Dinitrosyl Iron Complexes as a "Working Form" of Nitric Oxide in Living Organisms

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FOREWORD

The monograph offered for your attention concerns a new trend in biology, viz., the biology of nitric monoxide (NO). The discovery, in the second half of the last century, of the biological role of this simplest gas molecule as one of the most universal regulators of metabolic processes occurring in virtually all representatives of the living world, had a dramatic impact on the global scientific community: In 1992, the internationally known journal *Science* bestowed upon NO the awe-inspiring title "The Molecule of the Year". In the period that followed, it was found that other simple gas molecules, e.g., carbon monoxide (CO) and hydrogen sulfide (H_2S), also possess high biological activity and can exert regulatory effects similar to those of NO. So, the time is ripe to talk about the emergence of a radical new branch in biology, viz., the biology of gas mediators whose biological effects are not expressed by specific receptors as is the case with cytokines, hormones and other endogenous mediators of cell and tissue metabolism. The biological activities of gas mediators are manifested primarily in their direct effects on intracellular targets. The oxygen allotrope ozone, whose biological effects are not only long-known but also resemble many features of NO, is yet another typical representative of this class.

Dinitrosyl iron complexes (DNIC) with thiol-containing ligands were discovered in yeast cells by Robert Nalbandyan and myself in the period 1963–1964. Two years later, the presence of DNIC in animal tissues was first demonstrated by Lev Blumenfeld, Anatoly Chetverikov and myself. Both discoveries were made by using the electron paramagnetic resonance (EPR) method, which enabled the detection of the characteristic EPR signal of DNIC at $g_{aver.} = 2.03$ (the so-called 2.03 signal). This method proved to be unique in its ability to identify the nature of centres responsible for the 2.03 signal as a mononuclear form of protein-bound or low-molecular DNIC with thiol-containing ligands.

In the 1970s, it was established that the crucial role in the formation of DNIC with thiol-containing ligands is played by loosely bound iron as part of the "free" intracellular iron pool. As regards NO incorporation into DNIC, studies have shown that the generation of NO by living organisms is a result of nitrite reduction.

In the 1970−1980s, the main efforts of our research team were focused on studies of the origin and biological effects of newly discovered DNIC. Our pioneering activities in this area made us a laughing stock among colleagues and friends, who called the situation “a smart deadlock”. However, by the mid-1980s, when the number of publications on the issue was increasing with every passing month, I finally came to realize that as an indispensable component of DNIC, NO may be endowed with high biological activity. Supporting evidence for this hypothesis came from the discovery by Robert Furchgott (1980) of the endothelium-derived relaxing factor (EDRF), whose vasorelaxing effect on isolated blood vessels was very similar to that of nitrite under conditions of ultraviolet irradiation. More recent studies by Ferid Mured established that the heme-containing enzyme guanylate cyclase becomes activated by sodium nitroprusside, which is able to produce NO in living organisms − a fact I was already familiar with at that time. Inasmuch as NO possesses a high affinity for heme iron and guanylate cyclase contains heme groups, it was evident that the binding of NO to iron in the heme-containing groups of the enzyme would increase the guanylate cyclase activity. And, finally, the inhibiting effect of hemoglobin on EDRF-induced relaxation in cavernous tissues of the penis provided conclusive evidence that NO represents an active form of EDRF. As regards the ability of animal cells and tissues to produce NO, this was established in our earliest studies of DNIC.

The equivalence between EDRF and NO was first postulated by Salvador Moncada et al. in the article published in *Nature* in 1987. Studies conducted in the subsequent years established that NO is continuously generated in living organisms by L-arginine in the presence of NO synthases. Finally, after the discovery made in the 1990s that NO represents an active participant in the synthesis of DNIC with thiol-containing ligands first detected in our laboratory as early as the 1960s, the blind alley turned into a broad avenue, opening up entirely new (and adequately smart!) vistas for in-depth studies of DNIC and their role in the functional activity of NO.

The main conclusion we inferred from these studies is that DNIC have every right to be regarded as a “working form” of NO and one of the most universal regulators of metabolic processes in living organisms. DNIC with thiol-containing ligands, including protein-bound DNIC, create the necessary prerequisites for the stabilization and accumulation of NO and its biologically active species, viz., NO^+ ions. In addition, low-molecular DNIC are responsible for the transfer of NO and NO^+ from different body cells and tissues to their biological targets, e.g., heme- and thiol-containing proteins and enzymes.

In my book, which is synchronized with my life and takes stock of my more than 50-year research experience, I try to avoid a monotonous recitation of uninteresting events and uninspiring scientific facts for the benefit of a retrospective journey into the history of one or another discovery, beginning with the first successful registration of the EPR signal and a search for the centres responsible for it and ending with a design of new-generation remedies, including DNIC-based drugs with a broad range of therapeutic activities and other developments of crucial importance for the good of all mankind.

May I express the hope that the data described herein will convince my readers of the truth of my statement that DNIC with thiol-containing ligands can function in living organisms as a “working form” of NO and as one of the most universal regulators of metabolic processes occurring in various cells and tissues.

CHAPTER 1

THE OPENING STAGE IN THE STUDY OF DINITROSYL IRON COMPLEXES AND THEIR ROLE IN BIOLOGICAL SYSTEMS (1960−1980'S): HOW IT ALL STARTED

1.1. The discovery and identification of dinitrosyl iron complexes in baker's yeast and animal tissues (1960s–1970s)

The paradigm of the vital role of free radical species in the regulation of an immense diversity of metabolic processes occurring in living organisms emerged among the community of biophysicists, biochemists and other practitioners in general biology in the middle of the past century as a milestone event in the discovery, by academician N.N. Semenov, a famous Soviet physicochemist and a Nobel Prize winner, of active molecules (free radicals) endowed with an ability to control the progress and rates of chain chemical reactions. It was very tempting at that time to cogitate on the ability of highly reactive free radicals to control a vast array of biological processes occurring in human and animal organisms at relatively low ($\leq 40^0$C) temperatures. In his monograph entitled *Introduction to a Submolecular Biology* (1960), Albert Szent-Györgyi, a famous American biochemist with Hungarian roots, asserted, with a great deal of enthusiasm and spirit, that all living matter, including body cells and tissues, are literally swarming with free radicals (1). This viewpoint was consonant with the data obtained as long ago as the 1930−1940s by the German biochemist and physicochemist Leonor Michaelis, who was the first to call attention to the ability of many organic compounds to undergo redox conversions to reach a certain active semi-oxidized free-radical state which then determined their fate. The discovery and introduction of the electron paramagnetic resonance (EPR) method into routine research practice in the early 1950s gave a further powerful incentive to the free radical exhilaration.

The pioneering studies in this field were performed by Barry Commoner in the USA and Lev Blumenfeld in the USSR (2, 3). A comprehensive study of enzymatic reactions occurring in the EPR spectrometer resonator in the aqueous phase led Commoner and his coworkers to demonstrate, for the first time, that in the course of free-radical processes, active centres of enzymes and enzyme substrates pass into an EPR-detectable free-radical state characterized by the presence, in EPR spectra, of a symmetric EPR signal with a halfwidth of 1.2-1.5 mT and a g-factor at 2.005. Strange as it may seem, a group of Soviet researchers, led by Lev Blumenfeld, had to experiment with lyophilized cells and tissues because of the unavailability of adequate equipment to measure EPR signals in wet samples of biological materials. The incubation of dry tissue samples in a humid atmosphere triggered the appearance of a narrow EPR signal with a halfwidth of 0.7 mT. The exact identification of this signal in the 1980s led us to hypothesize that this EPR signal was produced by the semi-oxidized free-radical form of ascorbic acid (4). It is not out of place to mention here that in 1937, Albert Szent-Györgyi was awarded the Nobel Prize for Physiology and Medicine for the discovery of vitamin C and the biological oxidation in plant cells.

In the 1950−1960s, at the crest of the free-radical hullabaloo, the possibility of detecting hitherto unknown free radicals in living objects seemed to hold especially great promise. And it was particularly at that time that two young Moscow University (MSU) postgraduates, Robert Nalbandyan of the Faculty of Chemistry, and myself, Anatoly Vanin of the Faculty of Physics, took the bull by the horns. Under the guidance and following the recommendations of Professor Lev Blumenfeld, our scientific advisor, we conducted an in-depth EPR analysis of free-radical centres in samples of yeast cells. We proceeded from the conjecture that the concentration of free-radical centres varied depending on the rate of cell metabolism, being increased in the growth (log) phase or decreased during the transition to the terminal (steady-state) phase. In actual fact, this transition was accompanied by the appearance, along with the narrow symmetric EPR signal of organic free radicals at $g = 2.005$, of an additional EPR peak at $g = 2.04$, which was shifted to a distance of ~ 50 oersted from the EPR signal of free radicals in the direction of the low field (fig. 1).

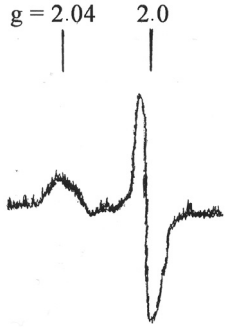

Fig. 1. The peak of an EPR signal first recorded in 1963 in dried preparations of yeast cells at g = 2.04

The 2.04 peak was discovered during the EPR analysis of yeast cells grown on a synthetic Reeder's medium supplemented with sodium chloride, phosphates, magnesium salts, glucose, and nitrates. After the substitution of this growth medium with a natural medium containing molasses isolated from fermented wort and sugar beet waste, the intensity of the EPR signal increased dramatically along with the appearance in the EPR spectra of an additional high-field peak at g = 2.014 with a peak at g = 2.04 (fig. 2, A). The fact that both components (2.04 and 2.014) of the EPR signal disappeared synchronously after the heating of dried yeast samples to 90−100^{0}C and displayed similar behavior during the microwave saturation of the EPR signal led us to hypothesize that both signals were nothing more than constituents of the same inhomogeneously broadened EPR signal. The latter represented an envelope of the spin packets with a halfwidth of 4 mT and was characterized, judging by its shape, by five bends on the first derivatives of the EPR absorption band and two values of the g-factor tensor ($g_{\perp}$ = 2.04 and $g_{||}$ = 2.014). Based on the average value of the g-factor (g_{aver} = 1/3 (2 $g_{\perp}$+ $g_{||}$)), this EPR signal was defined as the 2.03 signal.

The irreversible disappearance of the 2.03 signal at 90−100^{0}C (363 and 373K) (fig. 2 **B**) was suggestive of the binding of the paramagnetic centres responsible for the 2.03 signal to the protein globule denatured at high temperatures or, more specifically, of their protein origin. Supporting evidence for this conjecture was obtained in experiments where the 2.03 signal preserved its anisotropic shape after the moistening of dry yeast with water to a biologically active state (fig. 2 **C**). This finding unambiguously suggested the relatedness of the paramagnetic centres to

macromolecular compounds whose mobility was insufficient for averaging the anisotropy of the g-factor, as was the case with low-molecular compounds.

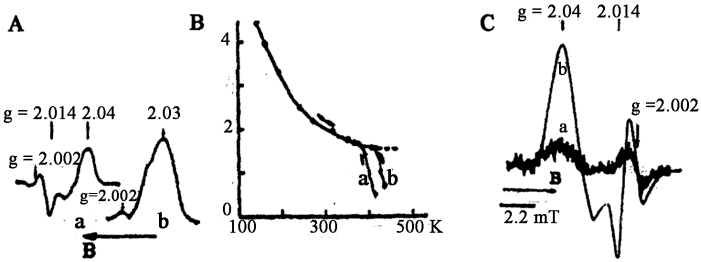

Fig. 2. **A:** The shape of the 2.03 signal recorded in dried yeast samples: the EPR absorption curve (**b**) and its first derivative (**a**). **B:** The dependence of the amplitude of the 2.03 signal on registration temperature (100−500K) (**a** and **b**) determined in yeast samples during their incubation in the air or in hermetically sealed ampoules, respectively. **C:** The shapes of the 2.03 signals recorded in samples of wet (**a**) and dried (**b**) yeast at ambient temperatures in 1965−1966 (6, 7).

It must be noted in this connection that in the year preceding this discovery, my coworker, Galina Morozova, succeeded in recording the 2.03 signal in samples of *Manilica murmanica* yeast residing in the waters of the Barents Sea. This finding is not documented anywhere but a reference thereto can be found in (6).

For the first time, the data concerning the discovery of the 2.03 signal in yeast cells and the early results of our studies of the nature of paramagnetic centres responsible for this signal (hereafter referred to as 2.03 complexes) were published in the *Proceedings of the All-Union Conference on Free Radicals* in 1964 and in 1966 (5) and in the Soviet journal *Biofizika* in 1965–1966 (6, 7).

Based on the data obtained in these studies, which took into account the significant deviation of the g-factor values of the 2.03 signal from the spin values (2.0024), we suggested that the 2.03 signal was generated by sulfur-containing free-radical centres. This hypothesis was subsequently confirmed in experiments on yeast samples treated with thiol group blockers, which led to the disappearance of the 2.03 signal (7). Some publications from that time reported on the detection of EPR signals generated by the paramagnetic centres of sulfur-containing compounds, the unpaired electron in which was localized on sulfur atoms (9−11). Some characteristics of these signals, including their g-factor values,

appeared to be very similar to those of the 2.03 signal (8) but their shapes, determined by the anisotropy of three different values of the g-factor tensor, were suggestive of the difference of these signals from the 2.03 signal characterized by two values of the g-factor tensor. For this reason, the statement that the 2.03 signal is generated by sulfur-containing paramagnetic centres can be accepted with certain limitations.

In 1965, the journal *Nature* published an article by a group of US investigators led by B. Commoner, one of the pioneers in EPR research, describing the successful attempt to record the 2.03 signal (or, more exactly, its constituent component at g = 2.035) in the liver of rats treated with various hepatocarcinogenic agents (12). This signal was detected in the liver samples of rats at ambient temperatures two weeks after treating the animals with hepatocarcinogenic agents and disappeared one month thereafter. Interestingly, in the publication by the British investigators J. Mallard and M. Kent, which had appeared in *Nature* a year before, in 1964 (13), the authors reported on the detection of a weak 2.03 signal in the livers of rats treated with one of the hepatocarcinogens used by Commoner et al. Curiously enough, the data was never cited by the authors of this discovery while Commoner and his colleagues continued to investigate this phenomenon for at least seven years.

At first, the presence of the 2.03 signal in malignantly transformed tissues was considered a specific marker of carcinogenesis. However, our studies dating back to 1966−1967, in which intense 2.03 signals were detected in the intact liver tissues of rabbits and pigeons (14) untreated with biologically active substances, fully disproved this hypothesis.

The first records of the 2.03 signal obtained simultaneously by our research team and by independent groups of US and British investigators are illustrated in fig. 3 (Panels A−D). The right panel depicts the results of EPR studies of activated cytotoxic animal macrophages, the signal 2.03 in which was recorded 25 years after its discovery (15). By that time, the capability of animal cells and tissues for continuous generation of nitric monoxide (NO), the simplest chemical compound and one of the most universal regulators of an immense diversity of metabolic processes, had become a well-established fact. NO represents a product of the enzymatic oxidation of the amino group in the guanidine residue of L-arginine by O_2 in the presence of constitutive isoforms of NO-synthases (NOS) or, more specifically, of endothelial and neuronal e- and n-NOS (16). NO can also be generated by activated immunocompetent cells as effectors of the system of cell-mediated immunity in the presence of an inducible isoform of NOS (iNOS). Based on our findings and the results obtained by other investigators suggesting that the 2.03 signal was hypothetically generated

by DNIC with thiol-containing ligands, J. Lancaster and J. Hibbs proposed that these DNIC were generated by activated macrophages in the presence of endogenous NO formed from L-arginine under the catalytic effect of iNO-synthase (iNOS) (15). Conclusive evidence in favour of this hypothesis was obtained in experiments on the treatment of activated rat macrophages with the iNOS inhibitor *N*-methyl-L-arginine, where the inhibition of DNIC synthesis was manifested in the attenuation of the 2.03 signal (fig. 3, right panel).

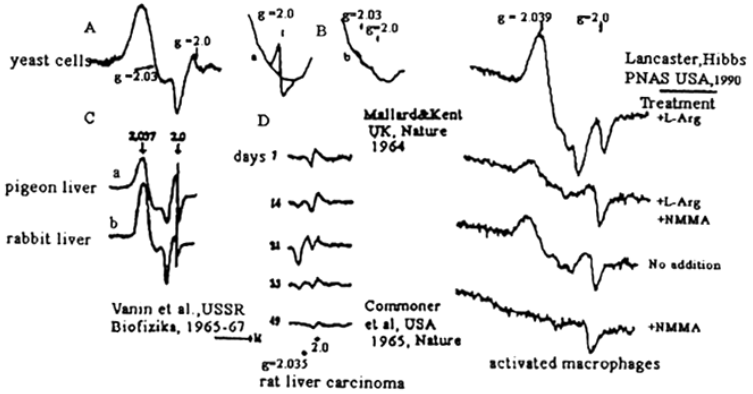

Fig. 3. The 2.03 signals recorded in the 1960s in: (A) yeast cells (6); (B, b) chemically induced rat hepatoma tissue (13); (C, a, b) pigeon and rabbit livers (14); (D) chemically induced rat hepatoma at different periods following treatment of rats with hepatocarcinogenic agents (12). Right panel: The 2.03 signals recorded in 1990 in activated rat macrophages in the presence of L-arginine, L-arginine + *N*-methyl-L-arginine, in the absence of L-arginine + *N*-methyl-L-arginine and in the presence of *N*-methyl-L-arginine alone (15).

A natural question arises: How did we establish the ability of paramagnetic centres of DNIC with thiol-containing ligands to generate the 2.03 signal in the state-of-the-art EPR technology in the 1960−70s? The answer to this question is simple enough: The major incentive for our studies was the article published in the Russian journal *Biofizika* in 1966 in which its authors, Yaroslav Azhipa, Lev Kayushin and Evgeny Nikishkin, reported on the successful registration of the 2.03 signal in the liver mitochondria of rats treated with an overdose of sodium nitrite used as a NO source (fig. 4, Panel B) (17). However, the EPR signals generated by nitrosyl complexes of hemoglobin and recorded by the authors in the blood of experimental animals had entirely different shapes and localizations (fig.

4, Panel A). This circumstance notwithstanding, and stipulating the position of the centre in the triplet hyperfine structure (HFS) of the EPR signal at g = 2.03 (fig. 4, Panel A), the authors suggested that the mitochondrial paramagnetic centres responsible for the 2.03 signal represent nitrosyl complexes of heme-containing proteins similar to hemoglobin-NO or, more exactly, to nitrosyl cytochrome complexes localized in the terminal segment of the mitochondrial respiratory chain.

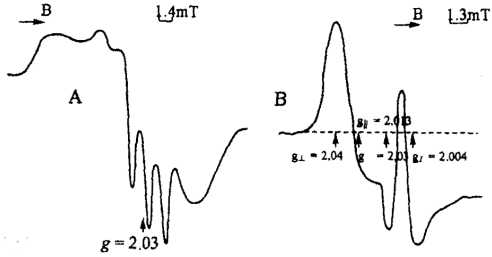

Fig. 4. The EPR spectra recorded in lyophilized preparations of the blood (A) and liver mitochondria (B) of rats treated intravenously with sodium nitrite at 77K (17).

Such identification of the paramagnetic centres responsible for the 2.03 signal (2.03 complexes) allowed Azhipa and his coworkers to claim the discovery of endogenous paramagnetic centres in living organisms. Strangely enough, in the 1970s, there was nothing extraordinary in the allowance of their appeal; further still, the claimants received a large cheque for their work. As for myself, I was all alone at that moment and as good as no-one: my then friend and coworker Robert Nalbandyan had turned his attention to other research objects. Furthermore, I was notified by someone from Azhipa's milieu that any claim for the invention on my part would be thwarted. Joking apart, Azhipa was a "big fish" in the Department of Science within the jurisdiction of the Central Committee of the USSR Communist Party.

Worthy of note is that the statement that the value of the g-factor (2.03) of the EPR signal of nitrosyl hemoglobin complexes (fig. 4, Panel A) which, in the authors' opinion, was a critical factor in identifying the nature of mitochondrial complexes, is not quite true. The authors acknowledged their inaccuracy later by declaring it an erratum (18), which gave us sufficient grounds to consider Azhipa et al.'s definition of 2.03 complexes as heme-nitrosyl complexes unfounded.

As for myself, I was fully aware that 2.03 signals must under no circumstances be associated with the nitrosyl complexes of heme-containing proteins, so plain was the difference between the EPR characteristics of these complexes and those of the 2.03 signal. However, the successful attempt by Azhipa et al. to record an intense 2.03 signal in mitochondria led me to conjecture that these cell organelles are endowed with an ability to generate nitrosyl complexes with the other iron-containing, e.g., iron-sulfur (Fe-S), proteins present in their respiratory chain. By that time (1965−1967), it was well established that in addition to the Fe^{2+} active centres of Fe-S, proteins contain inorganic and thiol sulfur and it was the presence of thiol sulfur in these active centres that strengthened my conviction that Fe-S proteins are responsible for the formation of 2.03 complexes (19). I was fortunate at that point: the authentication of my hypothesis did not require any additional equipment, experiment or treatment of isolated Fe-S proteins with nitrite or NO. Non-heme nitrosyl iron complexes with thiol-containing ligands had been synthesized and characterized as long ago as 1965 by a group of US investigators (C. McDonald, W. Philips and H. Mower) (20), who used EPR as the method of choice in the analysis of nitrosyl iron complexes. Besides, these authors succeeded in recording EPR signals of nitrosyl iron complexes with thiol-containing ligands (cysteine, its ethyl ether and β-mercaptoethanol) after the treatment of aqueous solutions of DNIC with gaseous NO in the presence of bivalent iron. However, the EPR signals of these complexes were recorded only at ambient temperatures, i.e., under conditions where all of them produced similar signals. One of such signals, viz., the EPR signal of nitrosyl iron complexes with cysteine, is shown in fig. 5, panel A.

This EPR signal represented a symmetric singlet with a halfwidth of ~ 7 oersted (0.7 mT) and a well-resolved 13-component HFS with a centre at g = 2.03. Its detailed analysis prompted the conclusion that this EPR signal was generated by the paramagnetic centres of dinitrosyl iron complexes (DNIC) with two thiol-containing ligands (formula **$[(RS^-)_2Fe(NO)_2]$,** the 13-component HFS in which was determined by the interaction of the unpaired electron with the nitrogen nucleus (^{14}N, nuclear spin I = 1) of two nitrosyl ligands and four protons (I = 1/2) in the methylene groups of two cysteine molecules incorporated in DNIC (fig. 5, panel B). Judging by the number of iron atoms in DNIC, the latter represented mononuclear DNIC (M-DNIC) (20). The main finding of this study was that the incorporation of the iron isotope ^{57}Fe in homologous DNIC with anionic ligands (e.g., hydroxyl ions) was accompanied by the doublet splitting of the singlet EPR signal of M-DNIC as a result of the hyperfine interaction of the

unpaired electron with the magnetic nucleus of ^{57}Fe (I = 1/2) (fig. 5, panel C).

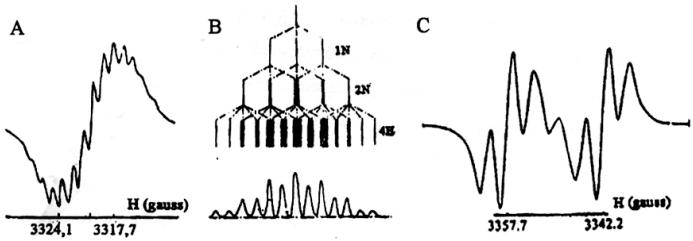

Fig. 5. **A:** The EPR signal of DNIC with cysteine recorded at ambient temperatures (20). **B**: The deciphering of the 13-component HFS of signal A: the triplet and the quintet HFS, respectively, from one and two nuclei of N^{14} (nuclear spin I = 1) of two nitrosyl ligands + additional HFS from four protons (I = 1/2) of the methylene group in two cysteine ligands of DNIC with cysteine. **C**: The doublet HFS of the Fe^{57} nucleus (I = 1/2) in DNIC with hydroxyl ligands (20). The triplet HFS was identified by the hyperfine interaction of the unpaired electron with two nuclei of N^{15} (I = 1/2) incorporated into nitrosyl ligands of DNIC (20).

The striking similarity between the value of the g-factor for the EPR signal of DNIC with cysteine (20) and the average value of the g-factor of the 2.03 signal generated in different cells and tissues prompted my idea to compare the values of their g-factor. The corresponding values for the 2.03 signal were found to be equal to 2.04 ($g_{\perp}$) and 2.014 ($g_{||}$), respectively, suggesting that the difference determining the halfwidth of the 2.03 signal (4 mT) was preserved during the registration of the 2.03 signal at ambient temperatures due to the protein origin of 2.03 complexes and very low mobility of protein molecules in the cell interior, insufficient for averaging the anisotropy of the g-factor. Such averaging is characteristic of low-molecular DNIC with cysteine and is determined by their high mobility in aqueous media. The EPR signals of these DNIC have only one value of g-factor (2.03), which represents the averaged value of the g-factor.

In order to determine the true value of this parameter, I thought it necessary to decrease the mobility of low-molecular DNIC in aqueous solutions to a level enabling the registration of their EPR signals at low temperatures, e.g., in frozen solutions. The results of these EPR studies were in complete agreement with the experiment and beyond all expectations, viz., the major physico-chemical characteristics of EPR signals recorded in frozen solutions of DNIC with cysteine at liquid nitrogen temperature appeared to be fully consistent with those of the 2.03

signal (fig. 6, d`) (21)! Precisely the same phenomenon was established in experiments on DNIC with other thiol-containing ligands; e.g., glutathione, ethylmercaptane and protein-bound thiols (21). After substituting these ligands for hydroxyl ions, H_2O or phosphate in frozen solutions of DNIC, the shapes of their EPR spectra recorded at 77K differed dramatically from the shape of the 2.03 signal (fig. 6, a`−c`).

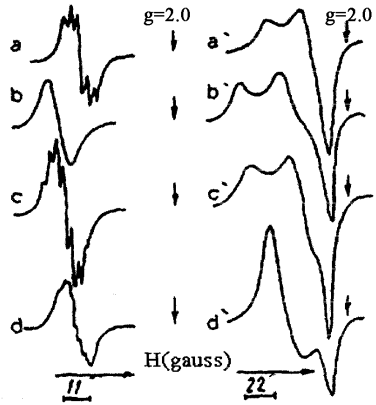

Fig. 6. The EPR spectra of DNIC with hydroxyl (pH 12.0) (curves a and a`), water (pH 7.0) (curves b and b`), phosphate (curves c and c`) and cysteine (curves d and d`) recorded at ambient temperatures (curves a−d) or at 77K (curves a`−d`) (21).

EPR signals that are similar or identical to the 2.03 signal were also recorded in lyophilized preparations of proteins treated with gaseous NO in the presence of exogenous bivalent iron (fig. 7, left panel) as well as in lyophilized preparations of mitochondria, yeast and the $NADH_2$-cytochrome *c* reductase + succinate-cytohrome *c* reductase mixture in the absence of exogenous iron (fig. 7, right panel).

A comparative analysis of 2.03 signals recorded in different biological objects (Figs. 8 and 9) demonstrated their absolute coincidence with the EPR signal of DNIC with cysteine (with regard to shape and other characteristics of the 2.03 signal) recorded in our more recent studies.

The results of a comparison of EPR signals generated by 2.03 complexes in the livers of mice kept on a 5-day drinking diet containing nitrite as a NO source + ^{57}Fe with citrate (curve a) or nitrite + ^{56}Fe with citrate (curve b) with EPR signals of DNIC with cysteine containing ^{57}Fe (curve c) or ^{56}Fe (curve d) recorded at 77K and at ambient temperatures are shown in fig. 8. As can be seen, in the course of registering the EPR

signal of DNIC with cysteine at ambient temperatures, the latter was transformed into a narrow doublet (curve f) and a singlet (curve g), respectively.

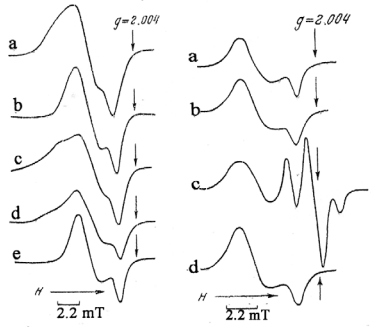

Fig. 7. Left panel: The EPR spectra of lyophilized preparations of proteins treated with NO in the presence of exogenous bivalent iron (a – actomyosin, b – aldolase, c – egg albumin, d and e – DNIC with cysteine (in the form of a lyophilized powder and as frozen solutions, respectively). Right panel: The EPR spectra of lyophilized preparations of mitochondria and yeast treated with NO in the absence of bivalent iron (a − $NADH_2$-cytochrome *c* reductase + succinate-cytohrome *c* reductase, b – yeast, c – bovine heart mitochondria, d – frozen solutions of DNIC with cysteine) (21).

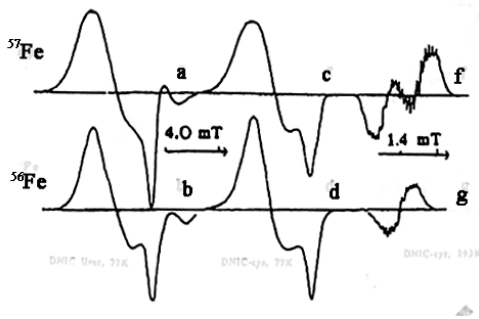

Fig. 8. The 2.03 signal recorded in the livers of mice kept on a 5-day drinking diet containing nitrite + ^{57}Fe with citrate (curve a) or nitrite + ^{56}Fe with citrate (curve b). The EPR signals of DNIC with cysteine containing ^{57}Fe (curves c and f) or ^{56}Fe (curves d and g). The 2.03 signals were recorded at 77K (curves a−d) or at ambient temperatures (curves f and g) (22).

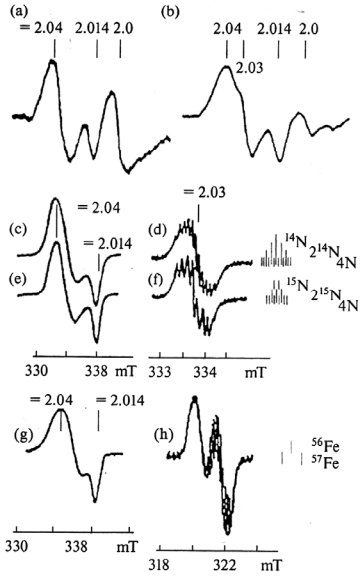

Fig. 9. The 2.03 signals recorded in wet preparations of rabbit liver (curve a) and yeasts (curve b) (23) and the EPR spectra of aqueous solutions of DNIC with cysteine containing ^{14}NO (curves c and d), ^{15}NO (curves e and f) or ^{57}Fe (curves g and h) (23, 24). The EPR signals were recorded at ambient temperatures (curves a, b, d, f and h) or at 77 K (curves c, e and g). Right panel: identification of characteristic HFS of DNIC with cysteine.

A similar correlation was established during a comparison of 2.03 signals recorded in wet preparations of rabbit liver and yeast (fig. 9, curves a and b) at ambient temperatures with EPR signals recorded in frozen solutions of DNIC-^{56}Fe with cysteine (fig. 9, curve c) (23, 24). No such correlation was found after substituting ^{56}Fe with ^{57}Fe, which caused a significant broadening of the EPR signal (fig. 9, curve g). Interestingly, substituting ^{14}N (I = 1) with^{15}N (I = 1/2) in nitrosyl ligands of DNIC with cysteine changed not only their HFS but also the shape of their EPR signal (in its central segment) during registration of their EPR spectra in frozen solutions of DNIC (fig. 9, curves c and e). At ambient temperatures, the difference between magnetic moments of nitrogen nuclei in nitrosyl ligands of DNIC with cysteine initiated the appearance, in the EPR spectra, of a 7-component HFS instead of a 13-component HFS of DNIC with ^{14}NO (fig. 9, curves d and f).

The deciphering of these HFSs (fig. 9, right panel) showed that both of them were formed as a result of the interaction between the unpaired electron and the nitrogen nuclei in two nitrosyl ligands and four protons in two methylene groups of two cysteine ligands adjacent to the thiol sulfur atom. The doublet splitting of the EPR signal resulting from substituting of ^{56}Fe with ^{57}Fe and the hyperfine interaction of the unpaired electron with the ^{57}Fe nucleus (I = 1/2) unambiguously testified to the presence of only one iron atom in DNIC.

These findings gave me sufficient grounds to hypothesize that paramagnetic centres able to generate 2.03 signals in biological objects are DNIC with thiol-containing ligands or, more particularly, DNIC containing the protein residues of cysteine. This hypothesis was consonant with the results of our previous studies (14) designed to compare the effects of compounds able to react selectively with iron (iron chelators), thiol groups of proteins (heavy metals) or compounds able to substitute thiol-containing ligands (xanthogenates) on DNIC with cysteine and 2.03 complexes of the yeast (fig. 10). In the latter case, the EPR signal with triplet HFS appeared in the EPR spectra instead of the 2.03 signal (fig. 10, curve d), most probably as a result of interaction between the unpaired electron and the nitrogen nucleus N^{14} in the *de novo* synthesized mononitrosyl iron complexes with ethyl xanthogenate. These studies demonstrated that the conversions of DNIC with cysteine and 2.03 complexes are mediated by the same mechanism, at least in yeast cells.

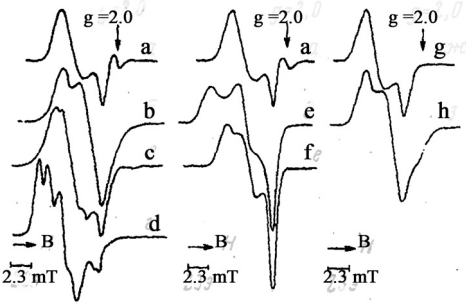

Fig. 10. A comparison of the EPR spectra recorded in samples of yeast cells able to generate the 2.03 signal (curve a) and DNIC with cysteine (curve g) after treatment with the Fe2+ chelator o-phenanthroline (curves b and h), the Fe3+ chelator dithizone (curve c), the thiocarbonate derivative ethyl xanthogenate (curve d), the thiol-specific reagent p-chloromercurybenzoate (curve e) and sodium dithionite (curve f). The measurements were performed 5 min. after treatment. All EPR spectra were recorded at 77K (14).

Despite the complete identification of the 2.03 signal recorded in different biological objects as the EPR signal produced by DNIC with thiol-containing ligands, I did not guess at that time that 2.03 complexes might contain NO. It seemed improbable that NO, generally recognized as an environmentally unfriendly (cytotoxic) compound and one of the major causes of acid rain, could be generated in living organisms by an endogenous route. This circumstance led me to a conjecture (which was later documented in my Ph.D. thesis (1968)) that the true reason for the complete identification of EPR signals of 2.03 complexes with those generated by DNIC with thiol-containing ligands is the presence, in cells and tissues, of a free-radical nitrogen-containing compound whose physico-chemical characteristics are similar to those of NO and relevant to the ability of the latter to produce non-heme paramagnetic iron complexes generating the 2.03 signal (25). The same conclusion was made in respect to paramagnetic centres detected at that time in malignantly transformed cells able to generate EPR signals whose characteristics were very similar to those of nitrosyl iron complexes with hemoglobin (fig. 11, curve d). Here it seems appropriate to refer to the work of the Russian investigators Nikolay Emanuel and Anatoly Saprin, who were the first to detect the EPR signal in tissue samples of some malignant tumours in humans and animals (26).

After the thermal treatment of yeast and animal cells hypothetically capable of generating the 2.03 signal, the latter transformed into a signal closely resembling that recorded in tumour tissues. It was assumed that this transformation was a result of a transfer of a hitherto unknown nitrogen-containing ligand from non-heme iron in 2.03 complexes to heme iron (27); i.e., the nitrogen-containing compound detected in tumour tissues had the same origin as 2.03 complexes.

A very simple experiment carried out in our laboratory reversed the situation. As mentioned earlier in this chapter, 2.03 signals could be recorded in yeast cells not only during their growth on complex molasses-enriched media but also during their culturing on a synthetic Reeder's medium containing calcium nitrate. Removal of the latter from the growth medium did not result in cell death but suppressed the synthesis of 2.03 complexes in them (28). This finding indicated that at least in yeast cells, 2.03 complexes were generated under the action of endogenous NO during reduction of nitrate anions released into the growth medium within the composition of calcium nitrate. This conjecture and the identification of 2.03 complexes as DNIC with thiol-containing ligands were the subjects of my plenary lecture at the 1969 EPR Conference devoted to the 25th anniversary of the discovery of electron paramagnetic resonance by

Evgeny Zavoisky (Kazan', Russia) (29). The fact that heme iron complexes detected in malignantly transformed tissues represented nitrosyl heme iron complexes was well established at that time.

It is not surprising that the next series of our EPR experiments were performed on the liver tissues of inbred mice kept on a one-day diet consisting of bread soaked with a saturated solution of potassium nitrate; control animals were given bread soaked with a solution of potassium chloride (30). The EPR spectra recorded in the liver samples of experimental mice 24h after sacrifice displayed the presence of a 2.03 signal superposed onto the EPR signal of nitrosyl complexes with hemoproteins, presumably with hemoglobin (fig. 11, curves b and c), while in the tissue samples of the control mice, this signal was absent (fig. 11, curve a) (30). The comparative characteristics of the EPR spectrum of the nitrosyl hemoglobin complex and the overall EPR spectra of this and the 2.03 complexes are shown in fig. 11 (curves d and e).

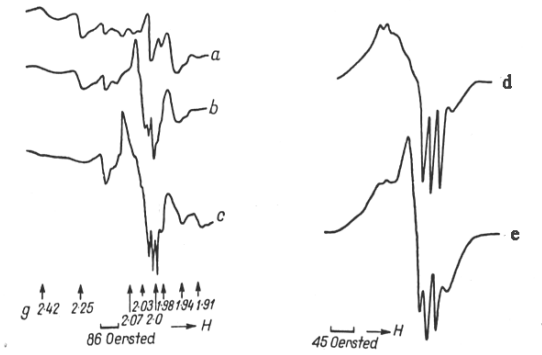

Fig. 11. The EPR spectra recorded in the liver samples of: (a) control mice; (b, c) experimental mice kept on a nitrate-rich drinking diet for one day; (d) the EPR spectrum of a nitrosyl hemoglobin complex obtained by treatment of the hemoglobin solution with gaseous NO; (e) the EPR signal of nitrosyl complexes of hemoglobin with a superposed 2.03 signal. The slope of the baseline reflects the EPR absorption of liquid oxygen present in liquid nitrogen as admixture. All EPR spectra were recorded at 77K (30).

Obviously, the formation of both types of paramagnetic complexes (2.03 complexes and nitrosyl complexes of hemoglobin) in animal tissues was induced by NO generated in the course of the enzymatic reduction of nitrate to nitrite and, further on, to NO. In animal tissues, this reaction usually proceeds under anaerobic conditions, as was demonstrated by

Richard Gscheidlen in his work *Studien über den Ursprung des Harnstoffs im Tierkörper* as long ago as 1874 (31) and corroborated in more recent studies by A. Stepanov (32), F. Bernheim and M. Dixon (33) in the first decades of the 20th century.

Based on these findings, it seemed expedient to examine the effect of nitrite on the formation of 2.03 complexes in animal tissues. Our experimental protocol included the addition of 0.3% sodium nitrite to the drinking diet of mice, which, after a lapse of three to four days, led to the appearance in the EPR spectra of their livers of a distinct 2.03 signal, whose intensity corresponded to the incorporation of iron (~ 0.3 μg per g of wet tissue) into 2.03 complexes (fig. 12, a). The kidney and blood levels of iron incorporated into 2.03 complexes showed a 5-fold decrease while the EPR spectra recorded in other organs of experimental mice did not contain any 2.03 signals (34).

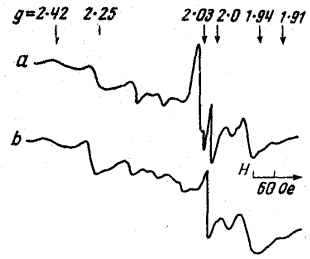

Fig. 12. The EPR spectra recorded at 77K in liver samples of: (a) experimental mice kept on a 4-day drinking diet containing 0.3% sodium nitrite; (b) control mice. The EPR signals at g = 2.42, 2.25 and 1.91, 2.00 and 1.94 were generated by cytochrome P-450, free radicals and reduced Fe-S proteins, respectively (34).

Our hypothesis on the iron-dinitrosyl origin of 2.03 complexes formed in animal tissues received further support in our experiments on the treatment of isolated mouse and rat tissues with gaseous NO, which confirmed the formation of both 2.03 complexes and nitrosyl complexes with hemoglobin.

In 1969, the results of these studies were submitted for publication in the Russian journal *Biofizika* but for reason unknown the article appeared as late as 1971 (30).

Further research in this area developed along similar lines. In 1968, a group of US investigators led by B. Commoner reproduced our experimental protocol but extended it further by including other anionic ligands of DNIC (amino acids, short-chain peptides, proteins) in the

experiment (35). The most remarkable result of these studies was that EPR signals whose characteristics fully coincided with those of the 2.03 signal were generated and recorded only in the frozen solutions of DNIC with cysteine (fig. 13, left panel, curve b). At ambient temperatures, a similar 2.03 signal was recorded for DNIC with aldolase, an enzyme containing cysteine residues (fig. 13, left panel, curve c). Binding DNIC to bovine serum albumin (BSA) was also accompanied by the generation of an EPR signal with a peak at g = 2.03; however, its shape differed drastically from the shape of the "classic" 2.03 signal and was characterized by three values of the g-factor suggestive of a lower (rhombic) symmetry of BSA-bound DNIC compared to the axial symmetry of 2.03 complexes (fig. 13, left panel, curve a).

A more recent publication by J. Woolum and B. Commoner (36) described a successful attempt to record the 2.03 signal in slices of rat liver tissue pre-incubated in a nitrate- or a nitrite-containing medium. This study established that DNIC with thiol-containing ligands are fully identical to the 2.03 complexes recorded in biological objects. The EPR spectra of slices of mice livers treated with nitrite, nitrate or NO recorded by these authors are shown in fig. 13 (right panel).

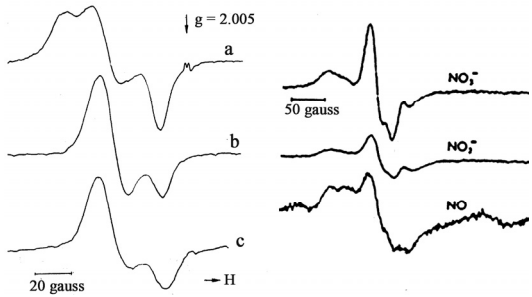

Fig. 13. Left panel: The EPR spectra of DNIC with bovine serum albumin (BSA) (curve a), cysteine (curve b) and aldolase (curve c) obtained after treatment of test solutions of BSA, cysteine or aldolase with Fe^{2+}, ascorbate + nitrite at pH 7.0. The EPR spectra were recorded at ambient temperatures (curves a and c) or at 77K (curve b) (35). Right panel: The EPR spectra recorded in slices of rats' livers treated with nitrate, nitrite or NO at 77K (36). The low-field signal superimposed onto the 2.03 signal was generated by nitrosyl complexes of hemoglobin.

Here it seems appropriate to cite the very interesting results obtained by B. Commoner and his coworkers concerning the relationship between the formation of 2.03 complexes in the livers of rats treated with various

hepatocarcinogenic agents and the presence of nitrites or nitrates in their drinking water (37). In the aforecited study, 2.03 complexes not only formed in the livers of experimental rats after the addition of nitrite to their drinking diet but were also detected in small amounts in the livers of control (non-treated) rats. It was also found that the difference between the concentrations of 2.03 complexes in tissue samples of rats treated with hepatocarcinogenic agents and those fed on tap water containing no additives was caused by the difference between the nitrate content in the drinking water of experimental and control rats. These findings led Commoner to conclude that "NO-Fe^{2+}-thiol complexes" (this term was used by the authors to describe *de novo* synthesized 2.03 complexes) not only had nothing to do with carcinogenesis but, rather, suppressed malignant growth in liver tissues.

1.2. The role of nitrite as a NO donor and weakly bound ("free") iron in the formation of 2.03 complexes (1970s studies)

In the 1970s, we decided to gain a deeper insight into the problem and performed a series of experiments designed to investigate the different effects of nitrite on the formation of 2.03 complexes in mice. These studies established that the simultaneous addition of sodium nitrite (0.3%), used as a NO donor, and bivalent iron within the composition of the Fe^{2+}-citrate complex (0.15% and 0.5%, respectively) to the drinking diet of experimental mice increased the intensity of the 2.03 signal generated in mouse liver 3-fold (to 1.5 μg/g of wet tissue as calculated per endogenous iron content in 2.03 complexes) (fig. 14) (38). The 2.03 signal was also detected in kidney tissues and circulating blood, where its intensity was five times lower than in the liver. In other internal organs (heart, spleen, lungs, skeletal muscle, etc.), the main contribution to the 2.03 signal was made by the circulating 2.03 complexes present in the residual blood of the mice. In the liver, their concentration decreased gradually (~2-fold within a 7-day observation period) as a result of the transfer of experimental mice to a drinking diet without nitrite or Fe^{2+} (fig. 14).

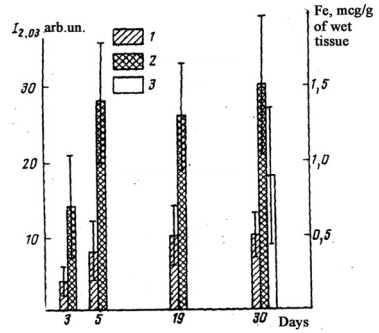

Fig. 14. The changes in the intensity of the 2.03 signal ($I_{2.03}$) recorded in liver samples of mice kept on a drinking diet containing nitrite (1) or nitrite + Fe^{2+} salt (2) for 3, 5, 19 or 20 days with a subsequent transfer of mice to a drinking diet without nitrite and Fe^{2+} (3). Right-side ordinate: the amount of Fe^{2+} incorporated into 2.03 complexes (38).

Based on these data, we suggested that iron added to the drinking diet of experimental mice initiated the formation of 2.03 complexes, or at least increased the probability of their formation. This hypothesis was confirmed in experiments that substituted the iron (^{56}Fe) added to the drinking diet of mice with the ^{57}Fe isotope with a nuclear spin I = 1/2 (39). It was found that ^{57}Fe was indeed incorporated into 2.03 complexes, as could be judged from the characteristic broadening of the EPR signal component, δ, at $g_{\perp}$ as a result of the hyperfine interaction of the unpaired electron with the ^{57}Fe nucleus (fig. 15, curves a and d). Treatment of ^{57}Fe- and ^{56}Fe-containing liver preparations with gaseous NO increased the intensity of 2.03 signals 3-fold as a result of the incorporation of endogenous ^{56}Fe into 2.03 complexes (fig. 15, curves b and e). This effect was manifested in the decrease of the value of the parameter δ, of the 2.03 signal originally broadened at the expense of HFS from exogenous ^{57}Fe.

A comparison of the δ values of the characteristic 2.03 signal generated in the livers of mice kept on a drinking diet containing nitrite and ^{57}Fe to the corresponding parameters of DNIC with cysteine and ^{57}Fe incorporated into them established that ~ 70% of ^{57}Fe was incorporated into the 2.03 complexes of the liver. Very similar results were obtained in experiments with forced *per os* treatment of mice with aqueous solutions of nitrite, iron and citrate. In these studies, the 2.03 signal was generated in the liver within 30 min. of the treatment beginning (40, 41).

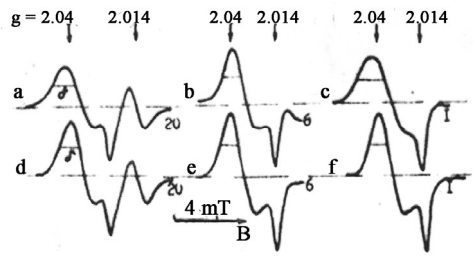

Fig. 15. The shapes of the 2.03 signals recorded in the livers of mice kept on a drinking diet containing sodium nitrite and a citrate complex with iron (^{57}Fe (a) or ^{56}Fe (d)). Signals (b) and (e) were generated in response to treatment of liver samples with NO. Signals (c) and (f) were recorded in liver homogenates of NO-treated mice in the presence of the ^{57}Fe- or the ^{56}Fe-citrate complex. The relative amplifications of the EPR spectrometer are indicated in the right part of the spectra. The value of δ (spectra a and d) defines t halfheight width of the EPR component at $g_{\perp}$; its increase in the presence of ^{57}Fe complexes was induced by unresolved HFS from the ^{57}Fe nucleus (39). All EPR spectra were recorded at 77K (39).

It may be inferred from these data that the formation of 2.03 complexes in the liver and other internal organs of experimental mice in the presence of exogenous iron is the result of the incorporation of low-molecular DNIC formed in the blood or the intercellular fluid into animal tissues and the subsequent transfer of $Fe(NO)_2$ groups of DNIC inside the cell. Our studies on the intraperitoneal (i/p) treatment of mice and rats with synthetic DNIC provided additional evidence in favour of this hypothesis (42). After such treatment, 2.03 complexes were found not only in the liver but also in the circulating blood irrespective of treatment with DNIC with thiol-containing ligands or low-molecular DNIC with phosphate, hydroxyl or thiosulfate (fig. 16). This series of experiments demonstrated that in all cases studied, 2.03 complexes were formed in animal organs as the result of a transfer of the $Fe(NO)_2$ fragment from DNIC with anionic ligands to thiol-containing proteins.

The substitution of ^{56}Fe with ^{57}Fe in the composition of the DNIC with cysteine added to animals was accompanied by a broadening of the 2.03 signal in the liver, kidney and blood caused by the HFS from the ^{57}Fe nucleus corresponding to the 100% substitution of ^{56}Fe in the 2.03 complexes with ^{57}Fe (fig. 17), suggesting that only iron in the composition of the added DNIC was responsible for the formation of 2.03 complexes in animal tissues.