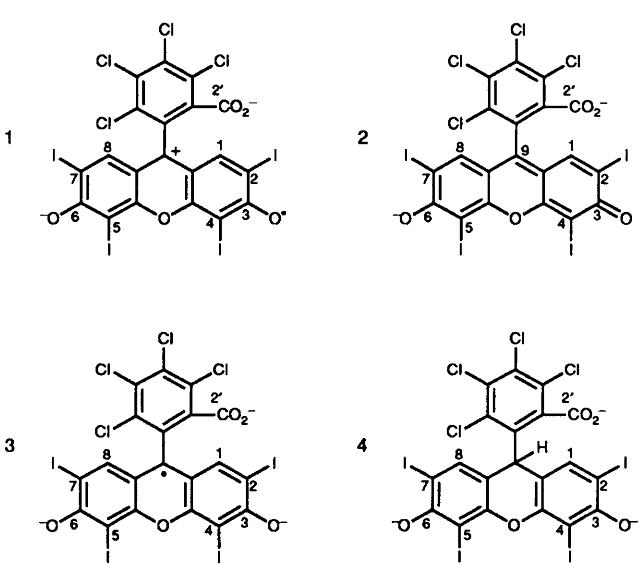

Fig. 2 Structures of rose bengal and its oxidation and reduction products: Monoanion semioxidised radical, $RB^{\bullet +}$(**1**); dianion from of RB(**2**); trianion semireduced radical, $RB^{\bullet -}$(**3**); and trianion fully reduced dihydroform, RB^{2-}(**4**)

mation of the $RB^{\bullet +}$, *i.e.* $k(RB + N_3^{\bullet})$ as 3.8×10^9 dm^3 mol^{-1} s^{-1}. Once again, simple charge-repulsion effects can account for the smaller rate constant involving the $CO_2^{\bullet -}$ radical.

As well as $CO_2^{\bullet -}$ reacting with RB to give $RB^{\bullet -}$, this species is also formed in a much faster step by direct reduction with the hydrated electron, *i.e.*

$$RB + e_{aq}^- \rightarrow RB^{\bullet -}$$

The rate constant for this process was determined by monitoring the enhanced decay of the electron at 680 nm and found to be 3.1×10^{10} dm^3 mol^{-1} s^{-1} [Fig. 3(*a*)], the resulting spectrum being the same as obtained with $CO_2^{\bullet -}$.

The disproportionation of $RB^{\bullet -}$ (as reported above) was determined in carefully deoxygenated solutions because of the rapid rate of electron transfer from $RB^{\bullet -}$ to O_2 to give the superoxide radical anion ($O_2^{\bullet -}$)

$$RB^{\bullet -} + O_2 \rightarrow RB + O_2^{\bullet -}$$

The rate constant for this process was found to be 1.5×10^8 dm^3 mol^{-1} s^{-1} as shown in Fig. 3(*b*). The transient was monitored at 420 nm and showed no residual absorption. This is a relatively large electron-transfer rate constant and, since the inherent lifetime of $RB^{\bullet -}$ is rather long (see above), it may imply that in the biological situation such electron transfers to yield the superoxide radical anion could be important. As a possible recent example Ciulla *et al.*[19] have described RB-photosensitised single strand breaks of super-coiled DNA and showed that this was not necessarily related to singlet oxygen formation. Thus, it is quite possible that $O_2^{\bullet -}$ (and other oxyradicals formed *via* Fe^{II}/Fe^{III}-mediated Fenton reaction) could be involved.

Redox metals such as iron and copper frequently arise in biological systems. We have studied, as a model process, the interaction of two Fe^{III} complexes, Fe^{III}/DTPA (this is frequently used in biological experiments because it prevents reduction of Fe^{III} by $O_2^{\bullet -}$) and Fe^{III}/EDTA. For both systems we have investigated the one-electron reduction by $RB^{\bullet -}$ to the Fe^{II} complex and obtained second-order rate constants of 8.6×10^8 and 4.5×10^8 dm^3 mol^{-1} s^{-1} for DTPA and EDTA complexes, respectively [Fig. 3(*c*)]. A control experiment showed that $RB^{\bullet -}$ also reacted with the chelators (the maximum second-order rate constant was $<2 \times 10^7$ dm^3 mol^{-1} s^{-1}). This was taken into account when studying the reaction of $RB^{\bullet -}$ with the Fe^{III} complexes.

In our pulse radiolysis work we have attempted to characterise $RB^{\bullet -}$ and $RB^{\bullet +}$ because such radicals may well arise in biological systems if RB is used as a photosensitiser. As a model of such a process we have used pulse radiolysis to generate the semireduced radical form of nicotinamide adenine dinucleotide ($NAD^{\bullet}$) and monitored the electron transfer from it to RB

$$NAD^{\bullet} + RB \rightarrow NAD^+ + RB^{\bullet -}$$

For this process we obtain a second-order rate constant of 9.6×10^8 dm^3 mol^{-1} s^{-1} [Fig. 3(*d*)]. The one-electron reduction potential of $NAD^+/NAD^{\bullet}$ is known to be -940 mV,[20] and since the reaction is very efficient, clearly the one-electron reduction potential of $RB/RB^{\bullet -}$ is substantially more positive. Pulse radiolysis can be used to obtain one-electron reduction potentials if an equilibrium can be established between a couple whose redox potential is already known and the unknown system. A typical transmission against time plot obtained under these conditions is shown in Fig. 4. We used paraquat as the redox indicator, the one-electron reduction potential of $PQ^{2+}/PQ^{\bullet +}$ being -448 mV.[21] For the process

$$RB^{\bullet -} + PQ^{2+} \rightleftharpoons RB + PQ^{\bullet +}$$

we obtain an equilibrium constant of 11.7 ± 8, and this leads to a determination of the one-electron reduction potential (at pH 7) of $RB^{\bullet -}$ as -511 ± 15 mV. This agrees well with a previously determined value[22] of -530 mV obtained by an electrochemical method.

In summary, we have unambiguously characterised the absorption spectra of the radical anion and cation of rose bengal in aqueous solution and obtained estimates of the $RB^{\bullet -}$ reactivity with biologically relevant molecules. In particular, we have shown an efficient electron transfer between $RB^{\bullet -}$ and oxygen to give the superoxide anion, $O_2^{\bullet -}$, and discussed the possible significance of this when RB is used as a photosensitiser in a biological system.

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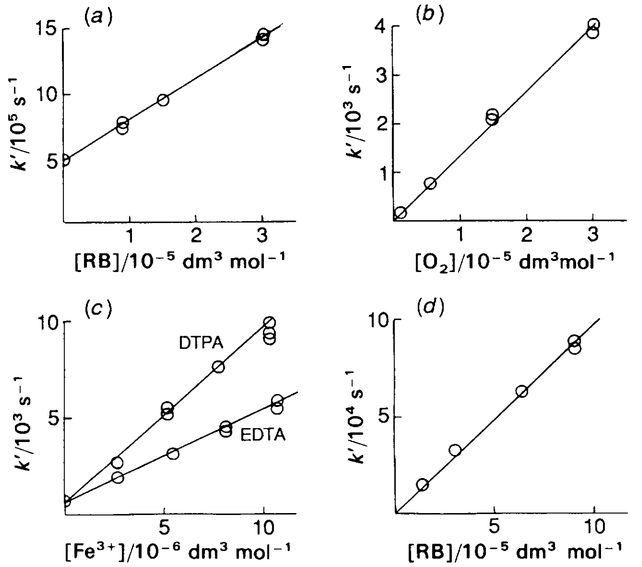

Fig. 3 Rate constant *vs.* concentration plots for the determination of second-order rate constants for: (*a*) $e_{aq}^- + RB$ (680 nm), (*b*) $RB^{\bullet -} + O_2$ (420 nm), (*c*) $RB^{\bullet -}$ + complexed Fe^{3+} (420 nm), (*d*) $NAD^{\bullet} + RB$ (420 nm). The monitoring wavelength is given in brackets

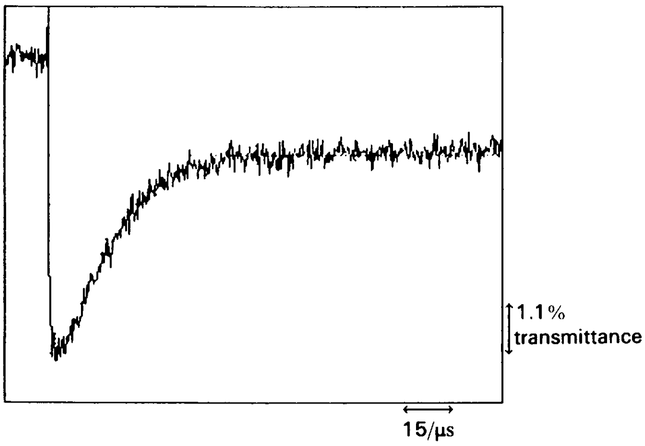

Fig. 4 Formation and decay of $RB^{\bullet -}$ to an equilibrium monitored at 420 nm. The solution contained RB (10^{-4} mol dm^{-3}), paraquat (1.9×10^{-5} mol dm^{-3}) and formate (0.1 mol dm^{-3}). The sample was saturated with argon. The dose was *ca.* 1.5 Gy

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