Thermodynamics of some long-chain biradicals studied by EPR spectroscopy

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Abstract. Four biradicals of differing chain lengths have been prepared by spin-labelling the sulphhydryl groups of dithiothreitol. Temperature-dependent EPR spectra of these biradicals indicate that the dynamics of biradicals is associated with both fast and slow exchanges of conformations. The three-conformational model incorporating cage configuration is found to be more appropriate. Certain thermodynamic parameters of conformational changes have been calculated from the experimental EPR parameters, obtained by the use of simulation procedures developed recently.

Keywords. Biradicals; EPR spectroscopy; simulation; thermodynamic parameters; conformational changes; fast and slow exchanges.

1. Introduction

In the chemistry of large molecules the intramolecular collisions of their functional groups are very significant. Macromolecules such as proteins and enzymes adopt various conformations in solutions depending upon the electrostatic environment and temperature. Conformational motion very often results in change of the distances between functional groups and also of their relative orientations. The rate of intramolecular collisions is also modulated resulting in the functional changes of the macromolecules. Spin exchange in biradicals can be used to study the dynamics of these intramolecular collisions because exchange interaction in a biradical depends on its conformation and transitions between different conformations. The dynamics of spins in a biradical thus typifies the dynamics of functional groups that collide in solution.

Electron paramagnetic resonance of biradicals has always been a complex problem due to the difficulty in understanding the line shape and linewidth. Depending upon the total number of conformations in a biradical, the magnitude of the exchange integral in each conformation and the rate of transition among various conformations, there are several extreme situations of spin dynamics in biradicals (Luckhurst 1966; Calvin *et al* 1969; Ferruti *et al* 1969; Luckhurst and Pedulli 1970; Metzner *et al* 1974, 1977; Parmon and Zhidomirov 1974, 1976; Parmon *et al* 1975, 1976). According to Parmon *et al* (1977), if the transitions between conformations occur less frequently

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OTT   Spin label   Biradical
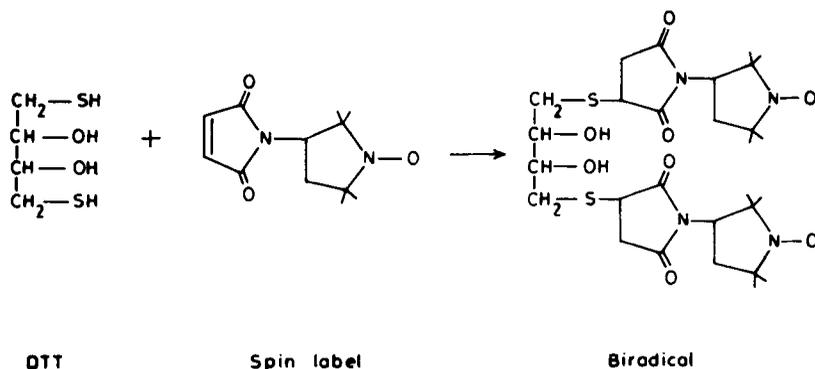
Scheme 1.

(slow-exchange phenomenon), then the overall EPR spectrum is the simple superposition of spectra of the two conformations. The superposition has conformations according to their relative concentrations. The widths of all the components of the spectrum for slow motion are practically independent of the frequency of exchange. But in the case of fast exchange, rapid intramolecular motion leads to the averaging of the EPR spectra of the two conformations resulting in the EPR spectrum of one *effective* conformation, with an average exchange integral. Fast exchange is associated not only with averaging of the exchange integral but also with another important phenomenon called *alternated broadening* of hyperfine splitting components of the biradical (Parmon et al 1977; Molin et al 1980).

In this paper we discuss EPR spectra of a series of long-chain biradicals (figure 1) in which the modulation of exchange interaction is shown to broaden the different hyperfine components differently. When the temperature is decreased, as can be seen from figure 2, the *biradical lines* (i.e. lines 2 and 2') broaden and also their relative intensities decrease till they disappear at the lowest temperature. Parmon and his coworkers (Parmon and Zhidomirov 1974; Parmon et al 1975, 1977) have studied this type of EPR behaviour both theoretically and experimentally. They have examined a multiconformational model for a hypothetical biradical and also developed adequate theory in calculating the EPR spectra using such a model. We have also developed a method based on their model but with a fast computational approach (Sankarapandi et al 1993). The simulation of EPR spectra of dynamic biradicals however still has some limitation because one does not know beforehand whether the spectrum is the superposition of the spectra of different conformations or whether it represents a picture averaged by rapid intramolecular motion. Moreover, simple inspection is not sufficient to determine the number of conformations the biradical possesses and hence the theoretical calculation of EPR spectra is difficult. Parmon et al (1977) put forward a semiquantitative analysis of the temperature-dependent EPR spectra under a three-conformational model which very successfully allows one to extract thermodynamical parameters for the dynamic process. We have adapted the same approach here to calculate the thermodynamic parameters for the conformational changes in the above biradicals.

According to this three-conformational model, the EPR spectra of long-chain biradicals represent the superposition of the spectra of the non-reacting radical moieties in the elongated or stretched-out conformation and that of a cage, where
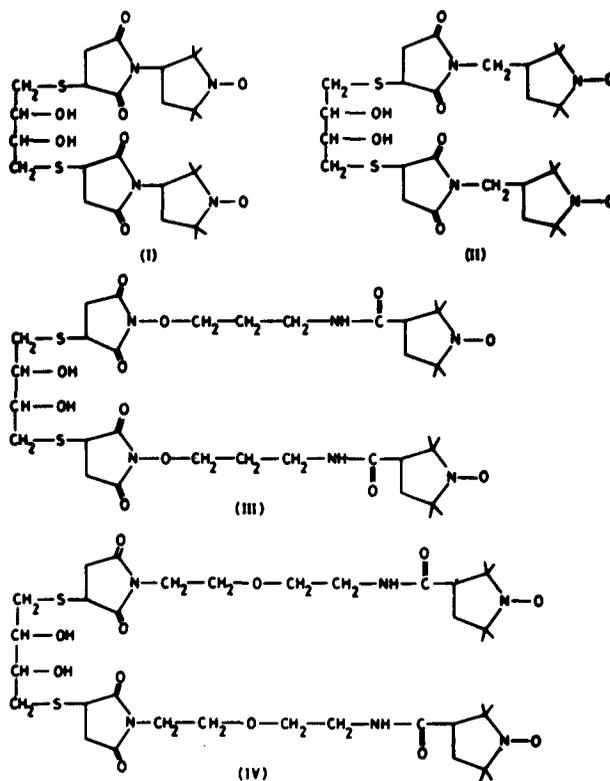
Figure 1. Structures of the four biradicals.

the radical moieties are close together and can interact with each other. Inside the cage a rapid intramolecular motion is observed between many conformations though they are characterized by two conformations as far as the strength of exchange interaction is concerned. Thermodynamic parameters for the cage as well as for the motion inside the cage are calculated from the experimental EPR spectra based on the work done by Parmon *et al* (1975).

2. Experimental

2.1 *Preparation of biradicals*

Four biradicals I-IV (figure 1) were prepared by spin-labelling dithiothreitol (DTT) using nitroxide spin labels with reactive maleimido groups. 1 mM of DTT (BRL, USA) and slightly more than 2 mM of the spin label (Sigma Chem. Co., USA) were dissolved in 10 ml of 5 mM of Na_2HPO_4/NaH_2PO_4 ($pH = 8.0$) buffer. The mixture was stirred gently for about 24 hours at 4°C. The mixture contained the biradical with small amounts of monolabelled DTT and the free reactants. The biradical and the impurities differ in molecular weight only by a few hundreds and all of them have identical solubility characteristics. So we purified the biradical by gel-permeation on Sephadex G-10 (Pharmacia), which can incorporate molecules of molecular
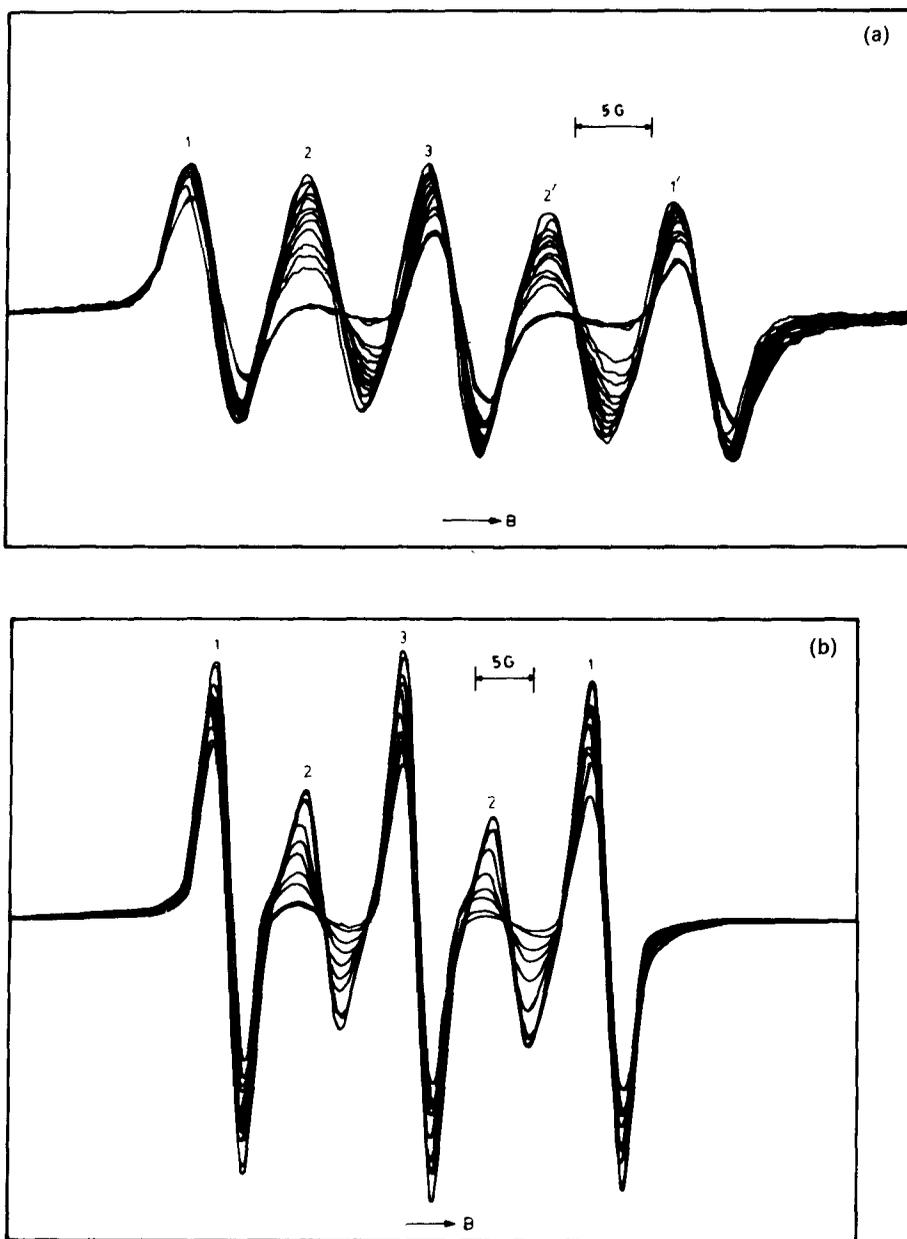
Figure 2.   (a and b)

weight below 650. A column of 1·5 cm diameter and 20 cm length was used for purification and fractions (each 15 drops) were collected by using a Gilson fraction collector. Each fraction was tested by EPR and the fractions containing the pure biradicals were pooled for complete EPR measurements. The concentration of the biradicals was $\approx 50\,\mu M$.
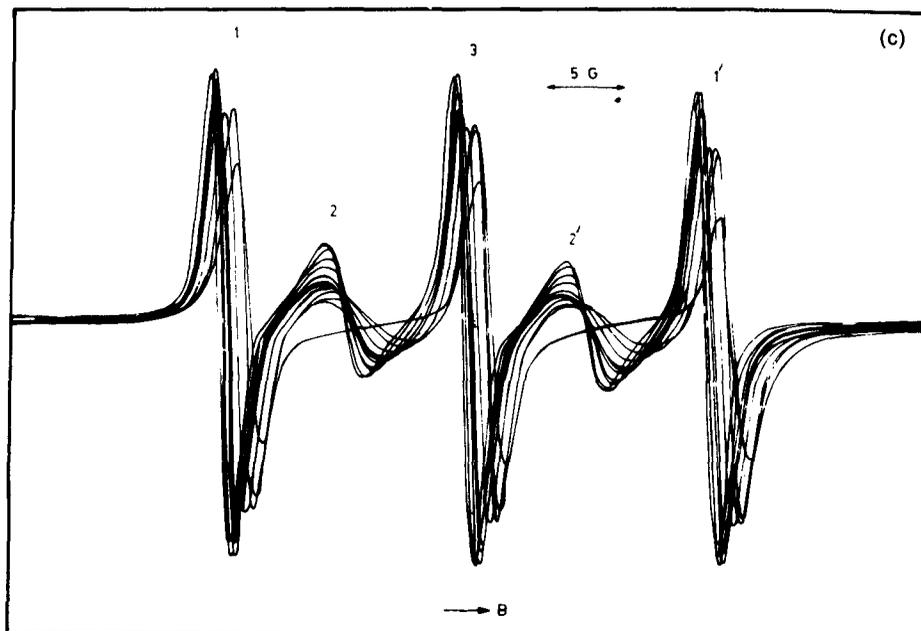
Figure 2. Temperature dependent EPR spectra of biradical I (a), and biradical II (b) in the range 28–75°C, and of biradical III in the range 36–70°C (c). The apparent shift in the spectra in (b) is due to the change in the tuning conditions.

2.2 *EPR measurements*

The EPR spectra were measured on a Varian E-112 spectrometer working at 9·5 GHz with 100 kHz modulation. The samples were taken in flat quartz aqueous cells for EPR measurements. Temperature-dependent measurements were made using a Varian E-257 variable temperature accessory. Temperatures were calibrated using a copper–constantan thermocouple and controlled to an accuracy of ± 1°C at the sample.

3. **Results and discussion**

3.1 *EPR spectra and the three-conformational model*

Typical changes in the EPR spectra of biradicals I-III at different temperatures are shown in figures 2 (a)–(c). Such a spectrum for biradical IV has already been discussed in our earlier work (Sankarapandi *et al* 1993). As the temperature increases, in all the biradicals the line widths of components 2 (or 2') decrease, whereas their relative intensities compared to lines 1 (or 1') increase. This fact leads to the inference that
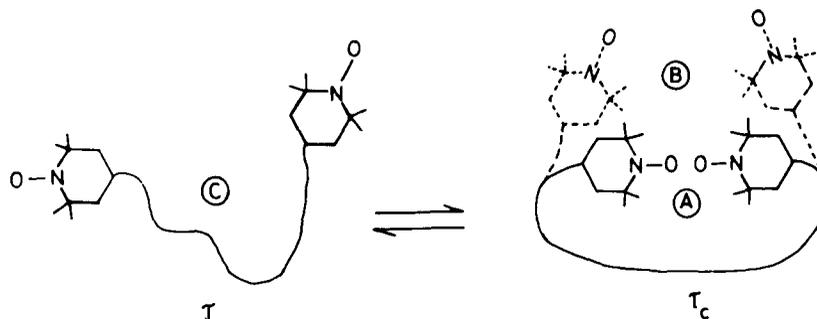
Figure 3. Three-conformational model: Two conformations (A) with $J_A \gg$ 'a', and (B) with $J_B \ll$ 'a' are in the state of fast exchange with each other while the third conformation (C) with $J_c \ll$ 'a' exchanges with the former two conformations rather slowly. (Redrawn from Parmon *et al* 1975.)

the features, which are typical both for the spectra with fast exchange and for those with slow exchange, are observed simultaneously in the EPR of the biradicals under consideration. This type of phenomenon was earlier observed by Parmon *et al* (1975) and they explained the EPR spectral behaviour by considering a model with more than two conformations, incorporating both slow and fast exchange. In other words, the ·EPR spectra can be considered as the superposition of two EPR spectra from *two states* of a biradical. The spectrum corresponding to one state is typical of slow exchange with the usual triplet pattern, whereas the one fitting the second state is a five-line spectrum with alternating line widths that characterizes fast exchange. Two conformations, (A) with $J_A \gg$ 'a' and (B) with $J_B \ll$ 'a', are in the state of fast exchange with each other while the third conformation (C) $J_C \ll$ 'a' exchanges with the former two conformations rather slowly (figure 3).

This model describes the state when the nitroxide fragments occur in the same *cage* of the solvent, but can either interact (the conformation A) or not (the conformation B) depending upon their mutual orientations. If the average lifetimes of the radical fragments inside the cage, τ_C, and outside the cage, τ, are sufficiently long (slow exchange), a superposition of the EPR spectrum of the cage and that of the separate radicals will be observed (with the statistical weights $\tau_C/(\tau + \tau_C)$ and $\tau/(\tau + \tau_C)$ respectively). If the movement of the radical fragments inside the cage is fast, the EPR spectrum of the cage will be described by the model of fast exchange (Parmon *et al* 1975).

In the framework of this three-conformational model the variation of the line widths of lines 2 and 2' with temperature can be attributed to the fast modulation conditions of the exchange interaction inside the cage, while the observed distribution of the line intensities can be explained as a result of the superposition of the EPR spectrum of the cage and that of the radicals outside the cage.

It has been established by earlier workers (Buchachencko and Vasserman 1973; Parmon *et al* 1975, 1976; Kokorin and Parmon 1978) that in the same solvents and at the same temperatures the widths of the lines 2 and 2' are the same for the biradicals of similar structures, though they have different chain lengths. This fact supports the conclusion that the widths of these lines are determined by the movement of the nitroxide fragments solely inside but not outside the cage. At the same time, the relative intensity of these lines decreases when the length of the chain increases in

agreement with the expected diminition of the ratio τ_C/τ and hence the statistical weightage of the cage conformations (Parmon *et al* 1975).

3.2 Calculation of thermodynamic parameters

The cage model allows one to obtain the thermodynamical parameters for the intramolecular processes in the long chain biradicals. This cage-type structure was first proposed by Rabinowitch (1937) to explain the kinetics of free-radical reactions in solution. According to him, in solution the surrounding solvent molecules form a *cage*, which holds the colliding spheres together and allows them to collide a number of times before they finally separate. In long-chain biradicals, the collisions of the radical fragments inside the solvent cage are caused by intramolecular rotations. The activation energy, ε, of such rotations depends strongly on the nature of the biradicals and on physical properties such as the viscosity and the polarity of the solvent. A typical value of time, τ_{eff}, between two collisions at room temperature is $\approx 10^{-9}$ s (Molin *et al* 1980). This is an example of a process that is often inaccessible to experimental methods other than magnetic resonance techniques. But EPR can be used to observe these rate processes by exploiting the variation in line width (Molin *et al* 1980).

It has been postulated for molecules in solution that the reorientation inside the cage is a thermally activated process with Arrhenius dependence between τ_{eff} and the temperature as,

$$\tau_{eff} = \tau_0 \exp(\varepsilon/R\,T), \tag{1}$$

where τ_0 is the pre-exponential factor which is a measure of the motions inside the cage. For the diffusion model of intramolecular collisions of paramagnetic fragments, τ_0, is of the order of 10^{-11} to 10^{-12} (Molin *et al* 1980).

The analysis of the narrowing of lines 2 and 2' with the temperature allows one to make conclusions about the motion of the radical fragments inside the cage. In the case of the fast modulation of the exchange interaction by the motions inside the cage, the exchange broadening $(1/T_2)_{ex}$ of the lines 2 and 2' is described by the expression (Rabinowitch *et al* 1937; Kaplan 1958; Johnson 1965; Parmon *et al* 1975; Parmon and Zhidomirov 1976)

$$(1/T_2)_{ex} = (1/4)a^2\tau_{eff}. \tag{2}$$

For the Lorentzian lines, the line width $1/T_2$ is given by

$$(1/T_2) = g\beta\,\Delta B_{1/2}/\hbar = (\sqrt{3}/2)(g\beta\,\Delta B_{pp}/\hbar). \tag{3}$$

where $\Delta B_{1/2}$ is full line width at half maximum for an absorption spectrum and ΔB_{pp} is peak to peak line width in the first derivative spectrum.

To find out the exchange broadening $(1/T_2)_{ex}$ of the lines 2 and 2', the difference in line widths of lines 2 and 1 can be used, since exchange interaction does not affect the line widths of lines 1 and 1'.

$$(1/T_2)_{ex} = (1/T_2^{(2')}) - (1/T_2^{(1')}). \tag{4}$$

From (2)–(4) one gets

$$\frac{1}{4}a^2\tau_{\text{eff}} = \frac{\sqrt{3}}{2}\frac{g\beta}{\hbar}\left[\Delta B_{pp}^{(2)} - \Delta B_{pp}^{(1)}\right],\tag{5}$$

or

$$a\tau_{\text{eff}} = \frac{2\sqrt{3}}{\hbar}\frac{g\beta}{a}\left[\Delta B_{pp}^{(2)} - \Delta B_{pp}^{(1)}\right].\tag{6}$$

Substituting for the constants (including $g = 2\cdot0036$), we get

$$a\tau_{\text{eff}} = 3\cdot46 \times \left[\Delta B_{pp}^{(2)} - \Delta B_{pp}^{(1)}\right]\Big/\Delta B_{\text{hf}}.\tag{7}$$

Here the hyperfine coupling constant is represented as ΔB_{hf} (in Gauss units) on the right hand side and as a (in rad s^{-1}) on the left hand side. ΔB_{pp} is in terms of Gauss.

By comparing (1) and (7), one can see that the plot of $\log(a\tau_{\text{eff}})$ against $1/T$ (Arrhenius plot) will be a straight line with slope, ε/R, and intercept $\log(a\tau_0)$.

Similar to the above calculation of thermodynamic parameters of the motion inside the cage, the thermodynamic parameters of the cage can be obtained from the ratio τ_c/τ (figure 3). Since the cage is described by the single effective conformation with the strong fast exchange, the biradical can be considered to exist in an equilibrium state of two conformations.

The equilibrium constant, K, can be expressed according to the Eyring equation,

$$K = \frac{k_1}{k_{-1}} = \exp\left[\frac{\Delta S}{R} - \frac{\Delta H}{RT}\right],\tag{8}$$

where k_1 and k_{-1} are the rate constants for the forward and the backward reactions respectively. For the present equilibrium $k_1 k_{-1} = \tau_c/\tau$ and hence

$$\tau_c/\tau = \exp\left[\frac{\Delta S}{R} - \frac{\Delta H}{RT}\right].\tag{9}$$

The ratio τ_c/τ can be easily calculated from the experimental EPR spectra by comparison with the integral intensities I_1 and I_2 of the lines 1, 1' and 2, 2' respectively.

If I is the total integrated intensity of the biradical system, the distribution of the line intensities of various lines among the two conformations can be calculated as follows.

Case (a) Outside the cage $(J = 0)$: The fraction of the intensity for the species outside the cage is given by $f^a(I)$, where $f^a(I) = \tau I/(\tau + \tau_c)$. This fraction is further distributed among the three lines in the ratio, 1:1:1, while the intensities of 1 (or 1') and 2 (or 2') are given by $I_1^a = \tau I/[3(\tau + \tau_c)]$ and $I_2^a = 0$. The superscript 'a' refers to case (a) and subscripts refer to the line number.

Case (b) Inside the cage $(J \gg a)$: The fraction of the intensity for the species inside the cage is given by $f^b(I) = \tau_c/(\tau + \tau_c)$. This fraction is further distributed among the five lines in the ratio, 1:2:3:2:1, while the intensities of lines 1 (or 1') and 2 (or 2') are given by $I_1^b = \tau I/[9(\tau + \tau_c)]$ and $I_2^b = 2\tau I_c/[9(\tau + \tau_c)]$. Hence the net observed intensities of line 1 (or 1') and line 2 (or 2'), I_1 and I_2, will be equal to

$(I_1^a + I_1^b)$ and $(I_2^a + I_2^b)$ respectively;

$$I_1 = (3\tau + \tau_c)I/[9(\tau + \tau_c)],$$ (10)

$$I_2 = 2\tau_c I/[9(\tau + \tau_c)].$$ (11)

From (10) and (11), one can get

$$\frac{\tau_c}{\tau} = \frac{3(I_2/I_1)}{2 - (I_2/I_1)}.$$ (12)

By comparing (9) and (12), one can see that the plot of $\log(\tau_c/\tau)$ vs $1/T$ (Arrhenius plot) will give a straight line of slope, $-\Delta H/R$, and intercept, $\Delta S/R$. The entropy, ΔS, and enthalpy, ΔH, of the cage can be obtained for the biradicals in solution.

The four biradicals under study are different only in the length of the chain. But the features of the EPR spectra, at a particular temperature, are different for them. The relative intensity of lines 2 and 2' (with respect to lines 1 and 1') decreases when the length of the chain increases in agreement with the expected diminution of the ratio $\tau_c\tau$. Also the widths of lines 2 and 2' are nearly the same for all the biradicals at a particular temperature. These factors are directly manifested in the nature of the EPR spectra of these four biradicals (figure 2). But when the temperature increases the relative intensity is found to increase whereas the width decreases. These results are in good agreement with the three-conformational model involving a cage structure. So, the thermodynamic parameters of the cage as well as thermodynamic parameters of the motion inside the cage are calculated for the four biradicals using the above equations.

In order to validate the assumption of the three-conformational model with different values of exchange energy, molecular mechanics calculations were performed for one of the four biradicals as an example. The IBM-compatible PC version of the molecular mechanics program MMX87 by Alinger (1977) was used. Many conformations of the biradical II were taken into consideration with different possible configurations around the two chiral carbon atoms of the DTT moiety. Out of those conformations, the ones with minimum MMX energy were identified and they are represented in figure 4. The energies and distances between the paramagnetic centres obtained from the molecular mechanics minimization are tabulated in table 1. Generally these calculations will be complete only when the solvent molecules are taken into account. But one can always compare the distances because energetically disfavoured conformations of the biradical molecule are prohibited here. It is clear from table 1 that the distances obtained for these conformations are in accordance

Table 1. Molecular mechanics calculations of biradical II.

Conformation	MMX energy (kcal mol^{-1})	d_{o-o} (Å)	d_{N-N} (Å)
A	104·05	6·9	9·1
B	98·73	13·6	12·9
C	99·89	14·1	14·0

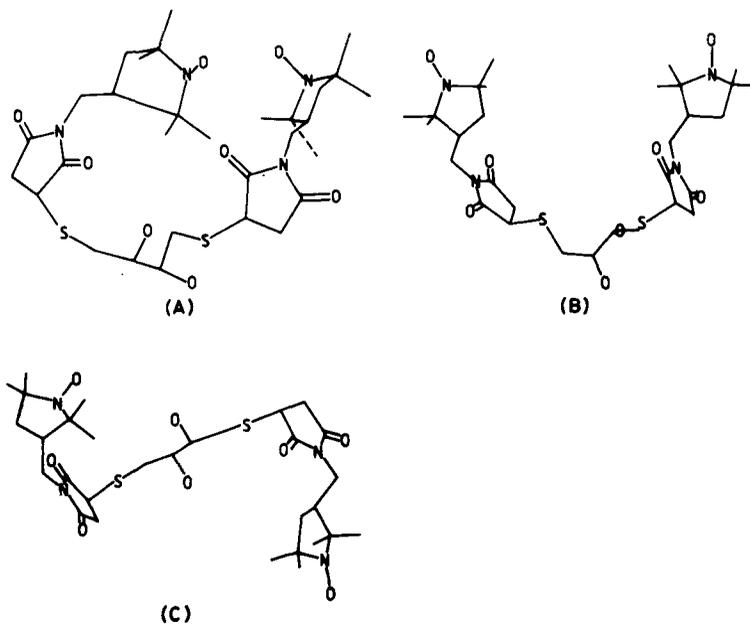**Figure 4.** Conformations of biradical II with minimum MMX energy as calculated from molecular mechanics.
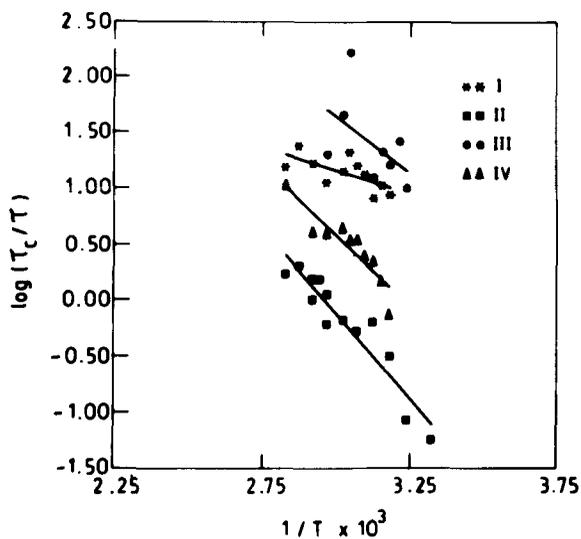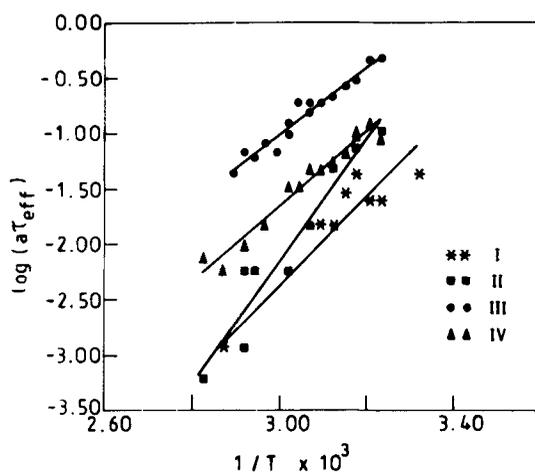

Figure 5. Arrhenius dependence of τ_c/τ.

with the strength of exchange interaction assumed for the three conformations A, B and C.

Figure 5 represents the plot of $\log(\tau_c/\tau)$ vs $1/T$. It can be seen from the plot that τ_c/τ increases slightly with temperature. This means that the relative energy of the elongated conformation is somewhat less than that of the 'collisional' one. The entropy

Table 2. Thermodynamic parameters of biradicals in water.

Biradical	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})	ε (kcal mol^{-1})	$-\log(\tau_0) \pm 1$ (τ_0 in s)
I	$1\cdot6 \pm 0\cdot2$	$7\cdot3 \pm 0\cdot8$	$7\cdot8 \pm 0\cdot5$	$14\cdot5$
II	$6\cdot0 \pm 0\cdot6$	$17\cdot8 \pm 1\cdot5$	$10\cdot8 \pm 1\cdot5$	$16\cdot1$
III	$4\cdot1 \pm 0\cdot3$	$15\cdot5 \pm 1\cdot3$	$5\cdot9 \pm 0\cdot4$	$12\cdot8$
IV	$5\cdot1 \pm 0\cdot4$	$16\cdot3 \pm 1\cdot3$	$6\cdot7 \pm 0\cdot4$	$13\cdot5$

Figure 6. Arrhenius dependence of $a\tau_{\text{eff}}$.

and the enthalpy of the cage are given in table 2. We can see a clear difference in ΔS for different biradicals due to the difference in their nature of solvation in water.

Figure 6 represents the Arrhenius plot of $\log(a\tau_{\text{eff}})$ vs $1/T$. When the temperature decreases, $\log(a\tau_{\text{eff}})$ increases, indicating that the motions inside the cage become slow leading to an increase in the effective correlation time inside the cage. Table 2 gives the values of the pre-exponential factor, τ_0, and activation energy, ε of the rotations inside the cage. It is worth mentioning here that the values obtained for τ_0 differ considerably enough for the various biradicals studied.

4. Conclusion

The three-conformational model proposed by Parmon and others is able to explain the EPR behaviour of long-chain biradicals and hence enables us to study quantitatively the dynamics of intramolecular collisions of molecular fragments separated by many chemical bonds. Such data seem to be important in understanding the mechanism of elementary chemical reactions of macromolecules. Thus, spin exchange between the specially introduced paramagnetic spin labels makes it possible to obtain

unique information on the kinetics and mechanism of intramolecular reactions in complex biological systems and in polymeric matrices in solution. The spin-exchange method may be used to study quantitatively the dependence of the rate of molecular collisions on such factors of importance for chemical kinetics as composition of solvents, spatial distribution of reagents etc. Interesting applications in the study of conformations of some cysteine tetrapeptides carried out in our laboratory will be published soon (Sankarapandi *et al* 1994).

Acknowledgements

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