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CO-ORDINATION BEHAVIOUR OF NUCLEIC ACID BASES AGAINST THE $Fe(I)(NO)_2$ GROUP IN SOLUTION. I. PURINES: ESR STUDY

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The binding sites of purine bases in the presence of the $Fe(I)(NO)_2$ group were investigated on the basis of the nuclear hyperfine structure of the electron spin resonance spectra. Selective isotopic substitution with ^{15}NO was used to clarify co-ordination. Coupling constants and different types of complexes were determined by means of computer-simulated ESR spectra.

Comparison was made with the binding sites of nucleotides and it was concluded that N-7, in the imidazole ring, is the preferred binding site. A structure with two base molecules bonded to the iron atom was proposed.

Finally 8-azaguanine, an antitumoral agent, was studied and the special behaviour of this antimetabolite was demonstrated in the biologically-interesting pH values.

1. Introduction

ESR spectroscopy provides the opportunity to get a deeper insight into the co-ordination of nucleobases in the presence of metal ions. This study is based upon the considerations that complexes participate in the regulation and control of metal uptake in biological systems and that conformational changes induced by binding of metal ions to DNA are attributed to interaction of purine sites with metal ions having d-electronic configuration [1,17]. Furthermore a great variety of purines exert an inhibitory effect on the growth of tumor cells or other chemotherapeutic action via chelation of metal ions [2,3].

The $Fe(I)(NO)_2$ group is a particularly suitable metal probe for such co-ordination studies as it presents relatively long electron spin relaxation times, allowing good resolution of the nuclear hyperfine structure [4,5]. Furthermore Fe–NO complexes are of noticeable importance in some biological processes [6,7]. Woolum and Commoner [8] revealed the presence of a paramagnetic complex in the liver of rats under carcinogenetic diet and the ESR analysis identified this compound as an iron-dinitrosyl-mercapto-protein complex.

The iron in the dinitrosyl complexes under study is in a d^7 low spin configuration ($S = \frac{1}{2}$), that is the same suggested for non-heme iron proteins [9]. Thus its formal oxidation state is +1.

The aim of our research is to analyze the Fe–NO complexes with both biological and non-biological nucleobases with the purpose of identifying the sites of co-ordination. Our attention is particularly focussed on the imidazole-nitrogens of purines, looking at the effects of substitution of various groups on the molecule rings.

To accomplish these objectives the following procedures were used:

(i) selective isotopic substitution with ^{15}N,

(ii) computer simulation of the ESR spectra in their derivative forms,

(iii) analysis of the equilibria between different types of complexes with changing pH.

2. Experimental

Alcoholic and hydro-alcoholic solutions of Fe(II) were saturated under a pure nitrogen stream, with NO prepared according to Blanchard's method [10] or via

reduction of $NaNO_2$ by ascorbic acid. The complexes were obtained adding the appropriate amount of nucleobase.

These paramagnetic systems were investigated by means of a Varian V-4502 X-band spectrometer operating with a 100 kc-field modulation. The g-factors were evaluated by comparison with an external reference standard (Fremy salt; $g = 2.0055$; $a_N = 13.0$ gauss). The coupling constants and the relative intensities were determined by comparison with the computer-simulated spectra giving suitable values to the linewidths ΔH associated with different values of given coupling constants. Pure lorentzian lineshapes were assumed. In the case of isotopic substitution the ratio between the nuclear g-factors was taken into account.

The nucleobases were obtained from Cyclo Chemicals, Los Angeles, and were used without further purification. $Na^{15}NO_2$ (99%) was supplied by ICN, Irvine, California.

3. Results and discussion

The purine bases of nucleic acids have two high-electron-density centers which are possible sites for metal ion chelation, that is C(6)–NH_2/C(6)–OH and N(7), or N(3) and N(9). Studies based on Calvin–Bjerrum potentiometric titration suggest [11] chelation in adenine via NH_2; more recent potentiometric results [12,13] assign chelate structures to complexes of adenine and other 6-substituted purines with divalent metal cations and suggest the participation of 2,6-NH_2-purine and 8-azaadenine via N(1). N(7) and/or C(6)–NH_2 positions appear to be the sites of binding in nucleotides according to a Mössbauer spectroscopy study on the Fe(III)-poly-(A)-system [14].

Infrared spectra of theophilina with methyl groups at the N(1) and N(3) positions lead to the hypothesis that the copper ion is bonded to N(7). Moreover N(7) is indicated as the preferred binding site in purine for both Cu(II) and Zn(II) [15,16] and in guanine for Mn(II) [17]. Eichhorn and coworkers affirm that in 3′ and 5′ nucleotides the binding of metal ion does occur at N(7) for adenine and guanine [18].

The problem is not yet unequivocally defined both in purines and nucleotides and very recent papers [19,20], based on ^{13}C and proton NMR data, lead to the conclusion that in adenine-nucleotides the metal ion holds near the N(7) base position or suggest an outer sphere complex involving a water molecule simultaneously coordinated by the metal ion and hydrogen bonded to N(7) of the adenine ring, as no direct evidence for metal-nitrogen bond or for inner sphere complex was reached.

On the other hand ESR nuclear hyperfine structures give direct evidence of the existence of the Fermi contact interaction, that is, of the nuclei bonded to the metal ion.

4. ESR spectra

Computer simulation of the ESR spectra was performed giving variable values to the coupling constants, linewidths, and relative intensities of the theoretical lines. Different types of complexes with one or more base molecules involved in the metal binding were considered. The interaction of the unpaired electron spin with non-chemically-equivalent nitrogen nuclei was taken into account. Namely the (1N+2N), (2N+2N) and (1N+1N+2N) types of spectra were simulated. Generally agreement is satisfactory with ^{15}N spectra presenting higher coupling constants; the imperfect fitting in the case of ^{14}N spectra could be attributed to the assumption of pure lorentzian lineshape for the simulated spectra. Furthermore, the presence of signals due to $Fe(NO)_2(H_2O)_2$ or to $Fe(NO)_2(OH)_2$ in some cases disturbs the shape of the ESR hyperfine structure [21].

Adenine gives rise to a nine-line spectrum, in the pH range 7–9.5. A comparison with the simulated spectra indicates a complex with two NO molecules and two equivalent base nitrogens bonded to the iron atom, that is, two adenine molecules involved in the metal coordination with the exclusion of the possibility of chelation. A septet is obtained using ^{15}NO, with apparent relative intensities 1:4:8:10:8:4:1; the agreement with the simulated spectrum is fairly good (fig. 1) assuming $a(^{15}NO) = 2.8$ gauss, $a_N = 2.7$ gauss and $\Delta H \approx 2.3$ gauss.

Furthermore, a chelate-type complex involving the -NH_2 group is ruled out on the basis of the experimental ESR spectra, which are identical to the adenine spectra, of 1-CH_3-adenine and 6-phenyl-NH-purine. A pure nine-line spectrum is also obtained with 6-CH_3-NH-purine.

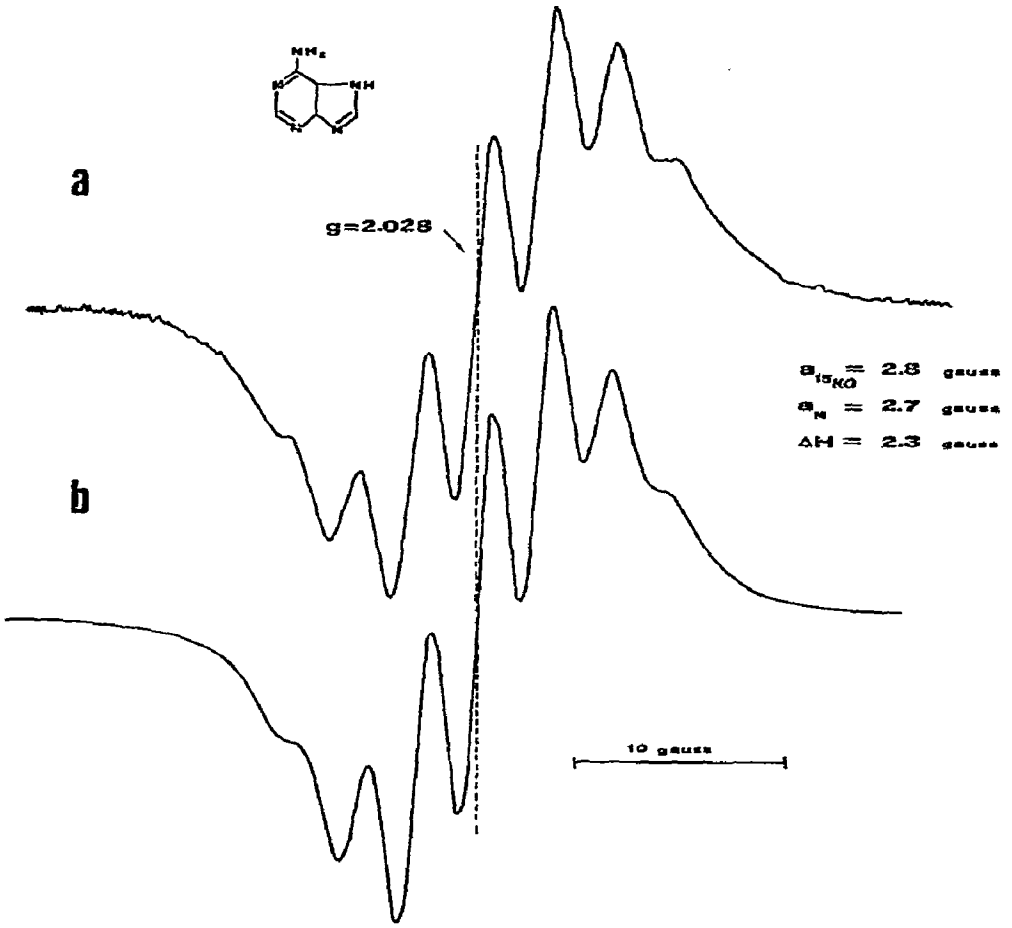


Fig. 1. (a) Experimental ESR spectrum of $Fe(^{15}NO)_2(adenine)_2$. (b) *Simulated* ESR spectrum.

The ESR spectrum of 2-NH_2-adenine is reported in fig. 2. This base, antagonist of adenine, was the first purine analogue used as an antitumoral agent [22]. Its antitumoral properties are presumably connected to the formation of adenine-coenzymes [3]. The presence of the NH_2 group in 2-position does not affect the ESR parameters, leading to the suggestion that the pyrimidine nitrogens are not involved in the binding. Similar behaviour results in the case of 2-isoguanine with the -OH group in 2-position.

At pH = 7, 3-CH_3-hypoxanthine is showing a nine-line ESR spectrum, which converts to a septet in the presence of ^{15}NO (fig. 3). These findings, together with the above-reported results on 1-CH_3-adenine, exclude the participation of N-1 and N-3 in the metal binding. Thus imidazole-nitrogens must be involved in the complex.

In order to confirm this hypothesis both experimental and simulated spectra were obtained with pure imidazole. As shown in fig. 4 the ESR pattern of the $Fe(^{15}NO)_2$-imidazole complex at pH = 7 is a septet of the type 1:4:8:10:8:4:1, and the simulation, based on the assumption of a coupling constant due to two NO molecules and of another coupling constant due to two imidazole molecules, fits the experimental results. Moreover the nine-line simulated spectrum fits the experimental one of the $Fe(^{14}NO)_2(imidazole)_2$ complex previously reported [23].

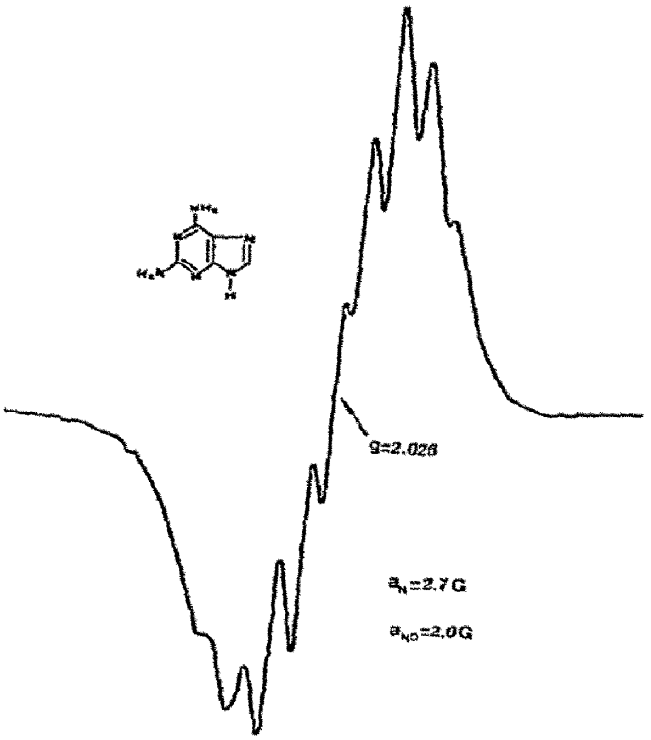
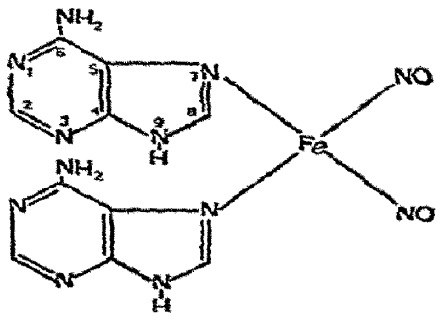


Fig. 2. Experimental ESR spectrum of $Fe(NO)_2(2\text{-}NH_2\text{-}adenine)_2$.

Thus both 9-line (^{14}NO) and 7-line (^{15}NO) spectra confirm the interaction of the metal unpaired spin with two base molecules via imidazole nitrogens, leading to the following structure for adenine and other purine bases:



Purine, guanine, xanthine, hypoxanthine, 6-Cl-purine and 6-I-purine give rise to ESR spectra identical

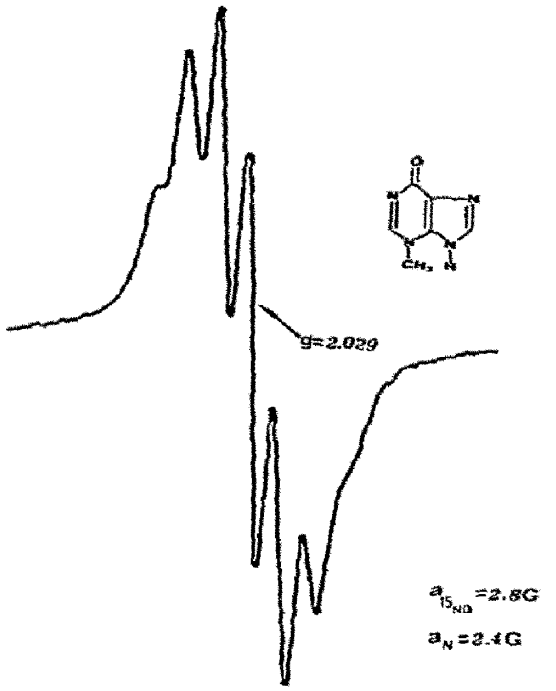


Fig. 3. Experimental ESR spectrum of $Fe(^{15}NO)_2(3\text{-}CH_3\text{-}hypoxanthine)_2$.

to those described above. The poor resolution obtained with 6-I-purine could be attributed to the steric hindrance of iodine.

In order to define whether nitrogen-7 or nitrogen-9 were involved in the complex formation, $7\text{-}CH_3$-adenine, $9\text{-}CH_3$-adenine, $7\text{-}CH_3$-hypoxanthine, $9\text{-}CH_3$-hypoxanthine and 9-cyclohexyl-adenine were studied. The ESR data display a series of spectra with 9 lines (^{14}NO) and 7 lines (^{15}NO) as shown in tables 1 and 2. We can conclude that the imidazole nitrogen coordinating the iron atom is always the one without the substituent group, that is, that close to the double bond.

In the pH range 7–9 the N-methyl-imidazole shows the same ESR patterns as imidazole does, further supporting the above conclusion.

Different behaviour is shown by $3\text{-}CH_3$-adenine and $3\text{-}CH_3$-xanthine. The experimental and simulated spectra, both in the presence of ^{14}NO and ^{15}NO, are reported in fig. 5. The ESR results can be explained in terms of a complex with only one base molecule and two NO groups, that is, a (1N+2N)-type spectrum.

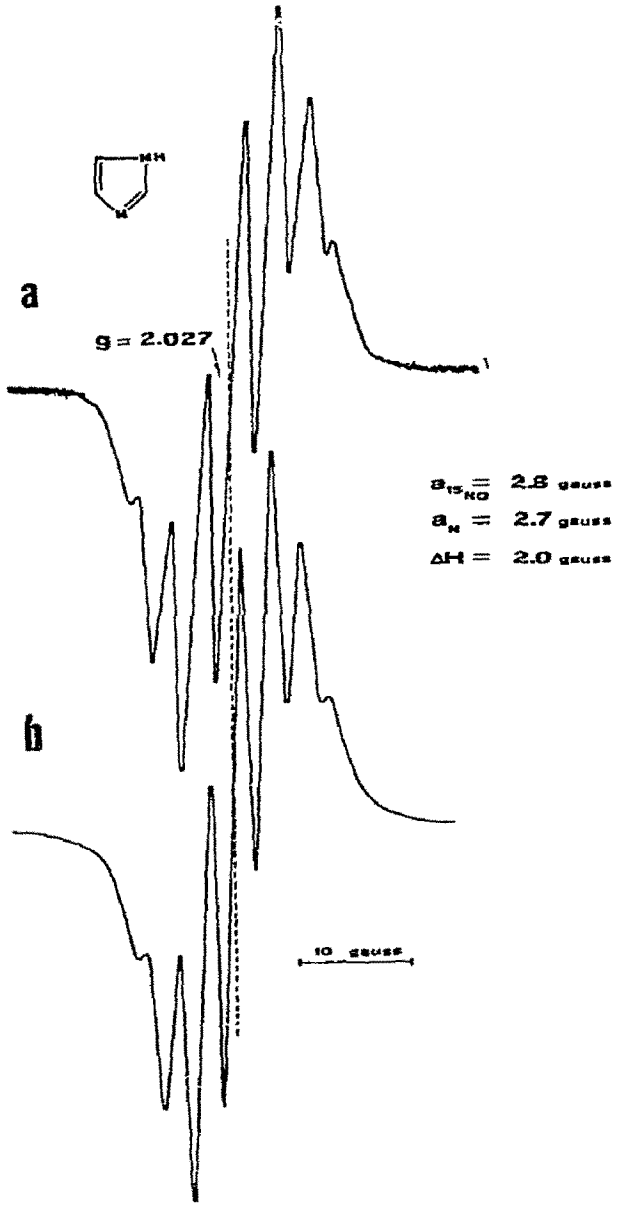


Fig. 4. (a) Experimental ESR spectrum of $Fe(^{15}NO)_2$(imidazole)$_2$. (b) Simulated ESR spectrum.

The 6-Cl-9-CH_3-purine, together with the usual sequence of spectra at pH 7–9, presents at pH = 5.5 a single line due to $[Fe(NO)_2(H_2O)_2]^+$ at $g = 2.032$ and a quintet at $g = 2.022$ (triplet with ^{15}NO) shown in fig. 6. The same characteristic features are present in

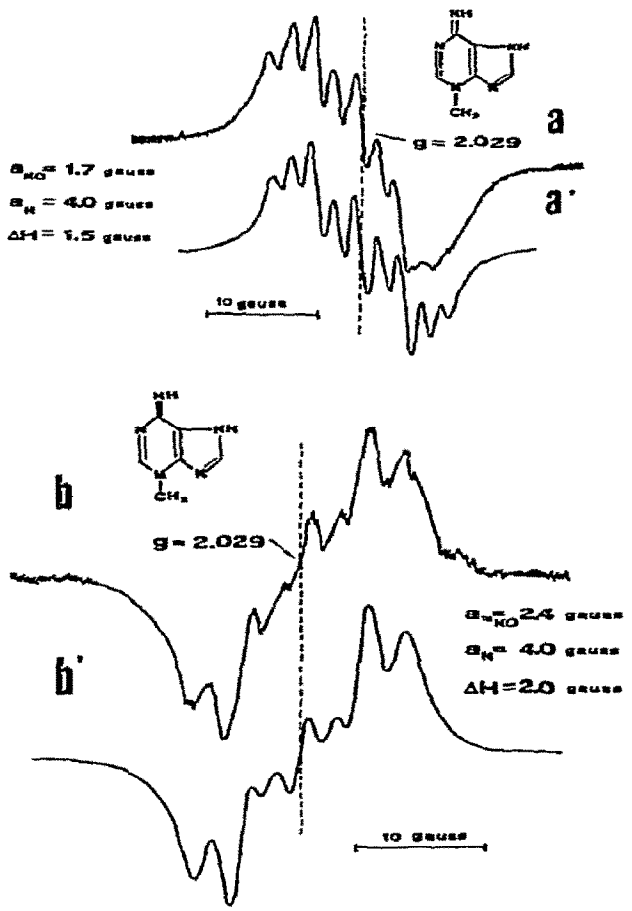


Fig. 5. (a) Experimental ESR spectrum of $Fe(NO)_2$(3-CH_3-adenine). (a') Simulated ESR spectrum. (b) Experimental ESR spectrum of $Fe(^{15}NO)_2$(3-CH_3-adenine). (b') Simulated ESR spectrum.

the case of 6-CH_3 and 7-CH_3 adenines (fig. 7). In the above cases it is possible to affirm that a complex is formed with the base also at acid pH, although on the basis of the nuclear hyperfine structure only the two NO groups are evidential and the binding site remains unknown.

The 8-azaguanine was then investigated, as this base presents a particular biological interest being an antimetabolite of guanine and is used in human cancer therapy.

The ESR behaviour of this base is completely different from the purines as it shows, in the pH range 6–9, a quintet spectrum ($g = 2.027$) which becomes a

Table 1

Nucleobase	g-factor	ΔH (gauss)	Number of lines (^{14}NO)	$a(^{14}NO)$ (gauss)	Number of lines (^{15}NO)	$a(^{15}NO)$ (gauss)	a(N) (gauss)	pH
imidazole	2.027	2	9	2.0	7	2.8	2.7	7–9
N-methyl-imidazole	2.028	2	9	2.0	7	2.8	2.7	7–9
adenine	2.028	2.3	9	2.0	7	2.8	2.7	7–9.5
1-methyl-adenine	2.028	1.7	9	2.1	–	–	2.7	7–9
6-phenyl-adenine	2.027	–	9	1.9	–	–	2.6	8–9.5
6-methyl-adenine	2.026	1.5	9	1.9	–	–	2.6	7.5–9.5
	2.021	–	5	2.8	3	4.0	–	5.5
2-amino-adenine	2.026	1.7	9	2.0	7	2.8	2.7	7.5–9.5
2-hydroxy-adenine (iso-guanine)	2.029	–	9	1.9	–	–	2.7	7–9
7-methyl-adenine	2.026	–	9	2.1	–	–	2.8	9
	2.022	–	5	2.8	3	4.0	–	5.5
9-methyl-adenine	2.027	–	9	2.1	–	–	2.7	7–9
3-methyl-adenine	2.029	1.5–2	9	1.7	7	2.4	4.0	6–9
9-cyclohexyl-adenine	2.026	2.2	9	2.0	–	–	2.7	7–9

Table 2

Nucleobase	g-factors	ΔH (gauss)	Number of lines (^{14}NO)	$a(^{14}NO)$ (gauss)	Number of lines (^{15}NO)	$a(^{15}NO)$ (gauss)	a(N) (gauss)	pH
purine	2.029	2.1	9	2.0	–	–	2.6	7.5–8.5
guanine	2.028	–	9	–	–	–	–	6–8
xanthine	2.029	–	9	2.1	–	–	2.6	8–9
hypoxanthine	2.029	2.2	9	2.1	–	–	2.7	7–9
7-methyl-hypoxanthine	2.026	–	9	–	–	–	–	8–9
9-methyl-hypoxanthine	2.026	–	9	–	–	–	–	8–9
6-chloro-purine	2.028	2	9	2.0	7	2.8	2.4	8.5–9
6-iodo-purine	2.027	2.2	9	2.0	–	–	2.4	8–10
6-chloro-9-methyl-purine	2.028	1.5	9	2.0	7	2.8	2.7	7–9
	2.022	–	5	2.8	3	4.0	–	5.5
3-methyl-xanthine	2.028	1.5–2	9	1.6	7	2.3	3.9	8–8.5
3-methyl-hypoxanthine	2.029	2	9	2.0	7	2.8	2.4	7–8.5
8-azaguanine	2.027	1.5	5	2.3	3	3.2	–	6–9

triplet after ^{15}N isotopic substitution (fig. 8). The coupling constant values (a(NO) = 2.3 gauss and $a(^{15}NO)$ = 3.2 gauss) suggest binding through the ionized -OH group in 6-position. This "sui generis" behaviour is easily understandable, as the imidazole is no longer present in this molecule.

All ESR parameters are summarized in tables 1 and 2. The reported nuclear coupling constants were always obtained on the basis of those best fitting the simulated spectra. Computer simulation gives values for the peak-to-peak width of each single line (ΔH) ranging between 1.5 and 2 gauss, that is, electron spin relaxation times of the order of magnitude of the free radicals. This explains the fairly good resolution of all ESR spectra.

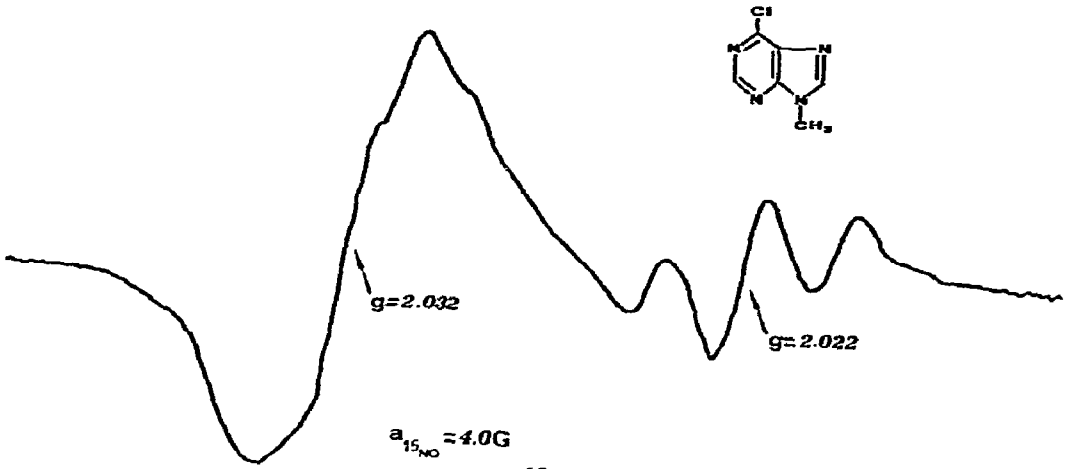


Fig. 6. Experimental ESR spectrum of $Fe(^{15}NO)_2$(6-Cl-9-CH_3-purine)$_2$ at pH = 5.5.

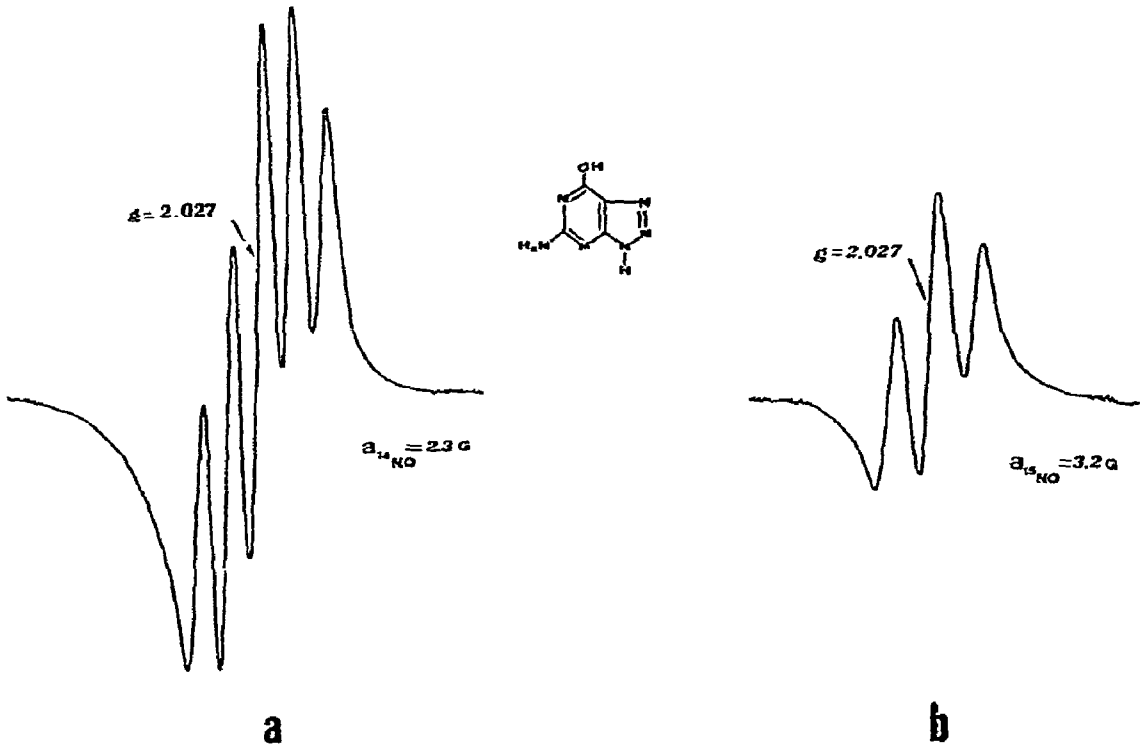


Fig. 8. (a) Experimental ESR spectrum of $Fe(NO)_2$(8-azaguanine)$_2$. (b) Experimental ESR spectrum of $Fe(^{15}NO)_2$(8-azaguanine)$_2$.

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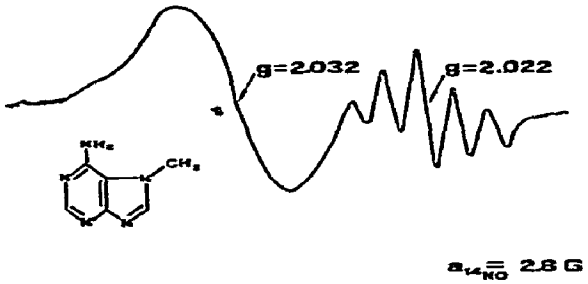

Fig. 7. Experimental ESR spectrum of $Fe(NO)_2(7\text{-}CH_3\text{-adenine})_2$ at pH = 5.5 (g = 2.022).

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