cyclohexadiene. Generally in cyclic π-hydrocarbon complexes such as **6** the 5-exo hydrogen appears as a doublet at higher field than 5-endo H, which shows a more complicated splitting pattern due to coupling to the olefinic hydrogens as well as 5-exo H. For compounds **6** one also expects[16] the vicinal coupling between 5-endo H and H-6 to be quite large and that between 5-exo H and H-6 to be small. Coupling of 5-exo H to the olefinic hydrogens should be small because of its nearly perpendicular orientation. For example, Figure 1 shows that for **6** (R = C_6H_5), *J* (5-exo H, 5-endo H) = 15 Hz, *J* (5-endo H, H-6) = 11 Hz, and *J* (5-endo H, H-4) = 4 Hz. Only the 15-Hz coupling could be resolved with 5-exo H.

Much to our surprise the preparation of **6** (R = C_6H_5) using $NaBD_4$ gave endo addition of deuteride (**6D**). Figure 1 shows that the 5-endo H is absent in the deuteride; the same result was obtained with **6D** (R = CH_3). The coupling constant arguments given above make it highly unlikely that the NMR assignments of 5-exo H and 5-endo H are incorrect. In particular the collapse of the 11-Hz coupling in the H-6 resonance in the deuteride is strong evidence that the addition was endo. $^2H\{^1H\}$ spectra of **6D** (R = CH_3, C_6H_5) confirmed that the selectivity was 100%, with no exo product being formed. Since at equilibrium the distribution of exo and endo deuteride should be near 1:1, it follows that the observed results refer to the kinetic product. Endo addition of nucleophiles to coordinated cyclic π hydrocarbons is very unusual. There are reported examples of initial exo products that can transform to endo species upon standing,[17,18] and some complexes of Pt(II) and Pd(II) are known to undergo endo addition via an initial ligand displacement step followed by migration to the ring.[19-21] Borodeuteride addition to (tropylium)$Mo(CO)_3^+$ is reported to yield a mixture of exo and endo products, with the exo predominating.[22,23] To our knowledge, the reaction reported herein is the first example of exclusive endo addition as the kinetic product. Experiments are underway to search for the obvious possible intermediates in this reaction, i.e., a species containing a M–H or M–CHO bond.

The phosphine nucleophiles reported in this study are believed to add exo as shown in **4**. Proof of this must await X-ray structure determination but is strongly indicated by the similarities in the NMR spectra to other coordinated cyclohexadiene phosphonium complexes.[15,24] The complexes **4** generally gave somewhat broadened NMR resonances due to trace paramagnetic impurities, but the complex **4** (R = CH_3; $PR_3' = PPh_3$) showed a well-resolved spectrum (Table II) with H-5 split into a triplet. This triplet probably arises from coupling of H-5 to phosphorus (*J* = 12 Hz) and to H-6 (*J* ≈ 12 Hz). Thus, the reasoning given above to show that deuteride adds endo suggests that PPh_3 adds exo, as expected.

In conclusion, this communication shows that double nucleophile addition to coordinated arene is facile provided the hydrocarbon

ring is reactivated by substitution of CO by NO^+ after the first nucleophile addition. The second addition is regioselective and stereoselective, with borodeuteride forming the endo product exclusively while other nucleophiles form exo products. Preliminary results show that simple carbanions such as $CH(CO_2Me)_2^-$ readily add to **3**, suggesting that the procedures reported herein may find usefulness in organic synthesis. X-ray structural determinations of **2**, **3**, and **4** are in progress.[25]

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Equilibrium and Kinetic CO Binding Parameters of T- and R-State Hemoglobin Chains Using Iron–Manganese Hybrids

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An understanding of hemoglobin (Hb) cooperativity requires knowledge of the protein's ligand affinity and binding rates, both in its low affinity (T) and high affinity (R) forms.[1] However, only indirect determinations of T-state CO binding properties have been available to date,[2-4] and there are no measurements of CO binding to the individual chains within R-state Hb. We report here the first directly obtained values of the CO affinities and on and off rate constants for CO binding to the individual chains within T- and R-state HbA. Comparison of the T- and R-state parameters gives unambiguous values for the amount by which the T state lowers the affinity of a chain, as well as for the relative contributions of the on and off rates in achieving the affinity reduction. The results, along with our recent kinetic study,[5] provide a complete set of parameters with which those of model compounds can be compared.[6]

We have measured CO binding to the ferrous iron subunits within mixed-metal [Mn, Fe] hybrid hemoglobins.[7] T-state hemoglobin binding parameters are obtained through the use of [Mn^{II}, Fe^{II}] hybrids, whereas the R-state parameters are obtained from the [Mn^{III}, Fe^{II}] hybrids. In both valency states of the hybrids the two ferrous iron chains bind CO, but the manganese-containing chains do not. Structural[8] and functional[9-11]

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Table I. T-State Equilibrium CO Binding Parameters[a,b]

	$P_{1/2}$, mmHg	Hill plot slope (n)	dimer-corrected $P_{1/2}$, mmHg[c]
β-Fe(II)	0.71	1.00	0.99
β-Fe(II) + IHP[d]	3.11	0.92	
α-Fe(II)	0.38	1.01	0.60
α-Fe(II) + IHP[d]	1.02	1.04	

[a] Obtained from [Mn^{II}, Fe^{II}] hybrids; conditions except as noted are 0.05 M Bistris·HCl, pH 6.6., $T = 25$ °C. [b] Estimated uncertainties: $P_{1/2}$, ±10%; n, ±0.05. [c] See ref 16. [d] 200 μM.

studies of fully substituted MnHb reveal that a Mn(II)-containing subunit is equivalent to an unliganded Fe(II) subunit. The [Mn^{II}, Fe^{II}] hybrids are therefore analogues of an unliganded T-state hemoglobin that can only bind two CO molecules, and thus CO binding to the ferrous chains is representative of the first two ligation steps in Hb. In contrast, a Mn(III)-containing subunit is similar in structure[8] and function[9,10] to a liganded subunit of met-Hb. Thus, the [Mn^{III}, Fe^{II}] hybrids are analogues of diliganded Hb intermediates, and CO binding to the ferrous chains is representative of the last two ligation steps of Hb.

The [Mn^{III}, Fe^{II}] hybrids were prepared by procedures similar to those of Yip et al.[12,13] Samples for equilibrium and kinetic measurements were 3–5 μM in heme in 0.05 M Bistris·HCl at pH 6.6. The Mn(II) form of the hybrids was prepared by the addition of dithionite to dearated sample. In addition, the (Mn^{II}, Fe^{II}) samples contained β-mercaptoethanol and methylene blue at concentrations of 0.1% and 1 μM, respectively. So that the binding equilibrium could be measured, small volumes either of CO or 4.988% CO in N$_2$ (primary standard, Matheson) were added to the unliganded [Mn^{II}, Fe^{II}] sample within a tonometer; after each addition the sample was equilibrated with stirring in a 25 °C water bath until a constant optical spectrum was obtained. The fractional saturation of the sample with CO was determined spectrophotometrically at wavelengths in the Soret region. Sample integrity within an experiment was confirmed by the observation of isosbestic points.

Figure 1 presents Hill plots for equilibrium CO binding to the ferrous iron chains of the [Mn^{II}, Fe^{II}] hybrids, and Table I gives $P_{1/2}$ values and Hill coefficients. In the presence of inositol hexaphosphate (IHP) at pH 6.6, both hybrids bind CO non-cooperatively (Hill constant, $n = 1$) and with low affinity, the affinity of the α-Fe hybrid being about 3-fold higher than that of the β-Fe hybrid (Table I). In the absence of IHP, the binding of CO remains noncooperative but the affinity of the β-Fe hybrid increases by a factor of ~4.4, while that of the α-Fe hybrid increases by a factor of only 2.7 (Table I). Significantly, at elevated pH each hybrid binds CO cooperatively and with increased affinity (Bohr effect), which confirms both their functionality and utility as models for intermediate liganded states of Hb; a full description of the pH dependence of equilibrium and kinetic CO binding will be presented elsewhere.[14]

Our recent kinetic studies[5] showed that the [Mn^{II}, Fe^{II}] hybrids in the presence of IHP at pH 6.6 are T-state tetramers, independent of whether the two Fe subunits have CO bound or not. The low affinity and lack of cooperativity observed in the equilibrium measurements confirm this conclusion. Since the protein does not change state during the binding process, the observed binding constant is precisely K_T^{IHP}, the CO binding constant[15]

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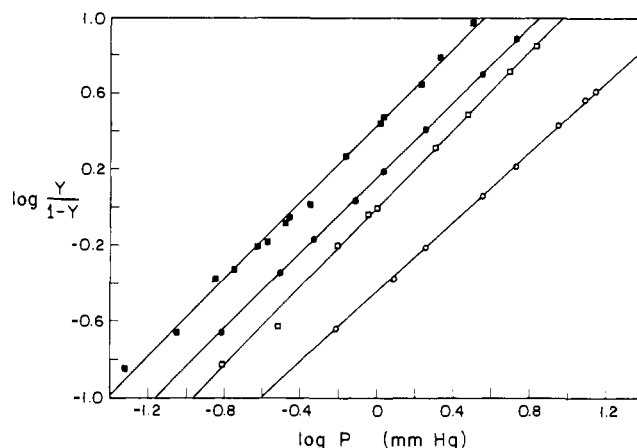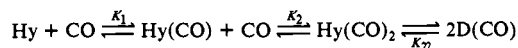
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Figure 1. Equilibrium CO binding to the ferrous chains within mixed-metal [Mn^{II}, Fe^{II}] hybrid hemoglobins: (ordinate) log $(Y/1 - Y)$, $Y =$ fraction of ligated heme; (abscissa) log (P_{CO}), with P_{CO} in mmHg. Conditions: 25 °C, pH 6.6, 0.05 M Bistris·HCl, solid symbols; in the presence of 200 μM IHP, open symbols; [α-Fe^{II}, β-Mn^{II}] ■, □; [α-Mn^{II}, β-Fe^{II}], ●, O. The lines represent least-squares fits to the data points. The slopes, n (the Hill constant), and intercepts, $P_{1/2}$, are listed in Table I.

for T-state Hb with IHP bound (Table II). The affinities of the IHP-bound T states are ~5 (α-Fe) to ~15 (β-Fe) times lower than those traditionally cited for T-state CO affinity (Table II). Since the CO binding reaction in this case appears to be simple ligand association, Table II lists CO off rates calculated from the affinity constants and the previously measured CO on rates,[5] which are also collected in Table II.

Flash photolysis experiments at pH 6.6 in the absence of IHP indicate that the unliganded and monoliganded [Mn^{II}, Fe^{II}] hybrids are completely in the T state but that the diliganded hybrids exist to a small extent as high-affinity R-state tetramers and dimers.[5] However, the present equilibrium measurements reveal that the extent of R-state tetramer and dimer formation is insufficient to produce measurably cooperative binding, namely a Hill coefficient of greater than unity. Moreover, the extent of dimer formation at the diliganded level is insufficient to appreciably increase the observed binding constant above that of the T state.[16] Thus, the directly observed $P_{1/2}$ listed in Table I should give a good approximation to K_T in the absence of IHP. Inclusion

(15) We have defined K_T and K_R as association constants.

(16) The binding of CO to the hybrids can be described by the following equilibria:

$$\text{Hy} + \text{CO} \xrightleftharpoons[]{K_1} \text{Hy(CO)} + \text{CO} \xrightleftharpoons[]{K_2} \text{Hy(CO)}_2 \xrightleftharpoons[K_{22}]{} 2\text{D(CO)}$$

where K_1 and K_2 are the stepwise CO association constants and K_{22} is the dimer–tetramer association constant of the diliganded species. The low affinity and lack of cooperativity require that within experimental error $K_1 = K_2 \simeq K_T$. The observed association constant cannot be precisely K_T, however, because of dimerization at the diliganded level. So that a dimer correction factor for the observed $P_{1/2}$ could be obtained, the above binding scheme was first solved for the fractional saturation (Y) as a function of CO concentration ([CO]) and total heme concentration [h$_0$].

$$\bar{Y} = \frac{4(1 + K_T[\text{CO}])^2 + 2aK_T[\text{CO}](1 + K_T[\text{CO}])}{2(1 + K_T[\text{CO}])^2(2 + a)}$$

$$a = \left[1 + \frac{8K_{22}[\text{h}_0](1 + K_T[\text{CO}])^2}{(K_T[\text{CO}])^2}\right]^{1/2} - 1$$

Setting $\bar{Y} = 0.5$ generates the following cubic equation in $y = K_T[\text{CO}]_{1/2}$:

$$y^3 - \frac{K_{22}[\text{h}_0] + 1}{K_{22}[\text{h}_0]}y^2 - y + 1 = 0$$

The approximate value $K_{22} \approx 10^6$ M^{-1} was obtained from flash photolysis experiments. Since h$_0$ is known, the cubic equation can be solved for the proper root by the Newton–Raphson method of approximating roots of functions. Division of the observed $P_{1/2}$ by y gives K_T^{-1}, the $P_{1/2}$ value in the absence of dimerization (column 3, Table I).

Table II. CO Binding Parameters of T- and R-State Hemoglobin and Model Compounds

system	kinetic constants		equilibrium constant[a]		ref
	$1'$, $M^{-1} s^{-1}$	1, s^{-1}	$P_{1/2}$, torr	K, M^{-1}	
Hb T state					
β-Fe[b,c]	1.3×10^5	0.16	0.99	8.0×10^5	this work, 5
β-Fe + IHP[b,c]	5.0×10^4	0.19	3.11	2.6×10^5	this work, 5
α-Fe	1.5×10^5	0.11	0.60	1.3×10^6	this work, 5
α-Fe + IHP[b,c]	1.1×10^5	0.14	1.02	7.8×10^5	this work, 5
Hb[d]	3.4×10^5	0.09	0.10–0.28	2.8–8.1×10^6	2–4
Hb R state					
β-Fe (Hb)[c,e]	6.5×10^6	0.009	0.0011	7.2×10^8	this work
β-Fe (isolated chains)[f]	5.5×10^6	0.011	0.0016	5.0×10^8	19
α-Fe (Hb)[c,e]	6.0×10^6	0.018	0.0024	3.3×10^8	this work
α-Fe (isolated chains)[f]	3.4×10^6	0.017	0.0040	2.0×10^8	19
Hb[g]	6.0×10^6	0.007–0.009 (0.011)[h]	8–14×10^{-4}	5.4–10×10^8	2, 20
Model Compounds					
T state					
mesoheme dimethylester[i] 2-MeIm, CTAB	4.8×10^5	0.12		4×10^6	6b
R state					
mesoheme dimethylester[i] 1-MeIm, CTAB	5.8×10^6	0.008		7×10^8	6b
chelated protoheme[j]	3.6×10^6	0.009		4×10^8	6a

[a] For convenience, the equilibrium constants are given both as a dissociation constant ($P_{1/2}$ (torr)) and as a binding constant (K (M^{-1})). [b] T-state constants were obtained from [Mn^{II}, Fe^{II}] hybrids. CO off rates for the T state were calculated from the measured T-state equilibrium constants and on-rate constants. T-state equilibrium constants are based on a CO solubility of 956 μM/atm at 25 °C. Estimated uncertainties: equilibrium constants, ±15%; on rates, ±0.2 × 10^5 M^{-1} s^{-1}; off-rates, ±0.02 s^{-1}. [c] Conditions except as noted: 0.05 M Bistris·HCl, pH 6.6, T = 25 °C. IHP when present was at 200 μM. [d] On rate, ref 3, 0.05 M Bistris, pH 6.0, 22 °C. Off rate, ref 2, 0.1 M phosphate, pH 7.0, 20 °C. Binding constants are estimated in ref 2 and 4. [e] R-state constants were obtained from the [Mn^{III}, Fe^{II}] hybrids. R-state equilibrium constants were calculated from the measured on- and off-rate constants. Estimated uncertainties: equilibrium constants, ±10%; on-rate constants, ±0.5 × 10^6 M^{-1} s^{-1}; off-rate constants, ±0.001 s^{-1}. [f] 0.1 M phosphate buffer, pH 7.0, 25 °C. [g] On rate, ref 20, 0.1 M Bistris, pH 6.6, 20 °C. Off rates, ref 2 and 20, 0.1 M Bistris, pH 6.6, 20 °C, and 0.1 M phosphate, pH 7.0, 20 °C. [h] This work, under conditions described in c. [i] 2.0% cetyltrimethylammonium bromide in 0.1 M, pH 9.5, phosphate buffer, 25 °C. [j] Suspended in 2% myristyltrimethylammonium bromide–phosphate buffer, pH 7.3, 20 °C.

of the tetramer–dimer equilibrium complicates the binding scheme but as may be seen in Table I, gives only minor corrections to the observed $P_{1/2}$.[16] The values listed in Tables I and II, both corrected and not, are lower than previous estimates obtained through indirect measurements,[2–4a] namely ~3-fold lower for α-Fe and ~5 fold lower for β-Fe. In addition, Table II lists the CO off rates for the T state in the absence of IHP. These were calculated from the dimer-corrected equilibrium constants and the previously measured on rates,[5] which are also collected in Table II.

R-state CO on rates were obtained from the [Mn^{III}, $Fe^{II}CO$] hybrids by using flash photolysis equipment previously described.[17] We used low levels of photolysis, which removes at most a single ligand to form the monoliganded hybrid. Since both the mono- and diliganded Mn(III) hybrids are totally R state,[14] the observed rapid, monophasic CO recombination is therefore representative of the R-state on rate. R-state CO off rates were determined in a standard fashion,[18] by using a Beckman Acta III spectrophotometer to follow the replacement of CO by NO. Table II gives the on and off rates for the individual chains within R-state hemoglobin as well as the R-state binding constants calculated from the rate ratios. The on rates for the two types of chains are indistinguishable, but the off rate for α chains is 2-fold higher and thus the calculated α affinity is 2-fold lower. These values are the first for individual chains within the R state. They are quite consistent with reported values for the average of the α and β chain constants obtained for R-state hemoglobin in this or previous studies and differ only slightly from values measured for isolated chains[19,20] (Table II).

Examination of binding parameters for the individual chains in the T and R states reveals the influence of quaternary structure on heme affinities and also shows that the individual chains within hemoglobin do not respond identically to this influence (Table II). Without IHP present, the affinity of the β chains is 900-fold lower in the T state than in the R, but the affinity of the α chains is only 250-fold lower. In both chains, the affinity decrease results primarily from a 40–50-fold decrease in the T-state on rate. In addition, an increase in the β-chain off rate contributes a factor of 18; the α-chain off rate contributes only a factor of 6. In the presence of IHP, the affinity and on rate of the T-state β chain is further reduced by a factor of ca. 3. Thus, although IHP primarily influences the T–R quaternary structure equilibrium, it also has a significant effect on the heme affinity of the β subunit within the T state. The T-state α chain affinity changes less than 2-fold when IHP is added.

These results comprise the first self-consistent set of CO binding parameters for individual chains within the two major quaternary states of hemoglobin. As such, they must form part of the underpinning to discussions of the mechanisms by which protein structure influences prosthetic group reactivity. For example, the CO affinities of unconstrained ferroporphyrins are very similar to those of the R-state chains (Table II). However, models for the T state are not as satisfactory. The best available T-state model employs 2-methylimidazole as the proximal base to provide tension to the imidazole–iron bond upon ligation.[1d,6a,b] This proximal strain decreases the CO affinity of the model compound by factors of 100–200, with the on and off rates contributing equally to the affinity reduction (Table II). However, the protein environment in the IHP-bound T state (β-Fe) reduces the heme

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affinity by ca. 3 × 10³, and the additional decrease in affinity of roughly 15-fold arises almost entirely from a reduction in the on-rates. Since proximal strain affects on and off rates equally, this suggests that other or additional mechanisms must be invoked. For example, steric hindrance on the heme distal side lowers the CO on-rate without affecting the off-rate.[6a,21] The lowered affinity of the T-state model more closely approaches that of the T state in the absence of IHP, but even in this case, the protein affinity is moderately lower (~4-fold) and the difference results primarily from a reduction in on rates. The level of success of the 2-MeIm-heme model for the T state may reflect a contribution of proximal strain or may be accidental, with the effects of this strain merely mimicking one or more of other postulated mechanisms.[6,7,21,22] In any case, a heme model does not yet exist that entirely reproduces the plasticity of hemoprotein ligation.

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Oscillating Chemiluminescence from the Reduction of Bromate by Malonic Acid Catalyzed by Tris(2,2'-bipyridine)ruthenium(II)

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We report the observation of oscillating chemiluminescence during the Belousov–Zhabotinskii (BZ) reaction[1,2] (reduction of bromate by malonic acid) when tris(2,2'-bipyridine)ruthenium(II), $Ru(bpy)_3^{2+}$, is used as a catalyst. The oscillating chemiluminescence, which is due to the reduction of $Ru(bpy)_3^{3+}$ by some strongly reducing radical with formation of the emitting excited state of $Ru(bpy)_3^{2+}$, shows a very peculiar time behavior. To our knowledge this is the first example of oscillating chemiluminescence in solution.

It is well established that certain types of chemical reactions, subject to appropriate conditions, organize themselves spontanesously to give rise to regular spatial patterns or to periodic rate fluctuations.[1–6] The best studied among the oscillating homogeneous processes is the "classical"[7] BZ reaction, in which a crucial role is played by an ox/red catalyst.[1–14] The usual catalyst of the BZ reaction is the Ce^{4+}/Ce^{3+} couple, but the Mn^{3+}/Mn^{2+} couple[6] and polypyridine complexes of Fe,[8,12] Ru,[8,15] Ag,[16] Os,[17]

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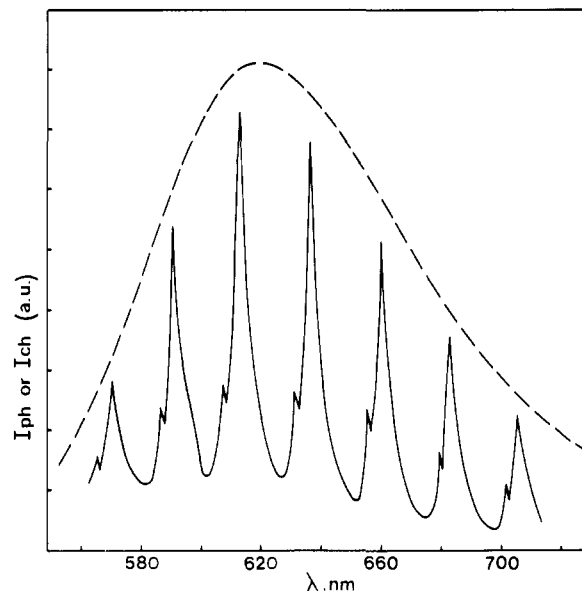(12) Smoes, M. L. *J. Chem. Phys.* **1979**, *71*, 4669.
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Figure 1. Oscillating chemiluminescence emission spectrum of the $Ru(bpy)_3^{2+}$-catalyzed BZ reaction (—) compared with the photoluminescence spectrum of $Ru(bpy)_3^{2+}$ (---) recorded by an MPF-3 Perkin-Elmer fluorimeter. Experimental conditions for chemiluminescence emission: $KBrO_3$, 0.06 M; malonic acid, 0.25 M; H_2SO_4, 1.5 M; $Ru(bpy)_3^{2+}$, 1.0 × 10⁻⁴ M; slit, 40 nm; sensitivity, ×30.

and Cr^{17} have also been used. The stoichiometry of the overall process is thought to be that of eq 1. The mechanism involves

$$2BrO_3^- + 3CH_2(COOH)_2 + 2H^+ \xrightarrow{ox/red}$$
$$2BrCH(COOH)_2 + 3CO_2 + 4H_2O \quad (1)$$
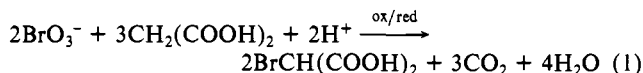
oxidation of the reduced form of the catalyst (red) by bromate and reduction of the oxidized form of the catalyst (ox) by malonic acid. Each of these mechanistic stages is complicated and involves many steps, some of which could be different when different catalysts are used.[7,8,12] With $Ru(bpy)_3^{2+}$ as a catalyst, oscillations had been previously observed in the rate of heat evolution,[8] in the rate of accumulation of monobromomalonic acid,[8] and in the UV-induced $Ru(bpy)_3^{2+}$ photoluminescence.[15]

In the last few years $Ru(bpy)_3^{2+}$ and other transition-metal complexes of the polypyridine family have been the object of much interest because of their peculiar photochemical and photophysical properties.[18–21] In particular, these complexes have been extensively used as light-absorption sensitizers[19–22] in artificial systems for the conversion of solar energy into chemical and/or electrical energy and as light-emission sensitizers[23–27] in artificial systems for the conversion of chemical and/or electrical energy into light.[28] The standard reduction potential of the $Ru(bpy)_3^{3+}/Ru(bpy)_3^{2+}$ couple in aqueous solution is +1.26 V vs. NHE, and the luminescent excited state of $Ru(bpy)_3^{2+}$, *$Ru(bpy)_3^{2+}$, is known to lie 2.12 eV above the ground state.[19–21] Thus, when $Ru(bpy)_3^{3+}$ is reduced by a reductant R^- having $E^0(R/R^-)$ <−0.86 V, either the ground state (eq. 2) or the excited state (eq

$$Ru(bpy)_3^{3+} + R^- \rightarrow Ru(bpy)_3^{2+} + R \quad (2)$$

$$Ru(bpy)_3^{3+} + R^- \rightarrow {}^*Ru(bpy)_3^{2+} + R \quad (3)$$
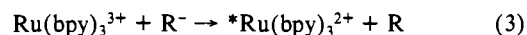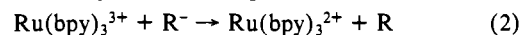
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