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Photon Transfer Reactions in Free Radicals. Spin pH Probes

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The kinetics and thermodynamics of proton transfer processes with participation of short-lived and stable radicals are surveyed. The extensive possibilities for the use of pH-sensitive nitroxy-radicals for the measurement of acidity in microheterogeneous chemical and biological systems, including cells, are demonstrated.
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I. INTRODUCTION

Proton transfer is one of the commonest reactions in organic chemistry.[1,2] The study of the proton transfer reaction is timely because of its important role in acid and alkaline catalysis[2,3] and in technological and biological processes.[4] The simplicity of these reactions, which proceed without the interaction of the electron shells of the reactants, making it possible to model the given reactions by methods of theoretical chemistry and promoting a deeper understanding of the nature and kinetics of the elementary step in condensed media, has also attracted physical chemists.[1,2,5] The large hydrogen isotope effect has stimulated numerous investigations of such effects in proton transfer reactions. They serve as an important test of the theory of isotope effects and yield significant information about the mechanisms of the processes considered.[5,6] As a consequence of the unique properties of the proton (the absence of an electron shell, anomalous mobility), proton transfer reactions are the fastest reactions occurring in solution; this gives rise to methodological complexities in the study of the kinetic parameters of such reactions. Relaxation methods (methods based on the abrupt change in temperature, pressure, the electric field pulse, etc.) as well as the NMR method (the study of the broadening of the ^1H and ^{17}O lines in the NMR spectra) are most often used to study the kinetics of proton transfer.[1] The relaxation methods are based on the observation of the kinetics of the return of the system to the equilibrium position (by optical or electrical procedures) after a rapid change in external parameters; their application is restricted to reversible proton transfer reactions. The NMR method is free from this limitation, since its application is based on the analysis of the form of the spectrum of the molecule participating in the reaction. However, when it is used, it is essential to have large concentrations of the reactants and, in the case of complex unresolved NMR spectra, the problem of the interpretation of the experimental data arises. Methods which make it possible to follow directly the state of the reacting species in the course of the proton exchange process (among the procedures indicated, the NMR method is of this kind) are of greatest interest. Evidently, the development of new methodological approaches which yield quantitative information about the kinetic and thermodynamic characteristics of proton exchange is a problem of extremely urgent current interest. The use of the EPR method, which is highly sensitive and makes it possible to observe directly the paramagnetic reactant and to obtain quantitative information about the kinetics of proton transfer, is promising. It may be expected that the application of the EPR method to proton transfer reactions in stable radicals will make it possible to employ such reactions as model processes for securing quantitative information about the influence of the steric factor, the solvent, and the electron-donating and electron-accepting substituents on the kinetics and equilibrium parameters of proton transfer. When a reversible proton transfer reaction takes place in radicals, the pH may influence the EPR spectra of the radicals. The possibility of measuring pH by the EPR method thus arises.

The measurement of pH is one of the most important problems in chemistry and biology, since the occurrence of an enormous number of chemical reactions and many processes involved in the vital activity of cells and cell organelles depends on this quantity. The electrometric method using glass electrodes is traditionally employed for this purpose.[7] Its main disadvantage is the large size of the glass electrodes and the impossibility of introducing them into closed structures (cell organelles, model membrane vesicles, etc.). Despite the recent development of microelectrode pH measurements,[8] this limitation in most cases precludes the use of glass electrodes for the measurement of local changes in pH (within the structures indicated, at interfaces, etc.). Fluorescent probes and the NMR method are therefore widely used nowadays in biological systems on the basis of the influence of the pH of the medium on specific characteristics of the spectra of these probles (fluorescence intensity, the chemical shift).[9-16] The application of fluorescent probes is restricted by the complexities in the interpretation of the fluoresecence intensity, which is sensitive to the interaction of the probe with the lipid membrane, to the type of buffer employed, to the ionic strength of the solution, etc. A significant limitation of the NMR method is its low sensitivity. The employment of stable nitroxy-radicals (NR) as pH probes is therefore promising, in view of the high sensitivity of the EPR method and the major advances in the organic chemistry of NR.[17,18] The considerable influence of pH on the EPR spectra of stable NR observed recently[19-21] makes it possible to take a fresh look at the prospects for their employment in the study of the mechanisms of proton transfer reactions and as pH probes.

The present review examines the results of studies of the influence of pH on the EPR spectra of short-lived and stable radicals of different types. The proton transfer in imidazoline and

imidazolidine radicals has been considered in especially great detail, because these radicals actually make it possible to obtain information about the mechanism of the proton transfer and are most promising for use as pH probes in various systems, including biological systems.[22]

II. THEORETICAL EXAMINATION OF PROTON TRANSFER REACTIONS IN RADICALS

The reversible proton transfer reaction (proton exchange) between the conjugate base (the $\dot{R}$ radical) and acid (BH^+) is described by the equation

$$\dot{R} + BH^+ \underset{k_r}{\overset{k_f}{\rightleftarrows}} \dot{R}H^+ + B \ . \qquad (1)$$

A necessary condition for the application of the EPR method in following the course of reaction (1) is a difference between the magnetic-resonance parameters of the radical in the $\dot{R}$ and $\dot{R}H^+$ forms. In this case, reaction (1) should lead to rapid exchange between the corresponding components of the EPR spectra of the radicals in the $\dot{R}$ and $\dot{R}H^+$ forms. The line shape of the EPR signal $G(X)$ in the case of rapid exchange involving two positions, corresponding to the $\dot{R}$ and $\dot{R}H^+$ forms of the radical, is described by the equation[23]

$$G(X) = I_m \left\{ iG_0 \frac{\tau_{RH^+} + \tau_R + \tau_{RH^+}\tau_R (p_{RH^+}\alpha_R + p_R\alpha_{RH^+})}{(1 + \alpha_R\tau_R)(1 + \alpha_{RH^+}\tau_{RH^+}) - 1} \right\} \ , \qquad (2)$$

where G_0 is a constant coefficient, τ_R and τ_{RH^+} are the lifetimes of the radical in the $\dot{R}$ and $\dot{R}H^+$ forms respectively, $p_R = \tau_R/(\tau_R + \tau_{RH^+})$, $p_{RH^+} = \tau_{RH^+}/(\tau_R + \tau_{RH^+})$, $\alpha_R = (1/T_2)_R - i\gamma X$, $\alpha_{RH^+} = (1/T_2)_{RH^+} - i\gamma(X + \Delta\omega/\gamma)$, $\Delta\omega$ is the difference between the frequencies of the corresponding lines in the EPR spectra of the radical in the $\dot{R}$ and $\dot{R}H^+$ forms, $(T_2)_R$ and $(T_2)_{RH^+}$ are the transverse relaxation times of the radicals $\dot{R}$ and $\dot{R}H^+$ respectively, and γ is the gyromagnetic ratio of the electron. The variable X is linked to the "permanent" magnetic field H_0 by the relation $X = H_0 - H_0^R$, where H_0^R is the field strength corresponding to the centre of the EPR signal of the radical in the form $\dot{R}$.

In conformity with Eqn.(2), different cases of exchange are realised experimentally: rapid ($1/\tau_R$, $1/\tau_{RH^+} \gg \Delta\omega$), slow ($1/\tau_R$, $1/\tau_{RH^+} \ll \Delta\omega$), or intermediate ($1/\tau_R$, $1/\tau_{RH^+} \simeq \Delta\omega$) in terms of the EPR time scale. Regardless of the frequency of the $\dot{R} \rightleftarrows \dot{R}H^+$ exchange, the ratio τ_R/τ_{RH^+} can be determined from the EPR spectra using Eqn.(2). On the other hand, analysis of Eqn.(2) shows that the determination of the lifetimes τ_R and τ_{RH^+} is possible only in the case of the intermediate exchange.[23] In the presence of well resolved lines in the EPR spectra, Eqn.(2) simplifies greatly and it is sufficient to know the positions and widths of the lines in order to find τ_R and τ_{RH^+}.[23] Knowing the ratio τ_R/τ_{RH^+}, it is possible to determine the equilibrium constant K:

$$K = k_r/k_f = \frac{[BH^+][\dot{R}]}{[B][\dot{R}H^+]} = \frac{\tau_R}{\tau_{RH^+}} \frac{[BH^+]}{[B]} \ . \qquad (3)$$

When reaction (1) describes the exchange with a solvent proton ($BH^+ \equiv H^+_{solv}$, i.e. a solvated proton), Eqn.(3) relates the equilibrium constant K to the concentration of protons $[H^+]$, i.e.

$$\left(K = \frac{\tau_R}{\tau_{RH^+}} [H^+]\right),$$

which leads to the possibility, in principle, of using radicals as pH probes.

The quantities τ_R and τ_{RH^+} determine the kinetic parameters of reaction (1), namely the rate constants k_f and k_r:

$$k_f = \frac{1}{\tau_R}[BH^+], \quad k_r = \frac{1}{\tau_{RH^+}}[B] \ . \qquad (4)$$

Thus in the cases indicated analysis of the form of the EPR signal makes it possible to obtain quantitative information about the thermodynamics and kinetics of the proton exchange in radicals.

In the case of irreversible proton transfer reactions in radicals, the difference between the magnetic-resonance parameters of the $\dot{R}$ and $\dot{R}H^+$ forms permits the direct recording by the EPR method of the accumulation (disappearance) of one of the forms of the radical. The characteristic times accessible to investigation by the EPR method are limited by the time required to "start" the reactions (for example, in the stopped flow method, the time taken to mix the reactants is $\gtrsim 10^{-3}$ s).

III. PROTON EXCHANGE IN SHORT-LIVED RADICALS

Changes in the EPR spectra of a series of alcohol radicals $\dot{R}OH$ in an acid medium have been observed.[24–26] These changes are manifested by the reversible merging of the lines of the doublet, which arises from the hyperfine interaction (HFI) with the hydroxyl proton, to a singlet as a consequence of the proton exchange:

$$\dot{R}OH + H^+ \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \dot{R}OH_2^+ \ . \qquad (5)$$

Reaction (5) alters the projection of the nuclear spin of the hydroxyl proton of the ROH radical and thus leads to frequency exchange between the lines of the doublet due to the HFI with the given proton. Analysis of the positions and widths of the lines in the EPR spectra of the $\dot{R}OH$ radicals made it possible to determine the proton exchange rate constant k_1, which is in the range 6.5×10^6 to 1.8×10^8 litre mol^{-1} s^{-1}. The temperature variation of the rate constant for the protonation of the hydroxymethyl radical k_1, characterised by the activation energy $\Delta E = 2.8$ kcal mol^{-1}, has also been found.[26]

Whereas the influence of pH on the EPR spectra is a consequence of the protonation of the OH group in hydroxyalkyl radicals in an acid medium in the studies[24–26] mentioned above, in other investigations[27–29] such influence was caused by the deprotonation of the OH group in accordance with the reaction

$$\dot{R}OH + OH^- \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \dot{R}O^- + H_2O \ . \qquad (6)$$

The difference between the EPR spectra of the $\dot{R}OH$ and $\dot{R}O^-$ forms made it possible to determine the pK of the radicals investigated and, in the two cases where intermediate exchange was observed, also the rate constants: pK = 11.51 and $k_1 = 7 \times 10^9$ litre mol^{-1} s^{-1} for the radical $CH_3\dot{C}HOH$ and pK = 1203 and $k_1 = 9 \times 10^9$ litre mol^{-1} s^{-1} for the radical $(CH_3)_2\dot{C}HOH$.[27–29] It is noteworthy that, in the case of slow and intermediate exchange, the EPR spectra of the test radicals[28] had a very complex form at pH $\simeq$ pK. Therefore 1 M orthophosphoric acid with pK = 12.32 was added to the solution of the radical. The rate of proton exchange then increases,[30,31] which simplifies the EPR spectrum. The essential feature of the proposed approach can be readily understood by considering all the proton exchange reactions with participation of the radical and the added buffer B:[30]

$$\begin{aligned} &\text{(a)}\ \dot{R} + H^+ \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \dot{R}H^+ \ , \\ &\text{(b)}\ B + H^+ \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} BH^+ \ , \\ &\text{(c)}\ \dot{R} + BH^+ \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \dot{R}H^+ + B \ . \end{aligned} \qquad (7)$$

The quantities pK_1, pK_2, and pK_3 are linked by the relation[31]

$$pK_3 = pK_1 - pK_2 . \quad (8)$$

The addition of a buffer to the system alters only the kinetic parameters of the $\dot{R} \rightleftarrows \dot{R}H^+$ exchange (τ_R, τ_{RH^+}), while the equilibrium in this reaction (τ_R/τ_{RH^+}) is undisturbed:

$$\begin{aligned} &(a)\ 1/\tau_R = k_3[BH^+] = k_3B_0/(1 + 10^{pH-pK_3}) , \\ &(b)\ 1/\tau_{RH^+} = k_{-3}[B] = k_{-3}B_0/(1 + 10^{pK_3-pH}) , \\ &(c)\ \tau_{RH^+}/\tau_R = [RH^+]/[R] = 10^{pK_1-pH} , \end{aligned} \quad (9)$$

where $B_0 = [B] + [BH^+]$ is the buffer concentration; here it is understood that $k_3[BH^+] \gg k_1[H^+]$, and $k_{-3}[B] \gg k_{-1}$. According to Eqns.(9), the most effective acceleration of the exchange (decrease in τ_{RH^+} and τ_R) is observed under conditions where $pK_1 \simeq pK_2$.

The proton exchange reaction between two forms of the glycine radical has been investigated:

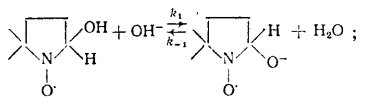
$$NH_2{-}\dot{C}H{-}COO^- + H_2PO_4^- \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} NH_2{-}\dot{C}H{-}COOH + HPO_4^{2-} . \quad (10)$$

In the absence of a buffer, the proton exchange via reaction (7a) leads to slow exchange between the corresponding lines of the EPR spectrum of the glycine radical. The addition of the phosphate buffer ($pK_2 = 6.4$) made it possible to pass to the region of the intermediate exchange and to calculate the rate constants for the proton exchange reaction (10): $k_3 = (2.5 \pm 0.5) \times 10^9$ litre mole^{-1} s^{-1} and $k_{-3} = (1.4 \pm 0.5) \times 10^9$ litre mol^{-1} s^{-1}. Rapid $\dot{R} \rightleftarrows \dot{R}H^+$ exchange occurs in 0.5 M phosphate buffer and $pK_1 = 6.65$ for the carboxy-group in the glycine radical was calculated from the pH dependence of the HFI constants: a_H^{CH}(pH) and a_N(pH). In the analysis of the a(pH) dependences use was made of Eqn.(11), which can be readily obtained for the case of rapid exchange from Eqns.(2) and (9c):

$$a(pH) = a_R \cdot p_R + a_{RH^+} \cdot p_{RH^+} = (a_R + a_{RH^+} \cdot 10^{pK_1-pH})/(1 + 10^{pK_1-pH}) , \quad (11)$$

where a_R and a_{RH^+} are the HFI constants of the radicals in the $\dot{R}$ and $\dot{R}H^+$ forms respectively.

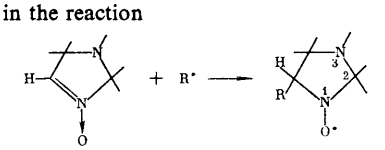
An interesting case of the manifestation of the deprotonation of the OH group has been observed[32] in a hydroxynitroxy-radical:



$a_\beta^H = a_N^{ROH} = 14.45$ G, $g = 2.0057$; $a_\beta^H = a_N^{RO^-} = 16.2$ G, $g = 2.0057$ (12)

The protonated ($\dot{R}OH$) and deprotonated ($\dot{R}O^-$) forms of the radical differed in their HFI constants. In the region where pH $\simeq$ pK, the rapid $\dot{R}OH \rightleftarrows \dot{R}O^-$ exchange was observed and the $a_N = a_\beta^H$(pH) dependence was described by Eqn.(11).

The influence of pH on the EPR spectra of a series of spin adducts formed in the reaction



(13)

R=OH, O_2^-(HO$_2$), CH_2OH, O_4^- .

has been observed.[33] The change in the EPR spectra of the short-lived NR formed via reaction (13) in the pH range 2–5 was explained by the authors of the above study by the protonation of the N(3) atom. A number of studies[34–38] have been devoted to the investigation by the EPR method of the proton exchange reactions in semiquinone, semidione, and other short-lived radicals.

Thus the manifestations of the proton exchange in the EPR spectra of short-lived radicals have been fairly thoroughly investigated. Analysis of these spectra makes it possible to measure both the equilibrium constants of the proton exchange reactions and the bimolecular protonation constants. It has been noted in a number of studies that the pK of the radicals are appreciably lower than those of the corresponding diamagnetic molecules,[27–31] which has been attributed to the electron-accepting influence of the radical centre. The protonation constants are in the range $5 \times 10^6 - 10^{10}$ litre mol^{-1} s^{-1}.[24–26,28,30,31,35–37]

The use of the radicals considered as pH probes is in general impossible as a consequence of their instability.

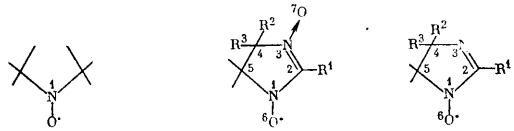
IV. PROTON EXCHANGE IN STABLE NITROXY-RADICALS

As can be seen from the previous Section, the presence of groups capable of being protonated (deprotonated) in the structure of the radical in the immediate vicinity of the radical centre leads to a difference between the EPR spectra of the protonated ($\dot{R}H^+$) and deprotonated ($\dot{R}$) forms of the radical. However, the simultaneous fulfillment of two requirements—the presence of a functional group near the radical centre and the stability of the radical—is not easy. The manifestation of proton exchange in the EPR spectra of stable radicals has therefore been recorded only in rare instances.

The greatest changes in the parameters of the EPR spectra of NR are to be expected when the latter are protonated at a radical centre where the main bulk of the spin density is localised.

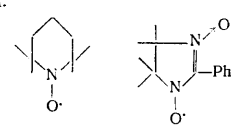
1. Protonation of Nitroxy-radicals at the Radical Centre

The spin density in di-t-alkylnitroxy-radicals is localised mainly on the nitroxy-fragmnent [$\rho_O^\pi \simeq 0.6$; $\rho_{N(1)}^\pi \simeq 0.4$ [39]], in nitronylnitroxy-radicals (NNR) it is localised on the two nitroxy-groups [$\rho_{N(1)}^\pi \simeq \rho_{N(3)}^\pi \simeq 0.22$; $\rho_{O(6)}^\pi \simeq \rho_{O(7)}^\pi \simeq 0.21$ [39]], while in iminonitroxy-radicals (INR) it is localised on the nitroxy-fragment and nitrogen of the imino-group [$\rho_{N(1)}^\pi \simeq 0.38$; $\rho_{O(6)}^\pi \simeq 0.4$; $\rho_{N(3)}^\pi \simeq 0.16$ [40]]:



di-t-alkyl nitroxide — nitronyl nitroxide — iminonitroxide

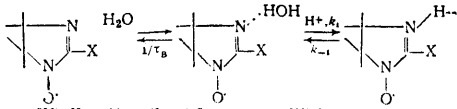
The N–O fragment in NR is protonated only in acid media.[41–43] Such protonation of the radical (I) in aqueous 80% sulphuric acid solution increases the constant $a_N(\Delta a_N = 5.7$ G) and decreases the g-factor ($\Delta g = 0.0013$) of the radical and also leads to the appearance of additional doublet splitting ($a_H = 3.12$ G) due to the HFI with the added proton:[43]



(I) $a_N = 15.87$G, $g = 2.0059$; (II) $a_{N(1)} = 7.5$G, $a_{N(3)} = 7.5$G

For the majority of NR, protonation at the oxygen atom of the N—O fragment cannot be observed, since in aqueous solutions this process is accompanied by the disproportionation of the NR to the corresponding oxoammonium and hydroxyammonium cations.[44] The protonation of the oxygen atom in the same fragment of NNR in aqueous solutions has not been observed as a consequence of the disproportionation of these radicals in an acid medium.[45] However, in benzene, in the presence of trifluoroacetic acid, it has been possible to observe the EPR spectrum of the radical (II) protonated at the oxygen atom of the nitroxy-group; the constants of the HFI with both nitrogen atoms then decreased (Δa_N = 1.8 G; $\Delta a'_N$ = 2.8 G) and an additional doublet splitting appeared due to the HFI with the added proton (a_H = 4.7 G).[45]

In contrast to the di-t-alkylnitroxy-radicals[41–43] and the nitronyl-nitroxy-radicals,[45] protonated at the nitrogen atom of the imino-group N(3), which is more basic than the nitroxy-group, has been observed for INR:[40,46]



(14)

(III) X = Ph; pK = 1.9; $a_{N(1)}$ = 9.9G; $a_{N(3)}$ = 4.5G
(IV) X = NH_2; pK = 6.4; (III)-H^+ $a_{N(1)}$ = 6.2G $a_{N(3)}$ = 4.9G
(V) X = $N(CH_3)_2$; pK = 6.8 a_H = 4.6 G

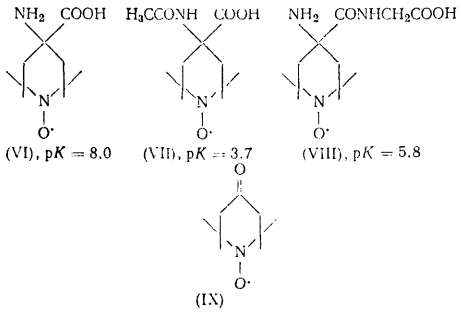
The large spin density in INR at the N(3) atom is responsible for the greater changes in the constants of the HFI with the nitrogen atom when the latter is protonated [Eqn.(14)]. The constant for the HFI with the added proton a_H in the protonated form of the radical (III) at pH > 1 is not observed as a consequence of the rapid (in terms of the EPR time scale) exchange [via reaction (14)]. The determination of the H^+ concentration at which additional doublet splitting due to the HFI with the added proton appears in the EPR spectrum of the radical form (III)-H^+ made it possible to estimate the lifetime of the $=N\cdots HOH$ hydrogen bond in the radical (III): $\tau_H \simeq 2 \times 10^{-10}$ s.[46] The condition for the rapid $\dot{R} \rightleftarrows \dot{R}H^+$ exchange in the radical (III) makes it possible to determine the lower limit to the bimolecular protonation constant: $k_1 \gg 5 \times 10^7$ litre mol^{-1} s^{-1}. According to other data,[40] the averaging of the signals of the $\dot{R}$ and $\dot{R}H^+$ forms of the radical (III) is incomplete and the exchange has a measureable effect on the position and width of the lines of the EPR signal; this made it possible to calculate the rate constant for the protonation of the radical (III): $k_1 \simeq 10^9$ litre mol^{-1} s^{-1}. The values of k_1 have also been obtained for three other INR and were found to lie in the range $3 \times 10^6 - 10^9$ litre mol^{-1} s^{-1}.[40]

Iminonitroxy-radicals can also form equilibrium complexes in aqueous solutions not only with the proton but also with a series of diamagnetic metals [Hg(II), Zn(II), Cd(II); Ag(I), etc.[40,47]]. Complex formation with participation of the N(3) atom in the INR and of the metal leads to a change in the HFI constants $a_{N(1)}$ and $a_{N(3)}$, which can be recorded by EPR, and to the appearance of HFI with the nucleus of the metal [the HFI constant $a_{Hg} \simeq 40$ G for Hg(II) [40]].

Among the radicals investigated in a series of studies,[40–43,45,46] only two [(IV) and (V)] can be used as pH probes in aqueous solutions in the pH range of practical interest. However, the interpretation of the EPR spectra of the radicals (IV) and (V) at pH $\simeq$ pK is extremely complex, since these spectra are formed as a result of the superposition of the signals of the $\dot{R}$ and $\dot{R}H^+$ forms, in each of which HFI with three non-equivalent nitrogen atoms and protons of the substituent X is manifested.[40]

2. Protonation of the Functional Groups of Nitroxy-radicals

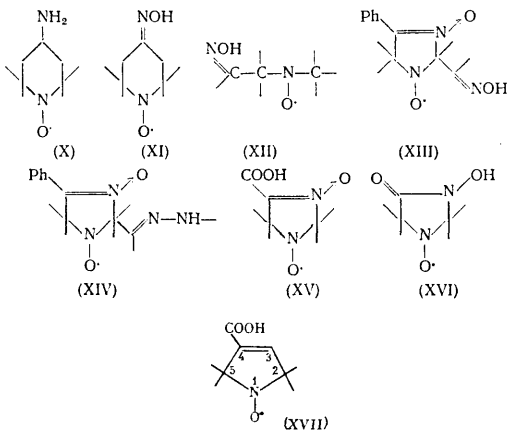
The influence of pH on the EPR spectra of radicals of the piperidine type [(VI)—(VIII)] containing functional groups capable of being protonated (deprotonated) at C(4) has been observed:[48]



(VI), pK = 8.0 (VII), pK = 3.7 (VIII), pK = 5.8

(IX)

A decrease in the HFI constant a_N(Δa_N = 0.15—0.3 G) and an increase in the g-factor ($\Delta g \simeq 0.0001$) have been observed for these radicals with decrease in pH. On the other hand, the EPR spectrum of the radical (IX), which has no group capable of protonation at C(4), did not change over the pH range 2—12. The authors[48] therefore explained the observed changes in the EPR spectra by the reversible protonation of the amino-group in the radicals (VI) and (VIII) and the deprotonation of the carboxy-group in the radical (VII). The slow exchange between the $\dot{R}$ and $\dot{R}H^+$ forms of the radicals (VI)—(VIII) leads to the asymmetry of the EPR spectra, which is manifested most markedly by the decreased intensity of the high-field component. It has been suggested that the pH dependence of the consant a_N and also of the parameters characterising the asymmetry of the signal (the ratio of the intensities of the central high-field components, etc.) be used to determine the pK of the radical by the EPR method. The pH dependence of a_N can be employed to determine the pK also in the absence of the asymmetry of the signal, as in the case of the rapid $\dot{R} \rightleftarrows \dot{R}H^+$ exchange. The sensitivity of the EPR spectrum of the radical (X) of the piperidine type, with the NH_2 group at C(4), to the pH has been reported.[49] However, the authors[49] did not interpret the observed slight increase in the HFI constant ($\Delta a_N \simeq 0.13$ G) in the alkaline pH range 8 to 10.5, which in our view is due to the deprotonation of the NH_2 group (pK = 9.5 [50]):



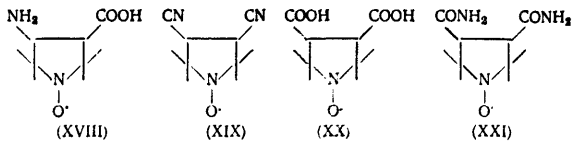
(X) (XI) (XII) (XIII)

(XIV) (XV) (XVI)

(XVII)

For another radical of the piperidine type with the =NOH group at C(4) [the radical (XI)], an increase in a_N in an alkaline medium has also been observed (Δa_N = 0.16 G).[51] The observed reversible

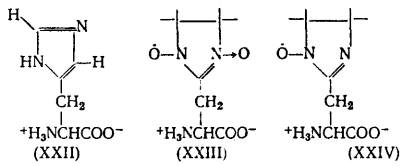
change in a_N over the pH range 10–12.8 was explained by the reversible deprotonation of the =NOH group. This is confirmed by the similar pK values found by the EPR method from the dependence of a_N on pH for three radicals of different types containing the =NOH group:[51] the piperidine type radical (XI) (pK = 11.35; Δa_N = 0.16 G), the acyclic di-t-alkylnitroxy-radical (XII) (pK = 11.55; Δa_N = 0.34 G), and the radical (XIII), which is of the 3-imidazoline 3-oxide type (pK = 10.7; Δa_N = 0.22 G). Much lower pK values have been found for the 3-imidazoline 3-oxide type radicals (XIV) and (XV) with other functional groups, amounting to 1.6 and 1.9 for the =N–NH group in the radical (XIV) (Δa_N = 0.47 G) and the COOH group in the radical (XV) (Δa_N = 0.15 G) respectively. A difference between the EPR spectra of the $\dot{R}$ and $\dot{R}H^+$ forms of the imidazolidine type radicals (XVI), which are analogues of hydroxamic acid (pK = 7.0; Δa_N = 0.24 G), and the pyrrolidine type radicals (XVII) with the carboxy-group at C(4) (pK = 3.5; Δa_N = 0.31 G) [51] has also been observed. The influence of pH on the EPR spectra of the pyrrolidine type radicals (XVIII)–(XXI) has been reported recently:[52]



(XVIII) (XIX) (XX) (XXI)

The spectra of these radicals at pH 2 and pH 12 differed in the values of the HFI constant (Δa_N = 0.3–0.5 G). However, in contrast to Nakie et al.[48] and Khramtsov et al.,[51] Matthew and Dodd[52] presented no data concerning the pH dependence of the parameters of the EPR spectra (a_N, g-factor), so that the question of the pK of the groups protonated in the radicals (XVIII)–(XXI) remains open. The prospects for the use of these radicals as pH probes are also obscure. We may likewise note that the difference between the EPR spectra of the $\dot{R}$ and $\dot{R}H^+$ forms can be determined by the influence of the protonation of the functional groups of the radical on its intermolecular or intramolecular interactions.

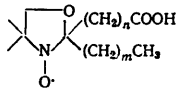
Changes in the EPR spectra of a series of radical analogues of histidine (XXII) have been observed as a function of pH:[53]



(XXII) (XXIII) (XXIV)

The protonation of the carboxy-group in the radicals (XXIII) and (XXIV) altered the constants for HFI with the non-equivalent β-protons of the methylene group. This was explained by the ion–dipole interaction between the electron-deficient nitronylnitroxy-ring [for the radical (XXIII)] or the iminonitroxy-ring [for the radical (XXIV)] and the carboxylate anion. The protonation of the carboxy-group disturbs such interaction and shifts the equilibrium between the conformations responsible for the non-equivalence of the constants for the HFI with the methylene protons. An analogous ion–dipole interaction between the imidazole ring and the carboxylate ion in histidine has been observed by NMR.[53]

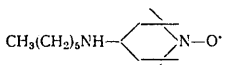
The pH influences the EPR spectra of the spin-labelled aliphatic acids (XXV) and (XXVI) in an aqueous lecithin suspension:[54]



(XXV) $m = 12$, $n = 3$
(XXVI) $m = 5$, $n = 10$

The spectra of the radicals (XXV) and (XXVI) have the form characteristic of immobilised radicals and the order parameter S, characterising the degree of hindrance of the rotation of the NR,[55] depended on the pH of the medium in the pH range 4–7. Since no influence of the pH on the EPR spectra of the methyl ester of the radical (XXVI) was observed under these conditions, the pH-dependent changes in the spectra of the radicals (XXV) and (XXVI) were explained[54] by the deprotonation of the carboxy-group. Since the carboxy-group is very remote from the N–O· radical fragment in the radicals (XXV) and (XXVI) [in the radical (XXVI) they are separated by a chain of 10 CH_2 groups], the observed differences between the EPR spectra of the protonated and deprotonated forms of the radicals are apparently entirely due to the interaction of both forms with the lipid phase, which leads to a difference between their rotational characteristics manifested in the EPR spectra.

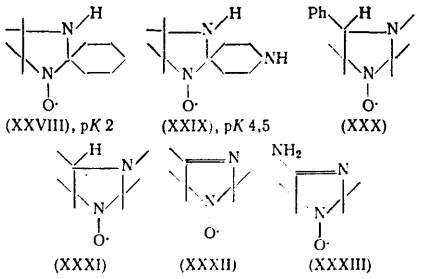
The influence of pH on the EPR spectrum of the NR (XXVII) in an aqueous suspension of lipid vesicles (liposomes) has been investigated:[56]



(XXVII)

In this medium, the radical (XXVII) is distributed between the aqueous and lipid phases, the EPR signal in the lipid phase being greatly broadened as a consequence of immobilisation. The partition coefficient of the radical (XXVII) between the aqueous and lipid phases varies by a factor of ~10 in the pH range 3.5–11.5, which has been explained by the difference between the partition coefficients of the form of the radical (XXVII) protonated at the third nitrogen atom linked to the C(4) atom and of its non-protonated form. The radicals (XXV) and (XXVII) have been used[57,58] to investigate the transmembrane proton transport through a model phospholipid membrane. The different permeabilities of the membranes to the $\dot{R}$ and $\dot{R}H^+$ forms of the radical (X) have been used to determined ΔpH on natural membranes.[50,59,60] Since the sensitivity of the EPR spectra of the radicals (XXV)–(XXVII) to pH is observed only when the solution contains the lipid phase, their use for the direct measurement of pH in general is not possible.

In 1982, the influence of pH on the EPR spectra of NR of the imidazoline and imidazolidine types was observed independently in a number of studies.[19–21] Rapid proton exchange between the $\dot{R}$ and $\dot{R}H^+$ forms of the radicals (XXVIII) and (XXIX), differing in the HFI constants ($\Delta a_N \simeq$ 1.2 G) was noted by Keana et al.[19] Khramtsov et al.[21] observed different cases of proton exchange (rapid or slow) for the radicals (XXX)–(XXXIII), whose $\dot{R}$ and $\dot{R}H^+$ forms differed both as regards the HFI constants (Δa_N = 0.8–1.2 G) and the g-factors (Δg = 0.0002–0.0003). As a consequence of such appreciable differences, the radicals of the imidazoline and imidazolidine types are most promising for the study of the mechanisms of the proton exchange reactions by EPR and also for use as pH probes:



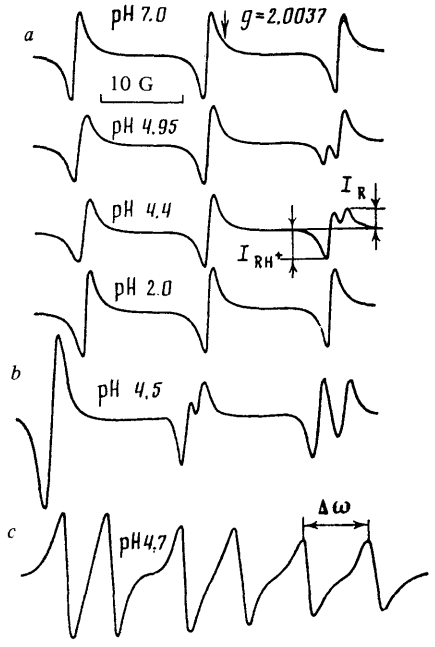
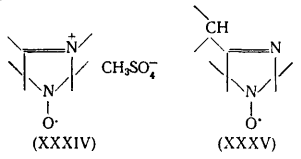


Figure 1. EPR spectra of an aqueous solution (at a concentration of 5×10^{-4} M) of the radical (XXXI) at different pH in the *X*-band (*a*), *Q*-band (*b*), and the 2-mm band (*c*) at 23 °C.[21]

observe the doublet splitting of two (in the *Q*-band) or all three (in the 2 mm range) components of the triplet (Fig.1) the EPR spectrum of the radical (XXXI). It has been shown[21] that the observed effect is associated with the protonation of the N(3) atom in the radical (XXXI) and with the difference between the HFI constants a_N and the *g*-factors of the Ṙ and $\dot{R}H^+$ forms.

This conclusion has been confirmed, firstly, by the similarity of the p*K* values for this radical measured potentiometrically and by EPR.[61] The dependence of the function $f = I_{RH^+}/(I_R + I_{RH^+})$ (I_{RH^+} and I_R are the intensities of the EPR signals of the $\dot{R}H^+$ and Ṙ forms respectively) on the pH was used to determine the p*K* by the EPR method (Fig.2). Previously this function had been employed[62] to describe the distribution of the radical (I) between the aqueous and lipid phases:



Secondly, the HFI constants and the *g*-factor of the radical (XXXI) in the $\dot{R}H^+$ form are close to the corresponding values for the imidazolinium salt (XXXIV) (Table 1). Thirdly, the effect of pH on the EPR spectra of the radicals (I) and (XXXIV), in which the N(3) atom capable of being protonated is absent, has not been observed.

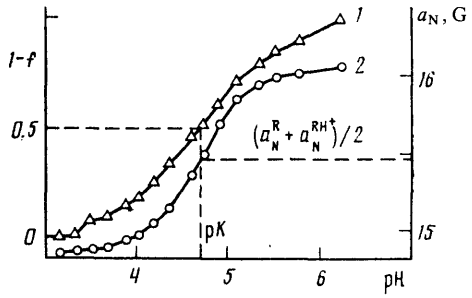


Figure 2. The pH dependence of the function $I-f = I_R/(I_R + I_{RH^+})$ (curve *1*) and of the HFI constant a_N (curve *2*) for the radical (XXXI).[61]

3. Proton Exchange in Nitroxy-radicals of the Imidazoline and Imidazolidine Types

(a) Manifestation of the protonation of the N(3) atom in radicals of the imidazoline and imidazolidine types in EPR spectra. The influence of pH on the EPR spectra of one of the radicals—an imidazolidine derivative (XXXI)—is illustrated in Fig.1.[21] In the pH range 3.5–6, the high-field component of the EPR spectrum in the *X*-range (Fig.1*a*) is split into two resolved components, whose relative intensities vary reversibly with pH. This form of the spectrum has been explained by the superposition of two triplet EPR spectra differing in the HFI constants and *g*-factors. Indeed, the use of spectrometers with a higher working frequency made it possible to

The p*K* found by the EPR method, the HFI constants, and the *g*-factors are listed in Table 1 for a series of radicals of the imidazoline and imidazolidine types.

The EPR spectrum of the radicals (XXXI) and (XXXIII) with $pK > 3$ in the region where $pH \simeq pK$ represents a superposition of the spectra of the Ṙ and $\dot{R}H^+$ forms of the radical, which is due to the slow $\dot{R} \rightleftarrows \dot{R}H^+$ exchange.[61] In this case, the p*K* of the radicals were determined using the pH dependence of *f* (Fig.2). It is noteworthy that the p*K* of the radicals determined in this way can differ in general from the true values as a consequence of the different widths of the high-field components of the spectra of the Ṙ and $\dot{R}H^+$ forms. However, for the radicals investigated (Table 1), these differences are small ($\lesssim 5\%$) and can lead to an error not greater than 0.1 in the measurement of the p*K*. In the *X*-range, the pH

dependence of the quantity a_N (Fig.2), measured as the distance between the first and second components of the triplet, can be used together with the functions f for the determination of the pK of the radical; here $a_N(pK) = (a_N^{\dot{R}H^+} + a_N^{\dot{R}})/2$ ($a_N^{\dot{R}H^+}$ and $a_N^{\dot{R}}$ are the HFI constants for the $\dot{R}H^+$ and $\dot{R}$ forms respectively). Two resolved signals in the third component of the EPR spectrum were not observed for the radicals (XXXII) and (XXXV) in the pH $\simeq$ pK region. The change in pH led to a reversible change in the parameters of the triplet EPR spectra of these radicals, namely the HFI constant and the g-factor, which can be explained by the rapid proton exchange $\dot{R} \rightleftarrows \dot{R}H^+$. In this case, the p$K$ of the radical can be calculated from the conditions leading to agreement between the experimental curve and the curve calculated by Eqn.(11) and also from the condition

$$a_N(pK) = (a_N^{\dot{R}H^+} + a_N^{\dot{R}})/2.$$

Table 1. The pK values, found by EPR, the calculated and experimental HFI constants a_N, and the g-factors for the $\dot{R}$ and $\dot{R}H^+$ forms of certain radicals of the immidazoline and imidazolidine types.[21,61,63]

Radical	pK (±0,1)	g-Factor* (±0.0001)		a_N (±0.05), G	
		$\dot{R}H^+$	$\dot{R}$	$\dot{R}H^+$	$\dot{R}$
(XXXI)	4.7	2.0057 2.0046**	2.0054 2.0044**	14.85 6.03**	16.10 7.29**
(XXXII)	1.3	2.0057 2.0047	2.0055 2.0037**	14.95 5.74** 10.22***	15.85 7.22** 11.22***
(XXXIII)	6.1	2.0057 2.0048	2.0054 2.0036	15.05 5.73**	15.80 7.26**
(XXXIV)	—	2.0057 2.0047**	—	14.80 5.78**	—
(XXXV)	0.8	2.0057 2.0046**	2.0054 2.0036**	15.00 5.79**	15.85 7.24**

* The experimental g-factors, determined by Khramtsov and coworkers,[21,61] are displaced relative to the tabulated values by the constant amount $\Delta g = 6 \times 10^{-4}$ as a consequence of a systematic apparatus error.

** Calculated values (without allowance for solvation effects).[61]

*** Calculated values (with allowance for solvation effects).[64]

As can be seen from Table 1, when the N(3) atom is protonated, the changes in the g-factor are $(2-3) \times 10^{-4}$ and those in the HFI constant are 0.8 – 1.2 G. Additional evidence for the possibility of such large changes in the parameters of the EPR spectra of NR of the imidazoline and imidazolidine types on protonation of the N(3) atom has been obtained by quantum-chemical methods.[61]

(b) Quantum-chemical calculations of the HFI constants and g-factors of radicals of the imidazoline and imidazolidine types in the protonated and non-protonated forms. Quantum-chemical calculations of the electronic structures of the radicals have been carried out[61] by the restricted and unrestricted Hartree – Fock methods in terms of the INDO approximation.[65] In the calculation of the g-factor by the restricted Hartree – Fock method, the molecular orbitals were calculated by Stone's method.[66] The available experimental data for the geometries of the analogous imidazoline radicals with the N-oxide linkage were used for the radicals (XXXII) – (XXXV).[67] In the calculation of the electronic structure of the radical (XXXI), the geometry of the corresponding hydroxypyrrolidine was attributed to the latter.[68]

The constants for the HFI with the N(1) atom (a_N) and the g-factors for the above radicals, calculated theoretically and found experimentally,[61] are compared in Table 1. The calculated constants a_N differ appreciably from the experimental values owing to the approximate nature of the INDO method used in the calculation.[65] However, the characteristics of the changes in the HFI constants on passing from uncharged to charged forms are reproduced quite satisfactorily. In these radicals, the spin density is localised mainly on the N – O· π orbital ($\rho_N^\pi \simeq 0.31$; $\rho_O^\pi \simeq 0.67$). When the N(3) atom is protonated, the splitting of the levels of the π orbitals of the N and O atoms increases under the influence of the field of the positive charge and the relative electronegativity of the oxygen atom diminishes: as a result of this, the antibonding orbital of the unpaired electron is localised to a greater extent on the oxygen atom and to a lesser extent on the nitrogen atom ($\rho_N^\pi = 0.26$; $\rho_O^\pi = 0.72$). The sum $\rho_N^\pi + \rho_O^\pi$ then remains almost constant. Since the constant a_N is linked to the π-electron spin populations by relations of the type[65] $a_N = Q_N^N \rho_N^\pi + Q_O^N \rho_O^\pi$, where $Q_N^N > 0$ and $Q_O^N < 0$, the above redistribution of spin density on protonation of the N(3) atom does in fact diminish the constants a_N.

The values of a_N for the $\dot{R}$ and $\dot{R}H^+$ forms of the radical (XXXII) have been calculated recently[64] taking into account solvation within the framework of a solvate model.[69] As can be seen from Table 1, allowance for solvation ensures the best agreement between the calculated and experimental values of a_N with retention at the same time of the difference between the a_N for the two forms of the radical, amounting to 1 G.

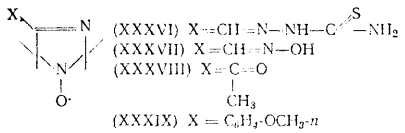
It follows from Table 1 that, in agreement with experiment, the g-factors of the protonated radicals are close to one another and do not differ from the g-factor of the imidazolinium salt (XXXIV). The non-protonated radicals are also characterised by a similar g-factor [with the exception of the radical (XXXI), whose geometry has been estimated tentatively by Khramtsov et al.[61]]. The g-factors of the protonated radicals are appreciably greater than those of the non-protonated radicals.

Analysis of the calculated data has shown that this increase in the g-factors is caused mainly by the increase in the component of the g-tensor $\Delta g_{zz} = 0.0034$ corresponding to the direction of the N – O· bond. The main contribution to this component ($\Delta g_{zz} = 0.0042$) comes from the transition from the molecular orbital corresponding approximately to the orbital of the unshared electron pair of oxygen n to the orbital of the unpaired electron $\pi\uparrow$, whose admixture in the ground state is determined mainly by the spin – orbital interaction at the oxygen atom.

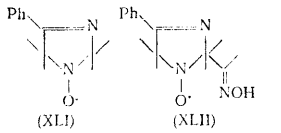
The above analysis has confirmed that the effects associated with the protonation of the N(3) atom can induce changes in the constants a_N and in the g-factor, which have been observed experimentally, and thus serves as yet another confirmation of the validity of the assignment of the EPR spectra by Khramtsov et al.[61]

(c) The equilibrium constants of the proton exchange in radicals and their relation with the character of the frequency exchange in the EPR spectra. The influence of the protonation of the N(3) atom on the EPR spectra of a large series of radicals of the imidazoline and imidazolidine types has been investigated.[51] Table 2 presents the pK,

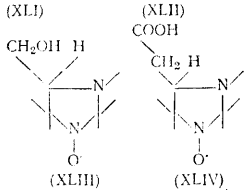
the HFI constants, and the *g*-factors for the radicals (XXXVI)–(LI) in the protonated and deprotonated forms found by EPR and also indicates the character of the $\dot{R} \rightleftarrows \dot{R}H^+$ exchange.



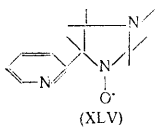
(XXXVI) X = CH=N–NH–C(=S)–NH$_2$
(XXXVII) X = CH=N–OH
(XXXVIII) X = C(=O)–CH$_3$
(XXXIX) X = C$_6$H$_3$-OCH$_3$-*n*
(XL) X = C(CH$_3$)=N–NH$_2$



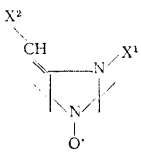
(XLI) (XLII)



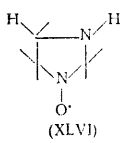
(XLIII) (XLIV)



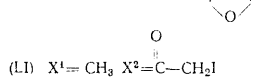
(XLV)



(XLVII) X^1 = H X^2 = NO$_2$
(XLVIII) X$_1$ = H X^2 = C(=O)–CH$_3$
(XLIX) X^1 = H X^2 = C(=O)–(CH$_2$)$_4$–COOH
(L) X^1 = CH$_3$ X^2 = C(=O)–CH$_2$–N

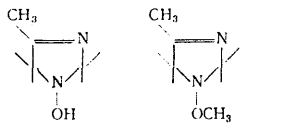


(XLVI)



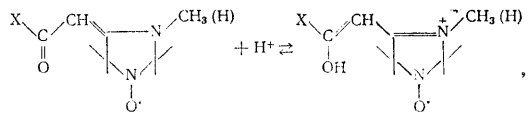
(LI) X^1 = CH$_3$ X^2 = C(=O)–CH$_2$I

The protonation of the N(3) atom in the given radicals is accompanied by appreciable changes in the HFI constants a_N (Δa_N = 0.8 to 1.3 G) and in the *g*-factor [$\Delta g = (2-3) \times 10^{-4}$]. The values of p$K$ for the imidazoline radicals correspond to the region of low pH values [pK < 3, with the exception of the radical (XXXIII), which has pK = 6.1]; they are smaller than the pK of the imidazolidine radicals [pK = 3.0–4.7 for the radicals (XXX), (XXXI), and (XLIII)–(XLVI)]. The smaller pK of the radicals compared with the pK of the corresponding diamagnetic molecules of imidazolines and imidazolidines[19,51] have been explained by the electron-accepting influence of the nitroxy-group. Indeed, pK = 1.9 for the radical (XXXII), which is appreciably less than the pK of the corresponding diamagnetic analogues (LII) and (LIII):[61]



(LII), pK = 3.85 (LIII), pK = 3.85

We may also note that pK = 11.15 for the =NOH group in the radical (XLII) [cf. the radical (XIII)] and pK = 2.0 for the COOH group in the radical (XLIV) [cf. the radical (XV)] [51] have been found by EPR. The high pK = 7.8, found by EPR for the imidazolidine radical (XLVII), is associated with its deprotonation in the 3-position. The deprotonation of this radical has been demonstrated chemically.[70] The deprotonation in the 3-position is also responsible for the change in the EPR spectra of the imidazolidine radicals (XLVIII) and (XLIX) in an alkaline medium (Table 2).[51] For the radicals (XLVIII) and (XLIX), yet another pK has been observed (Table 2) in the region of low pH values, which, as for the radicals (L) and (LI), has been explained by the protonation of the enamino-keto-group. It is known that the vast majority of enaminoketones are protonated at the oxygen atom.[71,72] The protonation of the radicals (XLVIII)–(LI) at the oxygen atom is described by the equation



(15)

where X are substituents. The positive charge on the $\dot{R}H^+$ form is then mainly localised at the N(3) atom, which explains the similarity of the values of Δa_N and Δg for the protonation of the radicals (XLVIII)–(LI) and of the N(3) atom in radicals of the imidazolidine type (Table 2).

Table 2. The values of pK, a_N, and *g*-factors for the $\dot{R}$ and $\dot{R}H^+$ forms of certain radicals of the imidazoline and imidazolidine types as well as the character of the $\dot{R} \rightleftarrows \dot{R}H^+$ frequency exchange.[51,63]

Radical pK (±0.1)		*g*-Factor (±0.0001)		a_N (±0.02), G		Character of exchange
		$\dot{R}H^+$	$\dot{R}$	$\dot{R}H^+$	$\dot{R}$	
(XXX)	3.0	2.0057**	2.0054**	14.85	16.20	s
(XXXVI)	–0.2	2.0057	2.0055	14.80	15.36	r
(XXXVII)	0.1	2.0057	2.0055	14.85	15.64	r
(XXXVIII)	0.2	2.0057	2.0055	14.93	15.81	r
(XXXIX)	1.2	2.0057**	2.0054**	15.00	15.85	r
(XL)	2.5	2.0057	2.0055	15.03	15.77	i
(XLI)	0.6	2.0057	2.0055	14.95	15.65	r
(XLII)	1.0	2.0058	2.0057	14.69	15.13	r
(XLII)	11.15	2.0057	2.0056	15.12	15.35	–
(XLIII)	3.85	2.0057	2.0055	14.89	15.96	s
(XLIV)	2.0	2.0058	2.0058	14.60	14.76	–
(XLIV)	4.3	2.0058	2.0055	14.76	15.92	s
(XLV)	4.5	2.0057	2.0055	14.72	15.89	s
(XLVI)	4.35	2.0057	2.0055	15.02	16.18	s
(XLVII)	7.8	2.0056	2.0055	15.29	15.75	s
(XLVIII)	1.1	2.0057	2.0056	15.03	15.46	r
(XLVIII)	13.25	2.0056	2.0055	15.47	15.98	r
(XLIX)	1.0	2.0057	2.0056	15.03	15.47	r
(XLIX)	13.5	2.0056	2.0065	15.46	15.92	r
(L)	–0.7	2.0058	2.0055	14.20	15.41	s
(LI)	0.2	2.0058	2.0055	14.40	15.57	s

*r = rapid, i = intermediate, and s = slow (in terms of the EPR time scale).
**See first footnote to Table 1.

The relation between the character of the $\dot{R} \rightleftarrows \dot{R}H^+$ exchange and the pK of the radical has attracted attention: for radicals with pK > 3 and also for the radicals (XLVIII) and (XLIX) with pK = 13.25 and 13.5 respectively, a rapid exchange has been observed, while in the case of the radicals with pK = 3–8 a slow exchange has been observed (Table 2). This has been explained in two investigations.[51,61] In aqueous solutions, the proton exchange between the $\dot{R}$ and $\dot{R}H^+$ forms is described by two mechanisms:

$$\dot{R} + H^+ \underset{k_{-1}^{H^+}}{\overset{k_1^{H^+}}{\rightleftarrows}} \dot{R}H^+; \qquad (16)$$

$$\dot{R}H^+ + OH^- \underset{k_{-1}^{OH^-}}{\overset{k_1^{OH^-}}{\rightleftarrows}} \dot{R} + H_2O\,. \tag{17}$$

The condition for slow exchange requires that the reciprocals of the lifetimes of the radicals in the $\dot{R}$ and $\dot{R}H^+$ forms ($1/\tau_R$ and $1/\tau_{RH^+}$ respectively) should be appreciably smaller than the difference between the frequencies of the EPR signals of these forms $\Delta\omega$:

$$1/\tau_R,\ 1/\tau_{RH^+} \ll \Delta\omega \tag{18}$$

For the mechanism (16), which is the main one in an acid medium, we have

$$1/\tau_{RH^+} = k_{-1}^{H^+} = k_1^{H^+}K_a = k_1^{H^+} \times 10^{-pK},$$
$$1/\tau_R = k_1^{H^+}[H^+] = k_1^{H^+} \times 10^{-pH}, \tag{19}$$

where $K_a = k_{-1}^{H^+}/k_1^{H^+}$ is the equilibrium constant of reaction (16) (by definition $K_a = 10^{-pK}$). For the mechanism (17), which is the main one in an alkaline medium, we have

$$1/\tau_{RH^+} = k_1^{OH^-}[OH^-] = k_1^{OH^-} \times 10^{pH-14}, \tag{20}$$
$$1/\tau_R = k_1^{OH^-} \times 10^{pK-14}.$$

On substituting Eqns.(19) and (20) in Eqn.(18), we obtain the condition for slow exchange:

$$\lg(k_1^{H^+}/\Delta\omega) < pK,\ pH < 14 - \lg(k_1^{OH^-}/\Delta\omega)\,. \tag{21}$$

For diffusion-controlled proton exchange reactions, $k_1^{H^+} \simeq k_1^{OH^-} \simeq 10^{10}$ litre mol^{-1} s^{-1} [1] and $\Delta\omega \simeq 10^7$ s^{-1} for the protonation of the N(3) atom (Fig.2). On substituting the values of $k_1^{H^+}$, $k_1^{OH^-}$, and $\Delta\omega$ in Eqn.(21), we obtain the condition for the slow frequency exchange in the EPR spectra for diffusion-controlled proton exchange reactions:[61]

$$3 < pK < 11\,. \tag{22}$$

This condition is satisfactorily consistent with the available experimental literature data. Indeed, for the radicals (III),[46] (XXVIII),[19] (XXXII), (XXXV)–(XXXIX), (XLI), and (XLII) [51] with $pK < 3$, the hydroxynitroxy-radical ($pK = 12.96$),[32] a series of alcohol radicals,[28] and the radicals (XLVIII) and (XLIX) [51] with $pK > 11$, a rapid $\dot{R} \rightleftarrows \dot{R}H^+$ proton exchange was observed. For the radicals (VI)–(VIII),[48] (XXX), (XXXI), (XXXIII),[62] (XLIII), (XLIV), (XLV), and (XLVII) [51] with pK in the range $3 < pK < 11$, a slow exchange was observed. Finally, for the radical (XL) [51] ($pK = 2.5$) and the radicals $CH_3\dot{C}HOH$ ($pK = 11.51$) and $(CH_3)_2\dot{C}OH$ ($pK = 12.03$),[28] the character of the exchange is intermediate. On the other hand, the rapid $\dot{R} \rightleftarrows \dot{R}H^+$ frequency exchange in the EPR spectra of the radical (XXIX) ($pK = 4.5$) [19] and the slow exchange in the EPR spectra of the radicals (L) ($pK = -0.7$) and (LI) ($pK = 0.2$),[51] conflict with condition (22). In order to account for these findings in conformity with condition (21), it is essential to postulate that $k_1^{H^+} \gg 10^{10}$ litre mol^{-1} s^{-1} for the radical (XXIX) (i.e. $k_1^{H^+} \simeq 10^{11}$ litre mol^{-1} s^{-1}) and $k_1^{H^+} \ll 10^{10}$ litre mol^{-1} s^{-1} (smaller by 3–4 orders of magnitude or more) for the radicals (L) and (LI). Appreciably lower bimolecular protonation constants $k_1^{H^+}$ compared with the corresponding value for diffusion-controlled reactions have been noted in the literature, for example for CH acids.[1] As mentioned above, the enaminoketo-group in the radicals (L) and (LI) is apparently protonated at the oxygen atom in accordance with Eqn.(15). The redistribution of electron density occurring under these conditions can lead to an appreciable increase in the activation energy for the chemical reaction and hence to a decrease in $k_1^{H^+}$. It is also possible that a decrease in the constants $k_1^{H^+}$ for the radicals (L) and (LI) can be explained by steric hindrance.[51]

(d) Kinetic characteristics of the reactions. The appreciable difference between the parameters of the EPR spectra of the $\dot{R}$ and $\dot{R}H^+$ forms of the imidazoline and imidazolidine radicals (Tables 1 and 2) enabled us[73,74] to propose a new approach to the study of the kinetics of proton exchange reactions by EPR. For the intermediate $\dot{R} \rightleftarrows \dot{R}H^+$ frequency exchange, all the parameters characterising the kinetics of the proton exchange reaction are susceptible to determination by EPR: the rate constants, the activation energies, and the kinetic isotope effects.[61,73,74]

According to Eqn.(22), the intermediate exchange should be observed for radicals with $pK \simeq 3$ and $pK \simeq 11$; among the radicals investigated (Tables 1 and 2), this condition is satisfied by the radicals (XXX) with $pK = 3.0$ and (XL) with $pK = 2.5$. Indeed, for $pH \simeq pK$, the high-field component of the radical (XL) is broadened as a result of the $\dot{R} \rightleftarrows \dot{R}H^+$ exchange.[72] In the case of tha radical (XXX), for increased temperatures spectral changes characteristic of the transition from slow to intermediate exchange are also observed.[61] In conformity with Eqn.(22), such spectral changes with increase in temperature have not been noted for the radical (XXXI) with $pK = 4.7$.

In order to determine the lifetimes τ_R and τ_{RH^+} and the corresponding proton exchange rate constants $k_1^{H^+}$ and $k_{-1}^{H^+}$, the high-field component in the EPR spectra of the radicals (XXX) and (XL) was modelled on a computer.[61,74] In calculating the spectra, use was made of Eqn.(22), describing the line shape of the EPR signal for rapid exchange involving two positions; the unresolved superhyperfine structure was taken into account in two ways: (a) by the direct allowance for the HFI with the methyl protons, whose contribution to the non-uniform broadening of the EPR signal is decisive,[61] and (b) by representing the form of the spectral line by a composite function comprising one calculated by Eqn.(2) and the Gaussian distribution function[73,74] [this is based on the insignificant difference ($\lesssim 5\%$) between the width of the Gaussian distributions in the $\dot{R}$ and $\dot{R}H^+$ forms of the radicals investigated (see also Lebedev and Muromtsev[75])].

The bimolecular protonation constants $k_1^{H^+}$ for the radical (XL), amounting to $3 \times 10^9 - 10^{10}$ litre mol^{-1} s^{-1} (Fig.3) in the experimental temperature range (274–300 K), are appreciably greater than for the radical (XXX) ($k_1^{H^+} \simeq 5 \times 10^8$ litre mole^{-1} s^{-1} at 20 °C); under these conditions, the temperature dependence of τ_R and τ_{RH^+} can be described by a linear function in terms of the Arrhenius parameters and, according to Eqn.(19), we have

$$k_1^{H^+} = 1{,}6 \cdot 10^{14} \exp(-5800/RT),\ \text{litre mol}^{-1}\,\text{s}^{-1};$$
$$k_{-1}^{H^+} = 1{,}0 \cdot 10^{14} \exp(-8900/RT),\ \text{s}^{-1}. \tag{23}$$

The high activation energy for the protonation of the radical ($\Delta E = 5.8 \pm 0.4$ kcal mol^{-1}) may indicate non-diffusional control of the reaction.

The possibility of obtaining quantitative information about the isotope effects in proton exchange reactions using pH-sensitive NR is of interest. A difference has been observed[74] between the EPR spectra of the radical (XL) in H_2O and D_2O (Fig.3). Therefore, in D_2O the temperature dependence of the lifetimes of the radical in the $\dot{R}$ and $\dot{R}D^+$ forms can also be described by a linear function in terms

of the Arrhenius parameters and the rates of the forward and reverse exchange reactions can be formulated as follows:

$$k_1^{D^+} = 9{,}9 \cdot 10^{13} \exp(-6100/RT), \text{ litre mol}^{-1}\text{ s}^{-1};$$
$$k_{-1}^{D^+} = 9{,}7 \cdot 10^{13} \exp(-10\,000/RT), \text{ s}^{-1}. \quad (24)$$

Comparison of Eqns.(23) and (24) shows that, for the given reaction, both thermodynamic (different equilibrium constants) and kinetic (different rate constants) isotope effects are observed. The thermodynamic isotope effect $\Delta pK = pK^{D_2O}/pK^{H_2O} = 0.4$ (at $T = 294$ K). This agrees with the fact that, for the majority of ionising groups, the pK corresponding to ionisation via the loss of a deuteron in D_2O are higher by $\sim 0.4-0.6$ of a unit than the pK for the ionisation of the same groups via the loss of a proton in H_2O.[76] The kinetic isotope effect has the usual value for reactions of this type ($k_1^{H^+}/k_1^{D^+} = 2.7 \pm 0.2$ and $k_{-1}^{H^+}/k_{-1}^{D^+} = 6.9 \pm 0.4$ at $T = 294$ K) and in order to account for it there is no need to resort to tunnelling effects.[5,6] This conclusion is confirmed by the satisfactory validity of the Arrhenius relation for k_1 (Fig.3). We may note that, in the case of the radical (XXX), where the interaction between the N(3) atom and the proton is additionally hindered by the presence of the bulky CH_3 group, the Arrhenius relation does not hold for k_1,[61] the rate constant k_1 being an order of magnitude smaller than for the radical (XL) (Table 3). Naturally the determination of the contribution of tunnelling on the basis of the temperature variation of the constant k_1 requires the measurement of the latter over a wider temperature range. Unfortunately, the proton exchange frequencies can be measured by EPR only when they are close to the difference between the Zeeman frequencies of the protonated and non-protonated forms of the radical, which limits the temperature range for the measurement of k_1 for the radicals (XXX) and (XL) to $\Delta T \simeq 40$ K.[61,74] We may point to the possibility, in principle, of extending the range of measurement of the proton exchange frequencies by EPR involving the use of spectrometers with different working frequencies. As can be seen from the EPR spectra of the radical (XXXI) presented in Fig.1, the difference between the frequencies of the $\dot{R}$ and $\dot{R}H^+$ forms in the X, Q, and 2 mm bands is significantly different (by a factor of ~ 4).

Table 3. The rate constants for the forward (k_f) and reverse (k_r) proton exchange reactions of certain radicals with the compounds BH^+ at 21 °C.[74]

Radical	BH+	$10^{-7}\,k_f$, litre mol^{-1} s^{-1}	$10^{-7}\,k_r$, litre mol^{-1} s^{-1}
(XL)	H_3O^+	850±90	2.7±0.3*
(XXX)	H_3O^+	50	0.05*
(XLVI)	CH_3COOH	20±1.5	53±3
(XXXI)	CH_3COOH	9.3±0.6	9.3±0.6
(XXXIII)	$H_2PO_4^-$	20±2	200±20

*The constant is expressed in s^{-1}.

Thus the EPR method makes it possible to determine all the kinetic parameters of the proton exchange reactions in nitroxy-radicals. However, this method is significantly limited, since it is applicable only to reactions in radicals with $pK \simeq 3$ and $pK \simeq 11$.

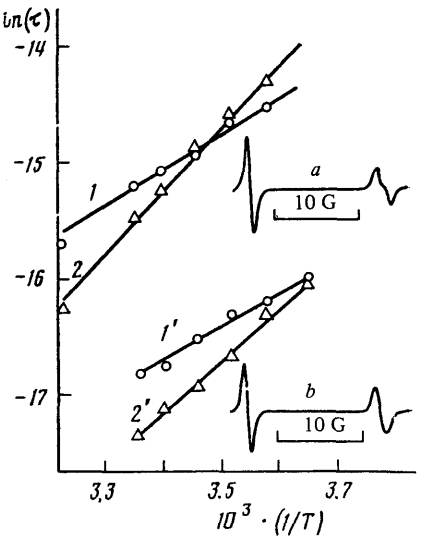


Figure 3. EPR spectra of 5×10^{-5} M solutions of the radical (XL) (the central and high-field components) at 278 K in D_2O (*a*) and H_2O (*b*); temperature variation of the lifetimes τ_R (line *1*) and τ_{RD^+} (line *2*) in D_2O and τ_R (line *1'*) and τ_{RH^+} (line *2'*) in H_2O.[74]

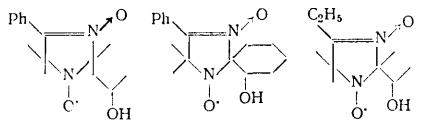
The methodological approach permitting an increase in the rate of the $\dot{R} \rightleftarrows \dot{R}H^+$ exchange without raising the temperature is therefore very useful for stable nitroxy-radicals.[73,74] Previously[30,31] this approach was realised for short-lived radicals by adding to the aqueous solution a buffer with pK_{buf} close to the pK of the radical, as a result of which reaction (7b) becomes the principal exchange mechanism. The influence of the buffer concentration on the rate of exchange has been investigated[73,74] for a series of pH-sensitive stable NR with $3 < pK < 11$, where a slow exchange is observed in the absence of a buffer. An increase in the buffer concentration led to spectral changes characteristic of the transition from the slow to the intermediate exchange and then to rapid exchange (Fig.4). In the region of the intermediate exchange, the lifetimes of the radicals τ_R and τ_{RH^+} and the corresponding rate constants k_f and k_r were calculated by modelling the EPR spectra (Table 3). Table 3 presents the rate constants for the forward and reverse proton exchange reactions of certain radicals with different partners. A correlation has been observed between the bimolecular protonation constant and the steric accessibility of the N(3) atom: in the exchange with a solvated proton in the radicals (XL) and (XXX), which have similar pK, the protonation constant is more than an order of magnitude smaller for the radical (XXX), which has the bulky CH_3 group at the N(3) atom; the replacement of the hydrogen atom at the N(3) atom in the radical (XLVI) by the CH_3 group [in the radical (XXXI)] also leads to a significant decrease in the bimolecular protonation constant in the proton exchange reaction with an acetic acid molecule.

Thus the EPR method is extremely effective in the study of the kinetics and thermodynamics of proton exchange reactions in solutions. The presence of an unpaired electron in the radical does not influence the rate of the chemical proton transfer reaction and makes it possible to follow it by EPR, which creates the possibility of using the proton exchange reactions in NR as model processes in

the study of various factors (the structure of the radical and the properties of the solvent) influencing the proton transfer. It is important to note that the organic chemistry of NR has been well developed and ensures the availability of different NR.[18,22]

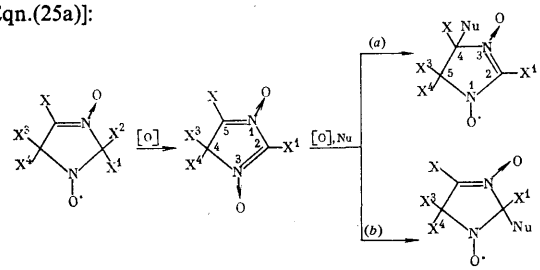
4. Proton Exchange in Nitronylnitroxy- and Iminonitroxy-radicals

(a) The transformations of 3-imidazoline 3-oxide nitroxy-radicals into nitronylnitroxy-radicals as a consequence of a change in pH. In the study of the influence of pH on the EPR spectra of stable 3-imidazoline 3-oxide radicals (LIV)–(LVI) with a hydroxyalkyl group in the 2-position in the heterocycle, it was observed unexpectedly[77,78] that at pH > 12 the spectra of aqueous solutions of these radicals are transformed irreversibly into more complex multiplet spectra typical of NNR:[46,79]



(LIV) (LV) (LVI)

It has been shown[77,78] that in this case the 3-imidazoline 3-oxide NR are indeed converted into NNR in accordance with a general scheme involving the intermediate formation of 4*H*-imidazole *N*-oxides [Eqn.(25a)]:



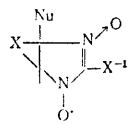
(25)

This reaction leads to extensive possibilities for the synthesis of new NNR; here it is possible to vary both the substituents X–X^4 and the nucleophile Nu (Nu = OH^-, OCH_3^-, NH_3, etc.) as a function of the reaction medium.

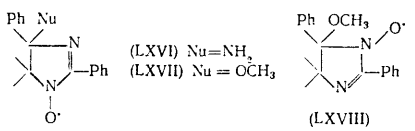
According to Eqn.(25b), nucleophilic attack can be directed not only to the 5-position in 4*H*-imidazole di-*N*-oxide, with formation of a NNR, but also to the 2-position, with formation of new 3-imidazoline 3-oxide NR.

By reducing with triphenylphosphine the NNR obtained by reaction (25a),[46] a series of new 3-imidazoline 3-oxide NR with a substituent (Nu) other than an alkyl substituent at C(2) (Nu = OCH_3 or NH_2) as well as INR have been obtained.[78,80]

Table 4 presents the parameters of the EPR spectra of a series of NNR and INR obtained in accordance with Scheme (25).



	(LXVII)	(LXVIII)	(LIX)	(LX)	(LXI)	(LXII)	(LXIII)	(LXIV)	(LXV)
Nu	OH	OH	OH	OH	NH_2	NH_2	OCH_3	OCH_3	OCH_3
X	Ph	Ph	Ph	C_2H_5	Ph	Ph	Ph	Ph	Ph
X^1	CH_3	$(CH_2)_4CHO$	Ph	CH_3	$(CH_2)_4CONH_2$	Ph	CH_3	$(CH_2)_4COOCH_3$	Ph



(LXVI) Nu=NH_2
(LXVII) Nu = OCH_3

(LXVIII)

Comparison of the HFI constants for the NNR (LVII) and (LVIII) with the corresponding values for radicals enriched in the ^{15}N isotope at N(3) made it possible to assign unambiguously the "nitrogen" HFI constants to the nuclei of the N(1) and N(3) atoms (Table 4).

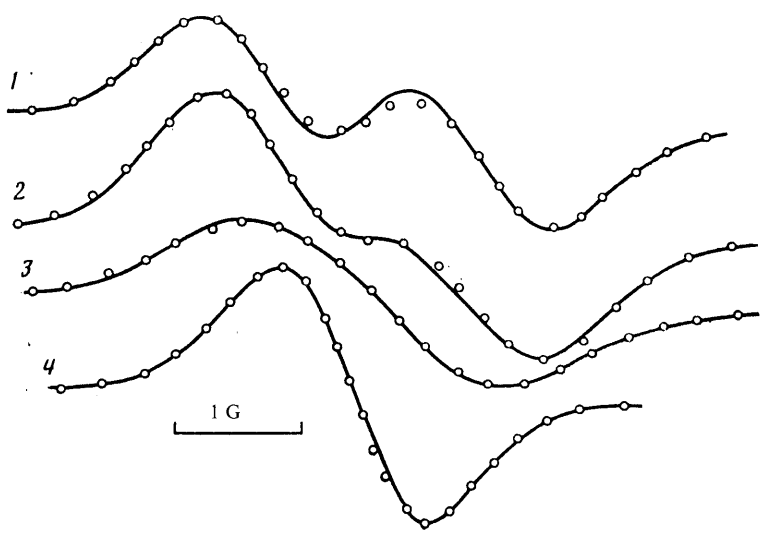


Figure 4. The high-field component of the calculated (circles) and observed (continuous lines) EPR spectra of a 5×10^{-5} M solution of the radical (XLVI) in the potassium acetate buffer (pH 4.35) at 294 K and different buffer concentrations (M): 1) 0; 2) 2.27×10^{-2}; 3) 5.76×10^{-2}; 4) 0.28.[74]

(b) The influence of pH on the EPR spectra of NNR and INR. Table 4 shows that the parameters of the EPR spectra of a series of NNR and INR change as the pH is varied, which has been explained by the protonation of NH_2 or the deprotonation of the OH group at the C(4) atom.[77,78] On the other hand, the EPR spectra of the NNR (LVIII)–(LXV) and the INR (LXVII) and (LXVIII), in the structure of which there are no such groups at C(4) capable of being protonated, did not change in the pH range 1–13.

The EPR spectra of the NNR (LVII)–(LX) in the pH range close to the pK represent a superposition of the spectra of the radicals and the deprotonated form; here p$K \simeq 9.2$ for the OH group in the radicals (LVII)–(LX).[78]

Table 4. The HFI* constants for a series of NNR and INR in H_2O (in gauss).[77,78]

Radical	pH > pK + 1.5					pH < pK − 1.5				
	$a_{N(1)}$	$a_{N(3)}$	a_H	$a_{N(NH_2)}$	$a_{H(OCH_3)}$	$a_{N(1)}$	$a_{N(3)}$	a_H	$a_{N(NH_3^+)}$	$a_{H(OCH_3)}$
(LVII)	8.67	8.28	3.13	—	—	8.00	8.00	3.13	—	—
(LVII)**	8.67	11.65	3.13	—	—	8.00	11.34	3.13	—	—
(LVIII)	8.73	8.22	1.93	—	—	8.00	8.00	1.93	—	—
(LVIII)**	8.73	11.57	1.93	—	—	8.00	11.35	1.93	—	—
(LIX)	8.50	8.00	—	—	—	7.93	7.93	—	—	—
(LX)	8.75	8.45	3.17	—	—	8.13	8.13	3.17	—	—
(LXI)	8.00	8.00	1.90	—	—	7.55	7.55	1.90	1.2	—
(LXII)	8.05	8.05	—	0.2	—	7.60	7.60	—	1.2	—
(LXIII)	7.68	7.52	1.80	—	0.60	7.68	7.52	1.80	—	0.60
(LXIV)	7.70	7.50	1.30	—	0.55	7.70	7.50	1.30	—	0.55
(LXV)	7.67	7.67	—	—	—	7.67	7.67	—	—	—
(LXVI)	9.26	4.27	—	0.5	—	8.37	3.9	—	1.6	—
(LXVII)	9.10	4.10	—	—	—	9.10	4.10	—	—	—
(LXVIII)	8.90	4.00	—	—	—	8.90	4.00	—	—	—

*The accuracy of the determination of the constants is to within 0.05 G.

The radicals (LXVII)* and (LXVIII) are the radicals (LXVII) and (LXVIII) respectively enriched in the ^{15}N isotope at N(3).

The lines in the EPR spectra of the NNR (LXI)–(LXII) initially broaden as the acidity of the medium increases to pH < 3 and beyond and are then split into three components with the intensity ratios 1 : 1 : 1.[78] The observed spectral changes have been explained by the rapid proton exchange in the given radicals and also by the significant increase in the constant for the HFI with the nitrogen nucleus of the NH_2 group when the latter is protonated. Since the relaxation time of the nuclear spin of nitrogen is high compared with the EPR time scale, in describing the experimental spectrum one may assume that proton exchange takes place between the $\dot{R}$ and $\dot{R}H^+$ forms of the radical with the same projection of the nuclear spin of nitrogen S_z^N. Thus, if the resonance frequencies of the $\dot{R}$ and $\dot{R}H^+$ forms of the radical are the same and equal to ω_0, then three lines for the following frequencies are observed in the EPR spectrum:

$$\bar{\omega}(S_z^N = 0, \pm 1) = (\omega_0 + S_z^N\delta_0)(1-f) + (\omega_0 + S_z^N\delta_+)f,$$

where f is the fraction of the $\dot{R}H^+$ form of the radical, δ_0 the HFI constant for the nitrogen of the NH_2 group, and δ_+ the HFI constant for the nitrogen of the NH_3^+ group. By modelling the central component of the EPR spectra of the radicals (LXI) and (LXII), the values of f at different pH were determined[78] and pK = 1.75 for the NH_2 group in these radicals was found.

The values p$K \simeq 9.2$ for the OH group in the radicals (LVII) to (LX) and p$K \simeq 1.75$ for the NH_2 group in the radicals (LXI) and (LXII) are smaller by approximately seven units than the pK of the corresponding aliphatic alcohols and amines,[81] which indicates a strong electron-accepting influence of the nitronylnitroxy-group. In conformity with this finding, deuterium exchange involving the methyl or methylene groups in the radicals (LVII) and (LVIII) is observed at pD $\gtrsim$ 12 in D_2O.[78]

It is essential to note that the character of the $\dot{R} \rightleftarrows \dot{R}H^+$ exchange in the NNR and INR investigated[77,78] is described by condition (22): in the radicals (LXI) and (LXII) with p$K \simeq 1.75$, the exchange is rapid, while in the radical (LXVI) with pK = 4.5 and in the radicals (LVII)–(LX) with p$K \simeq 9.2$ it is slow.

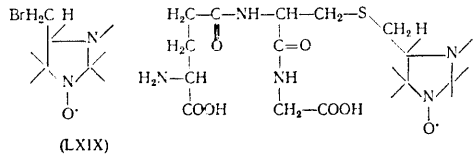
V. THE USE OF NITROXY-RADICALS AS pH PROBES

It follows from the preceding Sections that there are at present pH-sensitive NR which can be used as pH probes in the pH range 0–14. The measurement is then accurate to within 0.05 of a pH unit. This is especially important for biological systems including cell systems.[82,83]

1. Spin-labelled pH-Sensitive Proteins and Peptides

The spin-labelled pH-sensitive derivatives of the peptide hormone angiotension have been used[84,85] to study the conformational transitions and intramolecular interactions in the given octapeptide as a function of pH. The pH dependence of the EPR spectra of radical analogues of angiotensin containing NNR instead of the imidazole ring of histidine [similarly to the radical analogues of histidine (XXIII) and (XXIV)] has been investigated.[84] The observed spectral changes permitted the conclusion that an ion–dipole interaction takes place between the carboxylate ion and the C-end of the octapeptide and the nitronylnitroxy-ring. Such ion–dipole interaction promotes the stabilisation of the "loosened" structure of the hormone at neutral pH and its absence on protonation of the carboxy-group in an acid medium leads to a more compact "random" structure of angiotensin.[84] The above conformational transition has been recorded[85] from the change in the EPR spectra of the spin-labelled analogues of angiotensin, which the pH-sensitive radical (VI) was attached to the N-end, as a function of pH. It was concluded that the conformational transition is accompanied by the protonation of the amino-group at the N-end of the octapeptide.

When glutathione was modified by the radical (LXIX), a pH-sensitive spin analogue of the given tripeptide R-Glut was obtained:[63]



(LXIX) (*R*-Glut)

The parameters of the EPR spectrum of R-Glut in the pH range 1.5–4.5 varied to a much greater extent (Δa_N = 1.2 G, $\Delta g \simeq 0.0002$) than for the radical analogues of angiotensin ($\Delta a_N \lesssim 0.3$ G, $\Delta g \lesssim$ 0.0001). This made it possible to use R-Glut as the pH-probe in the

study of the mechanism of proton transport through model biomembranes.[86,87]

We may note that the alkylating radical (LXIX) is capable of binding covalently to different functional groups of the aminoacid residues of proteins (SH, NH_2, NH [88]) with formation of macromolecular pH probes. In the solution of many biological problems, the possibilities involving the application of macromolecular spin pH probes are much greater than for the low-molecular-weight probes. A macromolecular probe of this kind has been obtained by modifying protein—human serum albumin—by the radical (LXIX).[61] The changes in the spectral parameters of the spin-labelled albumin in the pH range 1.7—4.7 (Δa_N = 1.15 G, $\Delta g \simeq 0.0002$) are due to the protonation of the N(3) atom of the radical ring and are not associated with the possible conformational transformations of the protein.

2. Proton Transport Through Model Phospholipid Membranes

Proton transport plays an important role in the regulation of a number of cell functions,[82,89] which has stimulated numerous investigations of the mechanisms of the transmembrane proton transfer.[90–100] In the study of proton transfer, through phospholipid vesicles, molecular pH probes are widely used; in most cases this is based on the possibility of inserting them into the inner volume within the vesicles (liposomes) and of recording with their aid the pH within the liposome (pH_{in}).[99,100] The change in the characteristics of the pH probe (fluorescence intensity,[99] the chemical shift of the ^{31}P NMR signal[100]) after a transmembrane pH gradient has been established makes it possible to measure the kinetics of the variation of pH_{in} and thus the proton fluxes across the membrane.

A spin pH probe (R-Glut) has been used to record the kinetics of the variation of the intraliposomal pH.[63,86,87] It was shown that R-Glut does not flow out of the inner volume within phosphatidylcholine (PC) monolamellar liposomes. When the pH in 0.1 M potassium citrate buffer is altered, a shift in the lines of the triplet EPR spectra of the R-Glut radical is observed without change in the line shape (rapid $\dot{R} \rightleftarrows \dot{R}H^+$ exchange); the high-field component then shifts to the greatest extent. For this reason, the intensity of the R-Glut spectrum at the frequency $\omega(pK)$ corresponding to the centre of the high-field component at pH = pK = 3.0 is a convenient parameter for the investigation of the kinetics of the variation of pH_{in}.

When a transmembrane pH gradient was created by altering the HCl concentration in the external volume relative to the PC liposomes, a change in the intensity $I(t)$ of the EPR spectrum of R-Glut was observed at a frequency $\omega(pK)$; when the sign of the HCl gradient was changed and its magnitude was reduced, the sign of the change in $I(t)$ was altered and its rate was diminished[63] (Fig.5). By analysing the initial section of the $I(t)$ kinetic curve, the derivative $(dI/dt)_{t=0}$ was determined, its value permitting the calculation of the proton flux j_{H^+} through a unit surface of the membrane in the initial instant after the pH gradient has been established. Table 5 presents the values of j_{H^+} following the creation of the same transmembrane pH gradient by varying the concentrations of different acids in the external volume relative to PC liposomes. The constancy of the EPR signal (over a period of ~2 h) in the case of the H_2SO_4 gradient in 0.1 M potassium citrate buffer shows that the electrically non-compensated proton transport, the K^+-dependent proton transport, and the transport of H^+ in the form of an undissociated citric acid molecule are slow processes and that their contribution to the observed $I(t)$ relations in the presence of HCl, $HClO_4$, and HNO_3 concentration gradients may be disregarded. The changes in $I(t)$ in the cases indicated are therefore determined by the anion- or X^--dependent transport of H^+ (X^- = Cl^-, ClO_4^-, or NO_3^-) or(and) the transport of protons in the form of the undissociated acid HX. Table 5 shows that the proton flux, calculated from the $I(t)$ relation, increases after an H^+ gradient has been established with the aid of acids having a high pK value (in the series $HClO_4$, HCl, and HNO_3, the pK are respectively –8, –7, and –1.62 [81]). This fact permits the conclusion that protons are transported in an acid medium in the form of the undissociated acid HX; this has been confirmed by the absence of an effect on j_{H^+} of the surface charge of the membrane, varied by adding the negatively charged phosphatidic acid or the positively charged 3-cetyl(methyl)ammonium bromide, while the addition of cholesterol diminishes j_{H^+} significantly. It is noteworthy that, according to the data of Nichols and Deamer,[90] approximately the same changes in the surface charge of the PC membrane of liposomes led to a significant alteration of the overall permeability coefficient of the membrane for protons and hydroxide ions (by a factor of 3—4). The conclusion that protons are transported through PC membranes of liposomes in the region of pH ~ 3 in the form of the undissociated acid did in fact make it possible to calculate the permeability coefficients P_{HX} for HCl, $HClO_4$, and HNO_3 (only the upper limit was estimated for H_2SO_4) (Table 5). The values of P_{HCl} and P_{HNO_3} obtained by the EPR method are close to those measured[92] on planar lipid membranes ($P_{HCl} \simeq$ 3 cm s^{-1}, $P_{HNO_3} \simeq 10^{-3}$ cm s^{-1}); proton transport through the membrane was also not recorded[92] for H_2SO_4.

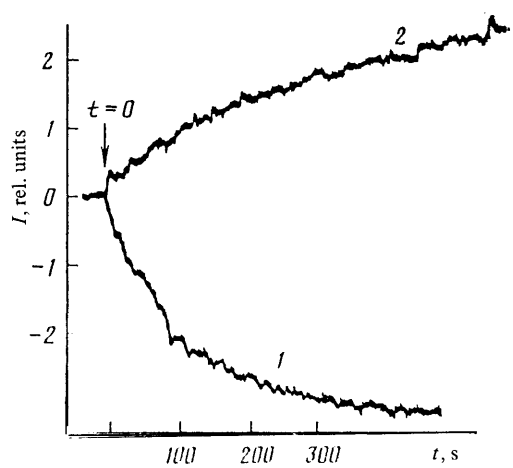


Figure 5. Time variation of the amplitude of the EPR spectrum of the R-Glut radical at the frequency $\omega(pK)$ after the establishment of a transmembrane HCl gradient:[63] 1) pH outside liposomes pH_{out} = 1.63, pH_{in} = 3.13; 2) pH_{out} = 4.63, pH_{in} = 3.13.

A new method for the investigation of the kinetics of proton transport through biomembranes, proposed in a number of studies,[63,86,87] can be extended to the region of neutral and alkaline

pH, since spin probes pH-sensitive in the given regions are now known.

Table 5. Proton fluxes j_{H^+} through the membrane of PC liposomes immediately after the establishment of a transmembrane pH gradient (pH_{out} = 1.63; pH_{in} = 3.13) using various acids and the permeability coefficients P_{HX} of the membrane for these acids.[63,86,87]

Acid	$HClO_4$	HCl	HNO_3	H_2SO_4
j_{H^+}, mol cm litre^{-1} s^{-1}	$2\cdot10^{-10}$	$1.8\cdot10^{-9}$	$3\cdot10^{-8}$	$\lesssim10^{-12}$
P_{HX}, cm s^{-1}	2.2 ± 1	2 ± 1	$(1.4\pm0.7)\cdot10^{-4}$	$\lesssim10^{-7}$

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As can be seen from the above discussion, the use of stable NR sensitive to pH makes it possible to obtain quantitative information about the thermodynamic and kinetic characteristics of the very simple proton transfer reaction. Major advances in the organic chemistry of stable radicals[18,101,102] have led to the possibility of a detailed investigation of the influence of steric factors, electron-donating and electron-accepting substituents, and the solvents on the characteristics of the proton transport. Unique experimental possibilities for the study of isotope effects have been created by the application of the EPR method in such systems, including systems in non-aqueous solvents in the absence of solvation effects.

The use of pH-sensitive NR as pH probes in chemical and biological systems is extremely promising. The possibility of introducing hydrophobic and hydrophilic groups into the NR permits their use for the determination of pH within microheterogeneous regions. The determination of pH within cells and certain cell structures, for example, liposomes, is of special interest. Measurement of the rate of proton transport through natural membranes by EPR may prove useful in the solution of many problems in bioenergetics.

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