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Review

Dedicated to the memory of
Prof. Petre T. FRANGOPOL (1933-2020)

SPIN LABEL STUDIES OF SOME BIOLOGICAL MATERIALS PERFORMED BY COLLABORATIVE WORK OF ROUMANIAN AND FOREIGN SCIENTISTS – A BRIEF REVIEW

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A brief review of Electron Spin Resonance (ESR) studies of some biological materials: serum albumin, liposomes, purified mitochondrial cytochrome oxidase complex, red blood cell membranes, liver subcellular membranes (mitochondria, microsomes) is presented. The work has been accomplished by collaboration of Roumanian and foreign (Russian, British and American) scientists. The review is dedicated to the memory of the late Professor Petre T. Frangopol, Dr. Mioara Frangopol, Professor Vasile V. Morariu and Professor Manase Dânşoreanu. The priorities of the research reviewed include: the first description of the theoretical and practical aspects of the spin label methodology in Romania, the synthesis at the Institute of Atomic Physics (in Roumanian Institutul de Fizică Atomică, abbreviated as IFA) Bucureşti-Măgurele of compounds used in ESR studies, the use of type ART-6 ESR spectrometer produced at IFA Bucureşti-Măgurele and the adaptation to this ESR spectrometer of a computerized system for the acquisition and processing of data.

INTRODUCTION

Electron spin resonance (ESR) or electron paramagnetic resonance (EPR) is a technique for detecting free radicals and/or other paramagnetic species. It was discovered by the Soviet physicist Evgeny Konstantinovich Zavoisky, described in his doctoral thesis in 1944 and reported in a publication in 1945.[1] The technique was later presented in a lot of books[2-6] and reviews.[7-12] The natural paramagnetism in biological systems is relatively low. Shun-ichi Ohnishi and Harden M. McConnell, working at Stanford University (U.S.A.) had the idea to introduce in biological systems (that do not have intrinsic paramagnetism) stable free radicals, either dispersed or covalently attached to biological molecules. The first pioneering paper, on the interaction of the radical ion of chlorpromazine with deoxyribonucleic acid (DNA), was published in 1965.[13] A review of the papers of H. M. McConnel was published later.[14] Such stable free radicals were called “spin labels” and the technique was named “spin-label technique” or ”spin-labeling technique”. In the

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scientific literature were used actually two terms: "spin label", for a stable free radical covalently bound to a macromolecule or biological system, and "spin probe" for a free radical dispersed in the biological system.[9] Today "spin labeling" refer almost exclusively to the use of stable free radicals containing the nitroxide moiety (the N-O group), which contain an unpaired electron localized on the nitrogen and oxigen atoms (Fig. 1).

The pioneer of the synthesis of nitroxide spin labels was the Russian chemist E. G. Rozantsev.[3,5]

Nitroxides, also called nitroxyl radicals are an important class of compounds, with a very long history. This is fully described in the first chapter of a book published in 2021,[16] chapter available on the publisher's website.

Nitroxides are unusually stable compounds. They can be handled and stored under ambient conditions, without any precaution of temperature, O_2, or moisture exposition. The conservation of the radical properties was early recognized and this key feature of nitroxide compounds explains the widespread research attention on their reactivity and applications as oxidation catalysts, imaging agents and probes in biomedicine and materials science, in energy storage etc.[16]

TEORETICAL AND PRACTICAL ASPECTS OF THE SPIN LABEL METHODOLOGY

Free radicals are atomic or molecular species which have the magnetic moment associated with an unpaired electron. Since electrons possess both spin and charge they behave like magnets. In a strong magnetic field unpaired electrons can exist in two states: either aligned parallel with the field, in a low energy state or antiparallel in a high energy state. Transitions between these energy states may be induced by application of electromagnetic radiation of the appropriate quantum of energy. If the electron is unpaired it is possible to apply electromagnetic radiation to make spin reversal (resonance) occur. It is easier in practice to irradiate the sample with a constant microwave frequency appropriate to the species being studied and vary the magnetic field until resonance occurs, resulting a peak absorption of the microwave frequency.[10]

ESR spectra become more complex when the impaired electron can interact with a magnetic nucleus as is often the case in spin labels. For example, ^{14}N has a nuclear spin of one unit and the magnetic moment of ^{14}N can be aligned parallel, antiparallel or perpendicular to the magnetic moment of the unpaired electron. Thus, each of the two electron energy levels is split into three. From six ESR transitions apparently possible only those which result in a change in the polarization of the nucleus moment are observable. Thus, within a first order approximation one would observe three ESR absorptions, whose separations are equal and represent the hyperfine splitting of the nitrogen nucleus. In practice the first order derivative of the absorption spectrum is recorded.[10]

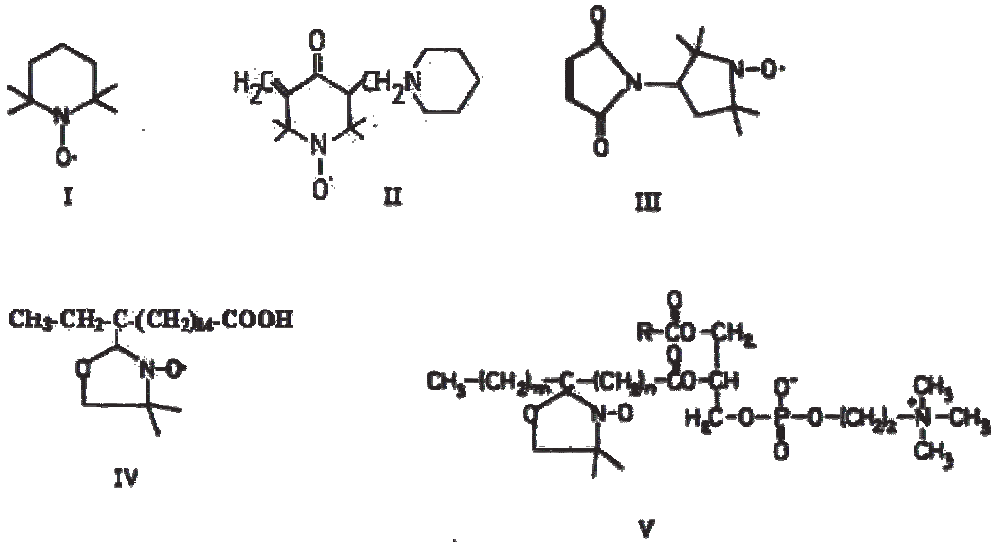

Fig. 1 – Formulas of some spin labels. I: TEMPO (2,2,6,6-tetramethyl-piperidin-1-oxyl; II: 3-methylen-5(piperidin-N-methyl)-4-oxo-2,2,6,6-tetramethyl-piperidin-1-oxyl; III: *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide (MSL); IV: N-oxyl-4'-4'-dimethyl-oxazolydine derivative of the ketostearic acid (16NS); V: a phospholipid spin label. The Figure is reproduced from reference,[15] with permission of *Rev. Chim.* (*Bucharest*), the Copyright holder.

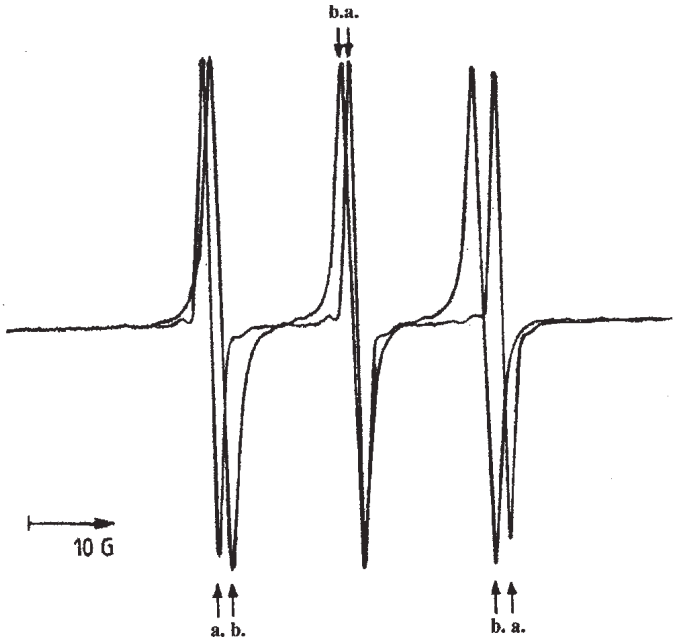

Fig. 2 – The ESR spectra of TEMPO (10^{-3}M) in water (a) and benzene (b). The spectra were obtained with an ART-6 ESR spectrometer produced at IFA Bucureşti-Măgurele. The Figure is reproduced from reference,[15] with permission of *Rev. Chim.* (*Bucharest*), the Copyright holder.

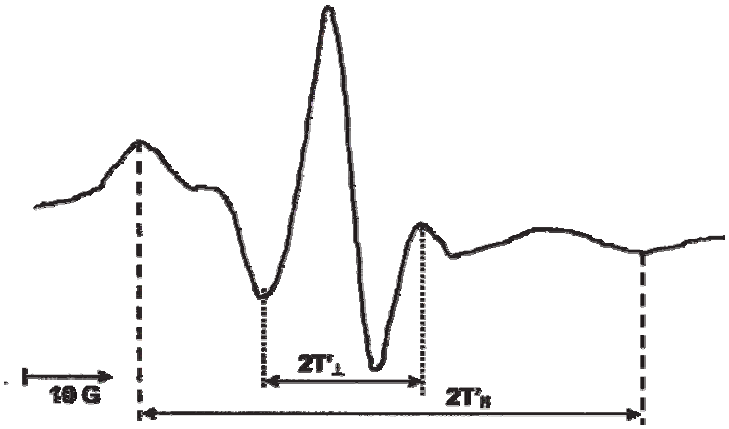

Fig. 3 – The ESR spectrum of a solution of bovine serum albumin (BSA) labeled with MSL after dialysis (24 h at 5 ^{0}C) against distilled water. The spectrum was obtained with an ART-6 ESR spectrometer produced at IFA Bucureşti-Măgurele. The Figure is reproduced from reference,[15] with permission of *Rev. Chim.* (*Bucharest*), the Copyright holder.

ESR spectra of spin labels are very sensitive to the rate at which the label is able to change its orientation. ESR spectra depend on the molecular motion of the nitroxide; consequently, one can evaluate the degree of mobility in the environment of the label.[7-12] An isotropic motion is completely random about all of the axes of the nitroxide. The rapid isotropically tumbling nitroxide gives rise to a spectrum of three sharp lines, called "mobile" spectrum, such as spectra in Fig. 2. The parameter used for estimation of the mobility of spin labels in case of fast isotropic motion is the rotational correlation time (τ_c).This can be defined as the time interval required for a molecule to rotate an angle of one radian.[8] Details regarding the calculations of this parameter may be found in several books and articles.[4,7,9-12] As an experimental parameter of spin label mobility in case of fast isotropic motion the ratio of heights of low field and central line or high field and central line may be used.

When the motion of the label is restricted (Fig. 3) it is difficult to estimate correlation times, particularly since the motion of the spin label is anisotropic. A useful experimental parameter in these cases is the separation between the two extreme components of the ESR spectrum which can be used as a qualitative estimate of the degree of immobilization of the spin label (the so called $2T_{II}$). A greater value of $2T_{II}$ reflects more restricted rotational motion.

There are situations in practice when spectra containing at least two discernable subspectra are observed (complex or composite spectra). For example in Fig. 4 are presented ESR spectra of BSA labeled with MSL. Subspectra corresponding to highly constrained (strongly immobilized), and partly constrained (weakly immobilized) spin labels are evident. Spectrum D is the most immobilized. There are regions in the composite ESR spectra in which one of the two types of spectra makes a contribution to the amplitude. The

ratio of these amplitudes (*b/a or c/a)* serves as a useful qualitative monitor of the conformational transitions of BSA.

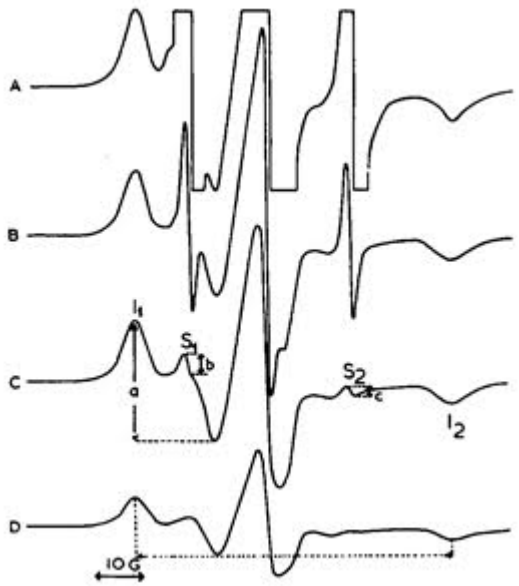

Fig. 4 – ESR spectra of MSL in a BSA solution by dialysis. 1.2 mg of solid MSL was added to 0.34 g BSA dissolved in 5 mL of 0.1 M phosphate buffer (pH 6.8). After stirring for 3 h at 0^0C spectrum A was recorded. The solution was dialysed against phosphate buffer at 5^0C for the following periods: 24 h (spectrum B), 3 days (spectrum C) and 6 days (spectrum D). Spectra were obtained with a Varian E 3 spectrometer in the Department of Chemistry, The University of Sheffield. The Figure is reproduced from reference,[17] with permission of Elsevier Publisher, the Copyright holder.

THE SYNTHESIS AT IFA BUCUREŞTI-MĂGURELE OF COMPOUNDS USED IN ESR STUDIES

Petre T. Frangopol presented in detail the history of IFA Bucureşti-Măgurele, founded in 1956 by Academician Horia Hulubei, under aegis of The Roumanian Academy (R.A.).[18] Petre and Mioara Frangopol begun the work on free radicals in 1960s when they were young researchers at IFA. Among the best young researchers of IFA was also Alexandru T. Balaban. Petre T. Frangopol with Alexandru T. Balaban founded together at IFA the Laboratory of Labelled Organic Compounds (LLOC). An important line of research of LLOC was the preparation and investigation of stable free radicals for use in ESR studies with the ART-6 ESR spectrometer made by electronics specialists at IFA.[9] It was first necessary to prepare the stable free radical 1,1-diphenyl-2-picrilhydrazyl (DPPH), which is used as standard of the position and intensity of ESR signals.[19] This was the starting point for the development of a new domain of basic research at IFA. Other stable free radicals have been prepared. Several articles were published in several journals: *Tetrahedron*,[20,22,23] *J. Chem. Soc.*,[21] *J. Organic Chem.*,[24] *Rev. Roum. Phys.*,[25] *Rev. Roum. Chim.*[26] The work on stable free radicals of the researchers at IFA gained soon international recognition, being cited in well known books by foreign researchers.[3,5,27,28]

In 1973 IFA was incorporated, together with other units of research from Roumania in The Central Institute of Physics (in Roumanian Institutul Central de Fizică, abbreviated as ICEFIZ). In 1977 ICEFIZ was reorganized and new institutes of research in physics and technology appeared, specialized on domains: Horia Hulubei Institute of Physics and Nuclear Technology Bucureşti-Măgurele (in Roumanian Institutul de Fizică şi Inginerie Nucleară Horia Hulubei, abbreviated as IFIN HH), Institute of Isotopical and Molecular Technology (in Roumanian Institutul de Tehnologie Izotopică şi Moleculară, abbreviated as ITIM Cluj-Napoca) etc.

THE INTERNATIONAL COLLABORATION OF PETRE AND MIOARA FRANGOPOL'S GROUP FROM BUCHAREST, GHEORGHE BENGA'S GROUP AND VASILE V. MORARIU'S GROUP FROM CLUJ-NAPOCA, WITH RUSSIAN, BRITISH AND AMERICAN SCIENTISTS

By the end of 1976 Petre T. Frangopol came to the Discipline of Biochemistry, Faculty of Medicine, The Institute of Medicine and Pharmacy (IMF) Cluj-Napoca (to become The "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca – abbreviated as UMF), where I was a young Lecturer, proposing me to collaborate with his group of researchers from IFA. Petre T. Frangopol has heard that I have just returned from UK, after 12 months of work (Sep. 1974- Sep. 1975) in the laboratory of Professor Dennis Chapman (3 months in Sheffield, 9 months in London). I studied protein-lipid interactions in biological membranes using several techniques including spin labeling ESR and the work was reported in several publications. [17,29-34]

Petre T. Frangopol was also aware that Dr. Vasile V. Morariu from ITIM and myself have an article accepted for publication in *Nature*, regarding a generalized membrane defect in children with

epilepsy, namely a decreased water permeability of red blood cells (RBCs), detected by NMR. [35]

I was honored that such an outstanding scientist as Petre T. Frangopol was inviting me to collaborate with him. After visiting his laboratory in București-Măgurele, where I met his wife, the distinguished Mioara Frangopol, I realized that actually at IFA there was the "Professor Petre T. Frangopol and Dr. Mioara Frangopol group". A very fruitful collaboration started between the "Frangopol group" in București-Măgurele and the two groups in Cluj-Napoca: V.V. Morariu's group at ITIM and Benga's group at UMF (where I was nominated in 1978 as Chief of the new Discipline of Cell Biology). Our groups have published many papers over several decades.[15,37-46]

The synthesis of a new spin label for SH groups in proteins was achieved by Petre and Mioara Frangopol in collaboration with the famous group of E. G. Rozantzev. Dr. V. D. Sholle, a member of this group came to Roumania (Bucharest and Cluj-Napoca) and we studied the labeling of BSA and RBC membranes.[36] The label (noted II in Fig.1) of the paramagnetic Mannich bases type, appeared to be suitable to study the conformational changes of BSA and of the RBC membrane proteins. The nitroxide group of the label was unstable in suspensions of RBC membranes and a decrease in ESR signal intensity was noted in time. We interpreted this as being due to the reduction of nitroxide group by RBC enzymes, since the decrease in ESR signal was more marked with intact RBCs than with RBC ghosts.

During the three months of work in Sheffield (1974) I had the chance to meet another very serious British scientist, Dr. William Ferdinand, Senior Lecturer at the Department of Biochemistry, who later came to Cluj-Napoca for a working visit. He collaborated to our studies of mitochondrial membranes.[29]

In 1976 I initiated a collaborative Roumanian-British "Program of Research on Biological Membranes with Medical Applications", between Chelsea College, University of London and IMF Cluj-Napoca. The program was included in the agreement of scientific collaboration between The Romanian National Council for Scientific Research and The British Council and facilitated exchange visits of British and Romanian scientists. The first visit was accomplished by Professor Dennis Chapman, who came in Romania in October 1975. Later I collaborated mainly with Dr. John Wrigglesworth, Senior Lecturer (later Reader), at The Department of Biochemistry, King's College, University of London (Chelsea College being integrated in King's College). For example we purified mitochondrial cytochrome oxidase and the water channel protein from the RBC membranes and performed biochemical, electron microscopic and spin label studies in London and in Cluj-Napoca.[38,39] Moreover, I arranged research visits to Roumania for Drs. John Wrigglesworth and William Ferdinand, and to King's College London for my coworkers Drs. Adriana Hodârnău and Victor I. Pop.

"Professor Petre T. Frangopol and Dr. Mioara Frangopol group" and my group in Cluj-Napoca performed spin label studies regarding the interactions of drugs with biomembranes.[41-43] Together with Mihai Ionescu (from the Frangopol group) and my coworkers (Octavian Popescu and Victor I. Pop) we studied the reduction by chlorpromazine (CPZ) of nitroxide group of the maleimide spin label (MSL, noted III in Fig. 1). We have analyzed by gel electrophoresis the changes in the RBC membrane proteins induced by CPZ. We found that CPZ had a weak solubilizing effect of RBC membrane proteins and actually preferentially reduces the mobile component of the ESR spectrum of MSL-labeled RBC ghosts. Consequently, the action of CPZ is to reduce the free radicals. This action may have consequences for patients receiving long-term treatment with phenothiazine derivatives.[44]

An important international collaboration was established with an American group led by Professor Fred A. Kummerow (Chief of Burnsides Research Laboratory, University of Illinois at Urbana-Champaign). He was a chemist interested in the medical effects of nutrition. In our collaborative work [45,46] we studied in parallel the effects of cholesterol and 25-hydroxycholesterol on egg yolk lecithin liposomes using as spin labels 5NS, 12NS and 16NS. We found that 25-hydroxycholesterol is decreasing the fluidity of the lipid bilayer at very small concentrations (0.5-1%), while cholesterol has a comparable effect at 10% concentration. 25-hydroxycholesterol, a compound angiotoxic and atherogenic, formed by oxidation of cholesterol in the food stored in the frozen state for a long time. Consequently, the use of such food needs to be avoided. Fred Kummerow became famous as he discovered the atherogenic effects of *trans* fatty acids. When he was 99 years old he sued the American Government for not mentioning the atherogenic effects of *trans* fatty acids and won

the lawsuit. Now in dozens of countries (including U.S.A. and E.U. countries) the amount of *trans* fatty acids has to be mentioned on the food packages. Fred Kummerow lived 101 years!

The collaboration between the "Frangopol group" in Bucureşti-Măgurele and the two groups in Cluj-Napoca: V. V. Morariu's group at ITIM and Benga's group at UMF was very fruitful. In addition to the many paper published over decades,[36-46] it was for us (Vasile Morariu and myself) the chance of becoming close friends to Petre Frangopol. We have learnt from him how to write applications to obtain financial support in Roumania, mainly from The *Academy of Medical Sciences* and *The National Council for Higher Education Scientific Research* (in Roumanian *Consiliul Naţional al Cercetării Ştiinţifice din Învăţăm□ntul Superior*, CNCSIS). Using funding from such grants Vasile V. Morariu could finance his group at ITIM Cluj-Napoca, while I succeeded to buy an ART-6 ESR spectrometer produced at IFA Bucureşti-Măgurele. The adaptation to this ESR spectrometer of a computerized system for the acquisition and processing of data was performed by Professor Manase Dânşoreanu (Chief of the Biophysics Department, UMF Cluj-Napoca). The system, as well as a summary of our spin label studies is presented in the last paper published in collaboration with Petre T. Frangopol and Mioara Frangopol.[15]

CONCLUSION

This review is dedicated to the late Professor Petre T. Frangopol (chemist, Honor Member of R.A.), his wife Dr. Mioara Frangopol (chemist), Professor Vasile V. Morariu (physicist) and Professor Manase Dânşoreanu (physician and biophysicist). All four were dedicated researchers, true scientists, who played a very important role in the life and achievements of many persons who had the chance to collaborate with them. Moreover, they were models from all points of vue to younger academics and researchers.

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