C O N T E N T S

C O N T E N T S

Foreword

The present volume hosts papers presented during the 9th edition of the „Metal Elements in Environment, Medicine and Biology" (M.E.E.M.B.) symposium, organized under the auspices of the Cluj-Napoca and Timisoara branches of the Romanian Academy. While all previous editions have taken place in Timisoara, this year the event has been organized at the „Babes-Bolyai" University in Cluj-Napoca, Romania. This series of symposia was initiated in 1993 by the „Working Group for Metal Research in Biological Systems", founded by Timisoara researchers in 1979. Past editions have allowed for inter-disciplinary interaction between researchers from various parts of the world, interested in the importance of metals for various aspects of our life. We thank this year's participants for their valuable contributions, and look forward for even more fruitful discussions during the future editions of this symposium.

THE ORGANIZING COMMITTEE

Acad. Ionel HAIDUC, PhD, Dr.h.c. - **Honorary President**
President of the Roumanian Academy

Prof. Ioan SILAGHI-DUMITRESCU, PhD, corresp. member Roum.Acad. – **Chairman**
„Babeş-Bolyai" University at Cluj-Napoca, Department of Chemistry and Chemical Technology

Gabriela GÂRBAN, MD, PhD - **Co-Chairman**
Institute of Public Health Timişoara

Prof. Zeno GÂRBAN, PhD - Founding member of „Working Group for Metal Research in Biological Systems" Timişoara (1979)
University of Agricultural Sciences and Veterinary Medicine Timişoara

Assoc. Prof. Radu SILAGHI-DUMITRESCU, Ph.D - **Secretary of 9th MEEMB Symposium**
„Babeş-Bolyai" University at Cluj-Napoca, Department of Chemistry and Chemical Technology

BORON CLUSTERS IN CANCER THERAPY

Sven STADLBAUER,[a] René FRANK,[a] Johannes KUNIG,[a] Verena ORTMANN,[b] Annette G. BECK-SICKINGER,[b] and Evamarie HEY-HAWKINS[a,*]

[a] *Universität Leipzig, Institute of Inorganic Chemistry, Johannisallee 29, D-04103 Leipzig, Germany, fax: +49-341-9739319; phone: +49-341-9736151; email: hey@rz.uni-leipzig.de*

[b] *Universität Leipzig, Institute of Biochemistry, Brüderstraße 34, D-04103 Leipzig, Germany*

Abstract

High and selective accumulation in tumor cells is an important requirement for a BNCT agent. Increasing the tumor selectivity of BNCT agents may be possible by the use of carbaborane-containing bis-phosphonates and incorporation of carbaborane-containing amino acids into breast cancer selective neuropeptide Y derivatives.

Keywords: BNCT, carbaboranes, phosphonates, tumor selectivity

INTRODUCTION

To date, the treatment of malignant tumors is always accompanied by extremely negative side effects. One potentially useful approach for selective destruction of tumor cells is boron neutron capture therapy (BNCT), a powerful form of radiotherapy involving preferential incorporation of ^{10}B-containing compounds into tumor cells, followed by irradiation of the tumor with thermal neutrons. The high-energy fission products which are formed on absorption of a neutron allow selective destruction of the tumor cells without affecting the surrounding healthy tissue. High and selective accumulation in tumor cells is one important requirement for a BNCT agent.[1] For successful treatment, a concentration of 30 µg ^{10}B per gram tumor must be achieved. The main problem to date is the availability of boron compounds which exhibit the necessary high selectivity, water solubility, and low toxicity in high concentrations.[2]

The synthesis of and bioactivity studies on the first phosphorus-containing boron cluster compounds bearing phosphate and pyrophosphate moieties were reported by Kaczmarczyk and Bechtold in 1975.[3] However, these compounds turned out to be highly toxic. Interestingly, some simple carbaboranyl bis-phosphonates exhibit high tumor selectivity and can be used in the treatment of calcifying tumors.[4] Oligomeric phosphate diesters which contain *closo*- or *nido*-carbaboranes show high accumulation in tumor tissue in BALB/c mice bearing EMT6 tumors.[5] However, comprehensive biological assessments of boron-containing phosphonates as potential tumor-targeting agents in BNCT are still rare.[6]

We have therefore devised efficient syntheses for novel boron compounds which provide a combined tumor-targeting system: The use of phosphonato groups as phosphate mimics and galactosyl groups for binding to lectins at the surface of a tumor cell.[7]

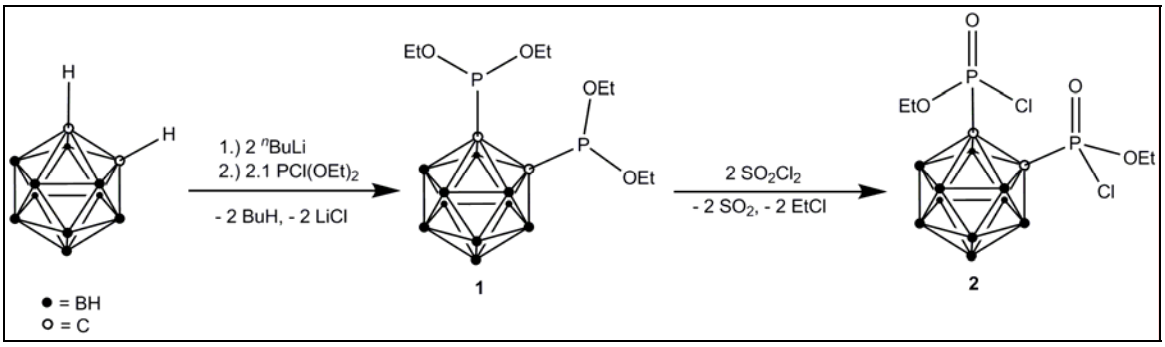
Transformation from a normal to a malignant breast cell is accompanied by overexpression of the Y1 receptor.[8] Beck-Sickinger and co-workers developed a modified neuropeptide Y derivative featuring high affinity for the Y1 receptor.[9] There-

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 3 - 10, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

fore, we synthesized carbaborane-containing amino acids for incorporation in neuropeptide Y to selectively target breast cancer cells.

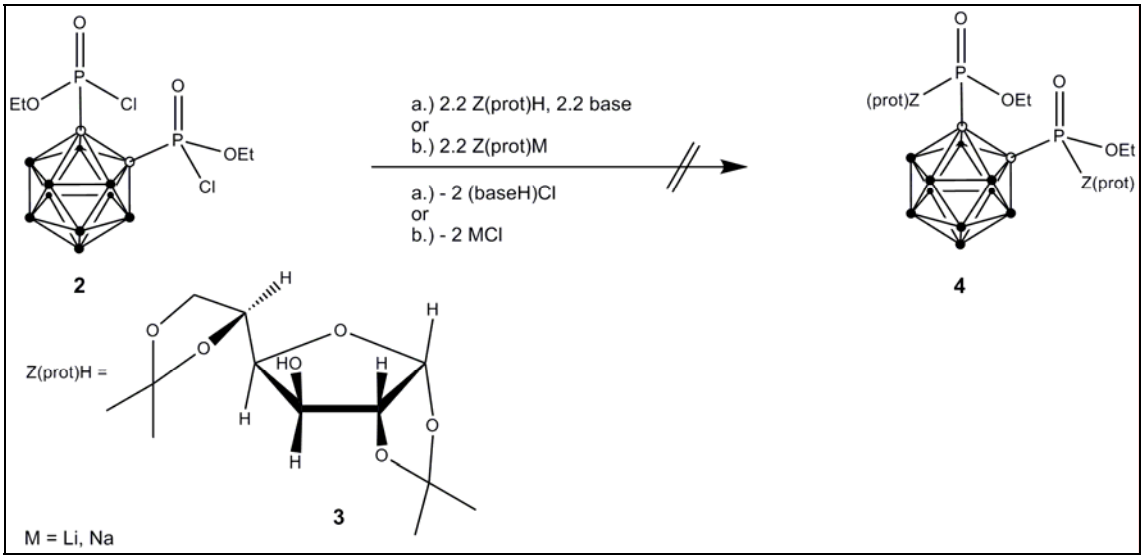
RESULTS AND DISCUSSION

The synthesis of sugar-containing carbaboranyl bis-phosphonates was attempted starting from carbaboranyl bis(chlorophosphonates) and a protected sugar (with one hydroxyl group still available). Thus, carbaboranyl bis(chlorophos-phonate) **2** was obtained from **1** by chlorination with sulfuryl chloride (Scheme 1).[10]



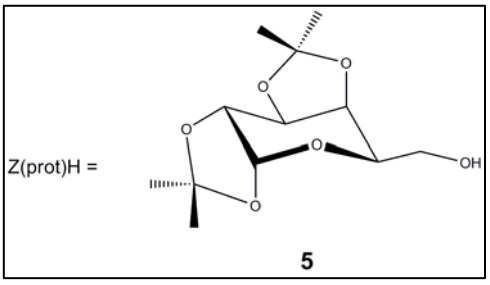
Scheme 1: Synthesis of **2**

However, we were unable to convert compound **2** to the target compound by reaction with 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**3**) under different reaction conditions; in all cases, only decomposition was observed (Scheme 2).



Scheme 2: Reaction of **2** with the sugar derivative **3** led to decomposition

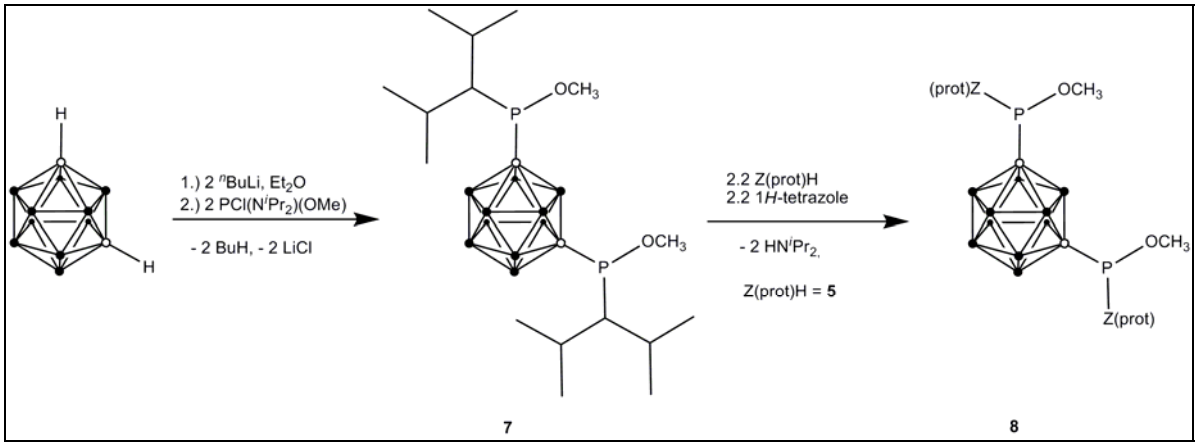
As phosphorus(V) sub-stituents on *ortho*-carbaborane exhibit only low reactivity, more reactive phosphorus(III) deriva-tives were employed. However, no reaction occurred between the dialkylamidohalophospho-nito-substituted *ortho*-carbabo-ranes 1,2-{$P(NR_2)X\}_2C_2B_{10}H_{10}$ (R = Me, ^{i}Pr, X = Cl, Br) and isopropylidene-protected galactose **5**. Employing *meta*-carbaborane instead of *ortho*-carbaborane was



also unsuccessful.

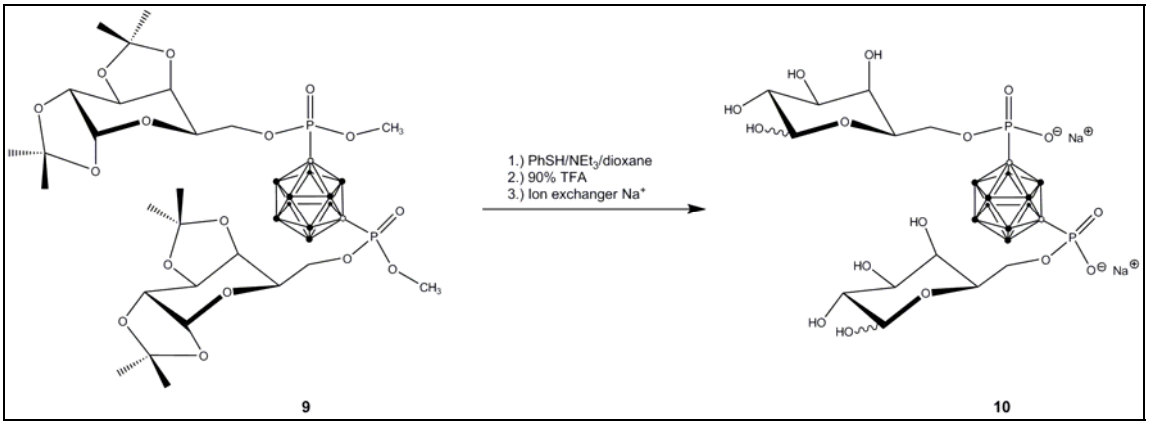
Attempts to employ the activation of a P–N bond by an acidic catalyst, which is well known from oligonucleotide synthesis, to replace the amido group in 1,2-{P(N^{i}Pr$_2$)(OMe)}$_2$C$_2$B$_{10}$H$_{10}$ (**6**) with sugar **5** was not successful when 1*H*-tetrazole was employed as catalyst. A reaction occurred at 140 °C, but with cleavage of the P–C$_{carbaborane}$ bond. The failure of the chloro and amido groups to act as leaving groups could be traced back to strong P–P interactions, caused by overlap of the lone pairs of the phosphorus atoms due to the 1,2-disubstitution pattern.[11] Theoretical calculations showed that methanolysis of {P(NMe$_2$)(Cl)}$_2$C$_2$B$_{10}$H$_{10}$ proceeds 262 times more slowly than for a monosubstituted derivative.

We therefore employed the analogous *meta*-carbaborane **7**, which could be galactosylated with **5** by catalysis by 1*H*-tetrazole (Scheme 3).



Scheme 3: Galactosylation of *meta*-carbaborane derivative **7**

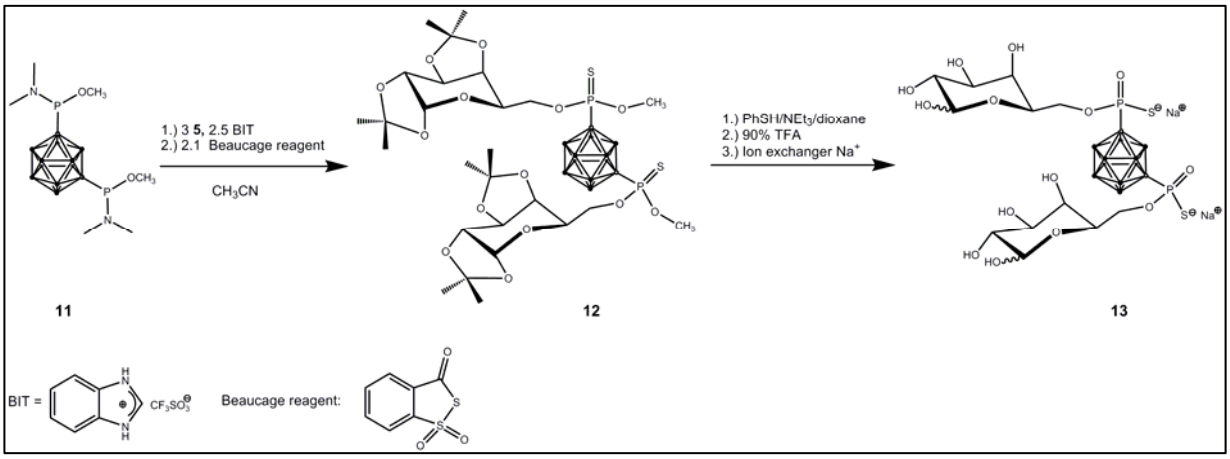
The synthesis of **8** could be improved by using benzimidazolium triflate (BIT) as catalyst under microwave conditions and by replacing the N^{i}Pr$_2$ group with the less sterically demanding NMe$_2$ group. Compound **8** was oxidized with *tert*-butyl hydroperoxide (TBHP) in situ to bis-phosphonate **9**, which was obtained as four diastereomers. Deprotection of the galactosyl and phosphonato groups with trifluoroacetic acid and conversion to the sodium salt gave highly water soluble compound **10** (910 g/L water; Scheme 4).[12]



Scheme 4: Deprotection of the galactosyl and phosphonato groups of **9**

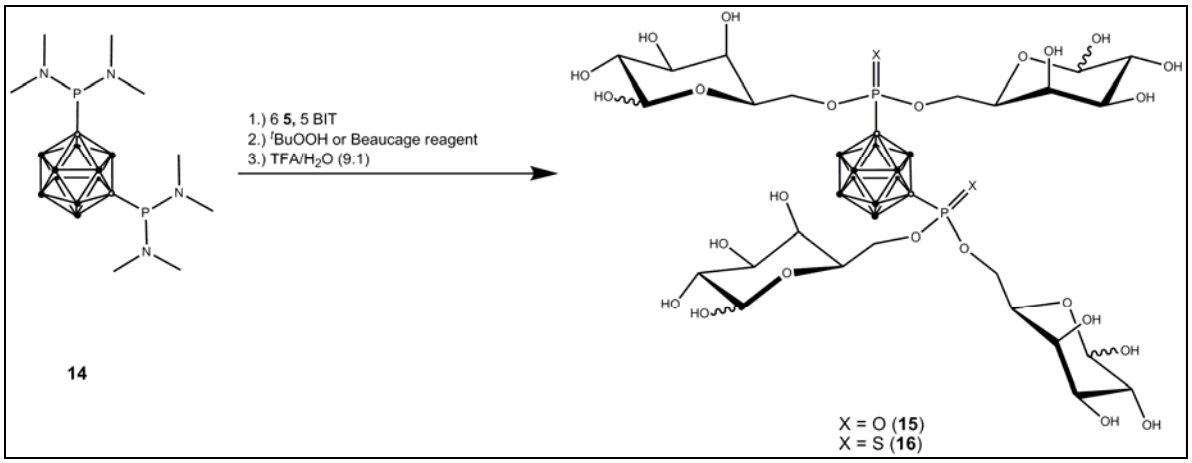
For improved in vivo stability towards phosphatases and phosphonate esterases the corresponding bis-phosphono-thioate **13** was prepared according to this

method using 3*H*-1,2-benzodithiol-3-one-1,1-dioxide, the so-called Beaucage reagent, instead of TBHP (Scheme 5). The water solubility of 830 g/L slightly lower than that of **10**.



Scheme 5: **Synthesis of bis-phosphonothioate** 13 **from** 11

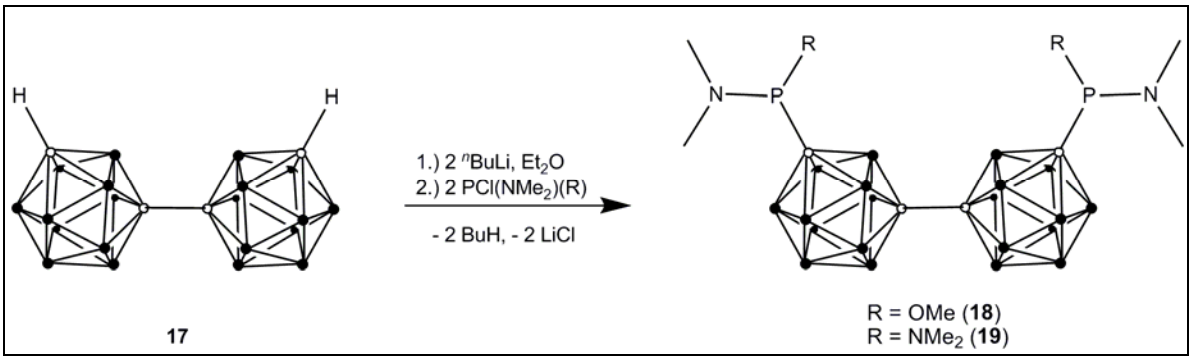
The synthesis of fully galactose substituted carbaboranyl bis-phosphonates **15** and **16** was achieved starting from **14** and **5** by employing the same protocol as described above (Scheme 6).



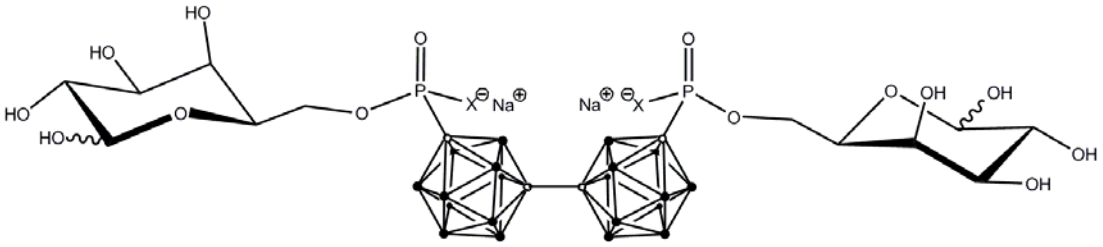
Scheme 6: Synthesis of tetrakis-galactosyl-substituted carbaboranyl bis-phosphonic acids

The biological activity of **10**, **13**, **15**, and **16** was tested on tumor cells of the line HeLa by employing the resazurin assay. Compounds **10** and **13** do not show cytotoxicity up to a concentration of 20 mM and are thus far less toxic than the boron compounds which are presently employed in BNCT, i.e., sodium mercaptoundecahydrododecaborane (BSH, IC_{50} 3.9 mM), and this makes them interesting candidates for BNCT. Compounds **15** and **16** show higher toxicity (EC_{50} ca. 29 mM (**15**), 14.0 mM (**16**)), which is, however, still lower than that of BSH. This can be attributed to the lower water solubility (790 g/L for **15** and 380 g/L for **16**) of these compounds.

Compounds with higher boron contents were obtained by copper-mediated C–C coupling of monolithiated *meta*-carba-borane. Functionalisation of the C–H groups of the resulting bis(*meta*-carbaborane) **17** with $PR(NMe_2)$ groups [R = NMe_2 (**18**), OMe (**19**); Scheme 7] followed by galactosylation, oxidation or sulfurization, deprotection, and conversion to the sodium salt gave galactosylphosphonato-substituted bis(*meta*-carba-borane)s **20, 21, 22, 23** (Fig. 1).[13]



Scheme 7: Synthesis of bis-phosphonito-substituted bis(*meta*-carbaborane) derivatives



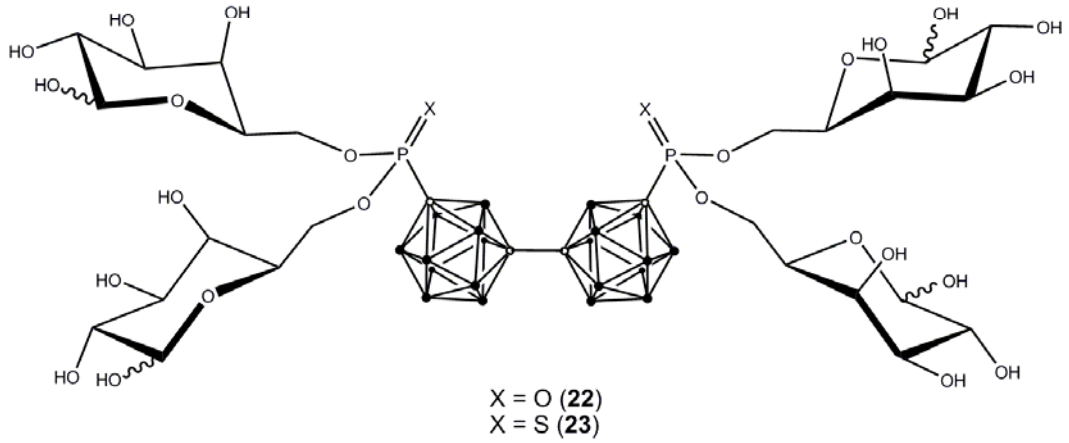


Fig. 1. Galactosylphosphonato-substituted bis(*meta*-carbaborane)s

The presence of the second lypophilic carbaborane cluster increases the cytotoxicity. Thus, bis-phosphonate **20** has an EC_{50} value of 19.5 mM, and bis-phosphonothioate **21** a value of 2.1 mM (Fig. 2). Compounds **22** and **23** have lower

water solubility than the other derivatives, which result in low EC_{50} values in HeLa cells [480.9 µM (**22**), 174.9 µM (**23**)]. Therefore, compounds **21**-**23** are unsuitable for application in BNCT.

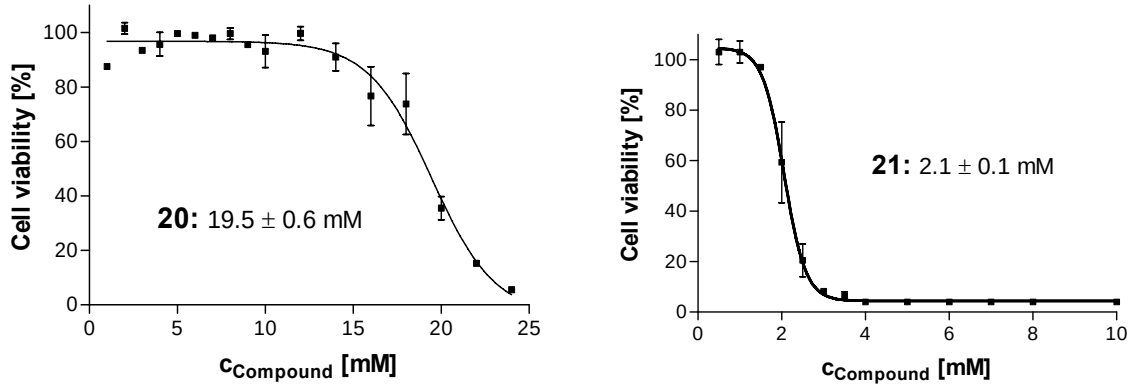
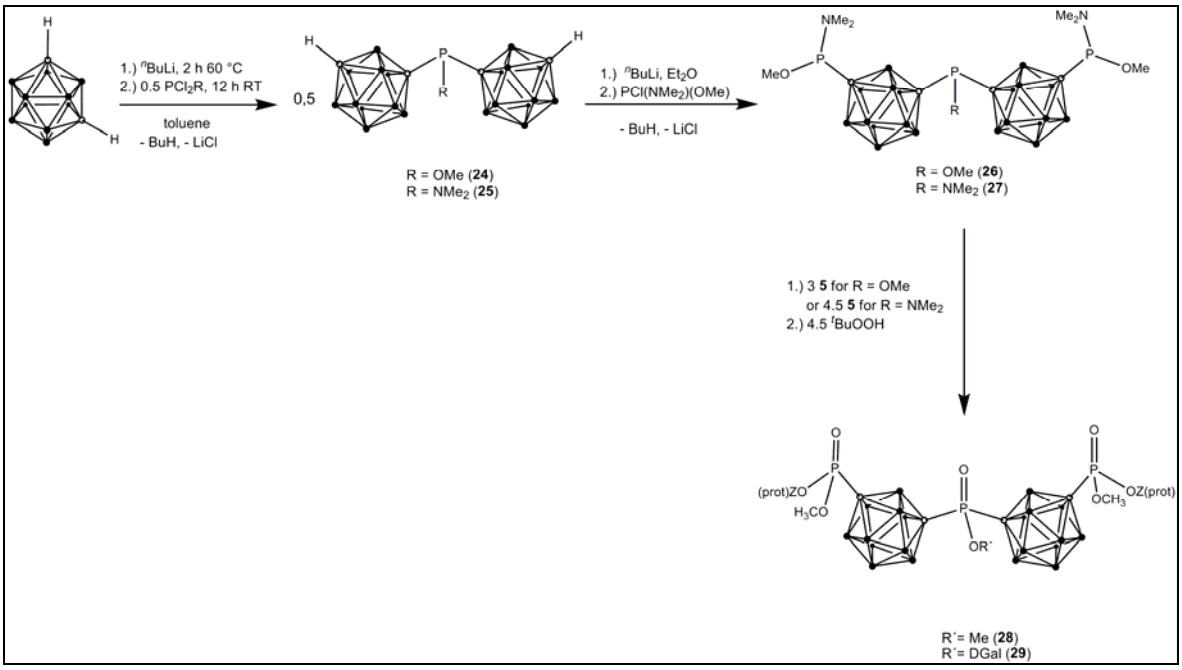


Fig. 2. Cell toxicity data for bis-phosphonate **20** and bis-phosphonothioate **21**

To increase the boron contents but to also improve the water solubility of bis-carbaboranyl derivatives, syntheses of compounds in which the two carbaboranyl clusters are bridged by a hydrophilic phosphinate group were attempted. Monolithiation of *meta*-carbaborane at high temperatures proved necessary to produce the bis-carbaboranyl phosphinites **24** and **25**.



Scheme 8: Synthesis of P-bridged bis(*meta*-carbaborane) derivatives

Conversion of the terminal CH groups to $P(NMe_2)(OMe)$ is readily achieved, but galactosylation of 1,1´-{7,7´-$[P(NMe_2)$-$(OMe)]_2[C_2B_{10}H_{10}]_2$}P(OMe) (**26**) followed by oxidation gave the desired product **28** only in low yield, while 1,1´-{7,7´-$[P(NMe_2)$-$(OMe)]_2[C_2B_{10}H_{10}]_2$}$P(NMe_2)$ (**27**) can be galactosylated only at the terminal P atoms

but not at the bridging P atom (Scheme 8). An improved synthesis for **29** remains to be developed.

Compounds **10**, **13**, **15**, **16**, and **20** exhibit low cytotoxicity and are thus suitable for studies on tumor selectivity. Preliminary studies were carried out in collaboration with Prof. Gabel, Universität Bremen.

The bis-phosphonothioates **13** and **16** were chosen as representative examples to study the in vivo toxicity in Swiss mice. It was shown that compound **16** (dosage 100 mg/kg boron) results in toxic side effects, while compound **13** was well tolerated and thus employed in further boron distribution studies. For these studies four female BALB/c mice with a CRL tumor were subjected to intraperitoneal treatment at a dosage of 100 mg/kg boron. The mice were euthanized after certain periods of time, and then frozen thin sections were made and subsequently irradiated. The results showed high concentrations of the compound in the kidneys, liver, and colon, and only minor concentrations in the tumor. Within 2 h the compound was excreted through the colon. In conclusion, this compound showed a lack of selectivity for this tumor type.

To incorporate carbaboranes in an Y1 receptor selective neuropeptide Y (NPY), *ortho*-carbaborane-containing lysine derivatives (Fig. 3) were synthesized. This lysine derivative was incorporated at the 4-position of [F7, P34]-NPY by manual coupling during peptide synthesis.

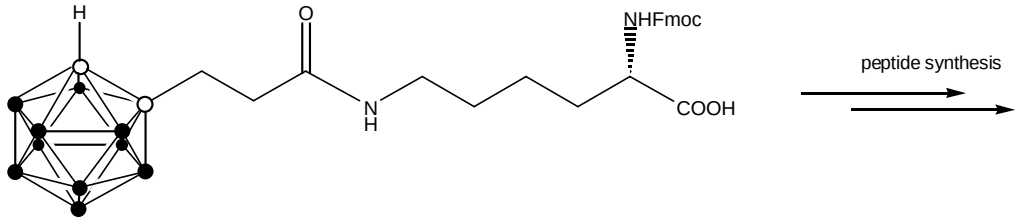


Fig. 3. Carbaborane-modified lysine derivative

A more convenient access with higher yields to carbaborane-containing lysine derivatives is linkage to an SH group at the 9-position of the carbaborane (Fig. 4).

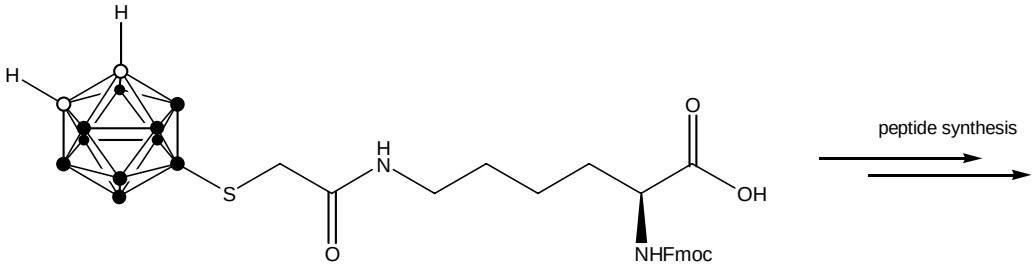


Fig. 4. B9-modified lysine derivative

Preliminary studies on carbaborane-modified NPY derivatives show only minor changes in receptor binding and signal transduction. Therefore these compounds are very promising for future in vivo studies.

SUMMARY

We have developed a suitable synthesis employing the phosphoramidite method to connect *meta*-carbaboranyl bis-phosphonites with the 6´-OH group of iso-

propylidene-protected galactose, followed by oxidation or sulfurization to give the corresponding bis-phosphonates. Deprotection yielded water-soluble compounds. The corresponding disodium salts exhibit especially low cytotoxicity. The fully galactosyl-substituted derivatives are, however, less water soluble and show higher cytotoxicity. Therefore, phosphinato-bridged bis(*meta*-carbaborane) derivatives seem to be more suitable also with respect to increasing the boron contents. In general, the bis-phosphono-thioate derivatives exhibit higher cytotoxicity than the bis-phosphonato derivatives. Preliminary results on the in vivo toxicity and biodistribution of two compounds in mice indicated a lack of selectivity for the CRL tumor chosen for the experiment.

Future studies will focus on different glycosides and their linkage with the phosphorus atom. Thus, glucose, mannose, or disaccharides such as lactose, amino sugars such as galactosamine, and different connectivities, e.g., via the anomeric position, will be employed, and their biological activity will be evaluated.

For the incorporation of carbaboranes into breast tumor selective neuropeptides, a synthesis of carbaborane-modified lysine derivatives was developed. Linkage of the lysine to the boron cluster was achieved by using a propanoic acid spacer. A more convenient synthesis includes the linkage of the lysine to the thiol group at the B9 position of the carbaborane unit. Incorporation of the amino acid derivatives into NPY by peptide synthesis was successful. Preliminary studies show no significant changes in receptor binding affinity and signal transduction.

ACKNOWLEDGEMENTS

We are very grateful to Prof. Dr. Peter Welzel, Universität Leipzig, Institute of Organic Chemistry, for extremely helpful discussions. We are grateful to Prof. Dr. Detlef Gabel, Universität Bremen, for enabling us to carry out the boron distribution studies in his group. We thank Dr. Peter Lönnecke for X-ray structure determinations.

REFERENCES

1 (a) Hawthorne, M. F. *Angew. Chem. Int. Ed.* **1993**, *32*, 950-984; (b) Soloway, A. H.; Tjarks, W.; Barnum, B. A.; Rong, F.-G.; Barth, R. F.; Codogni, I. M.; Wilson, J. G. *Chem. Rev.* **1998**, *98*, 1515-1562; (c) Valliant, J. F.; Guenther, K. J.; King, A. S.; Morel, P.; Schaffer, P.; Sogbein, O. O.; Stephenson, K. A. *Coord. Chem. Rev.* **2002**, *232*, 173-230.
2 Hawthorne, M. F.; Lee, M. W. *J. Neurooncol.* **2003**, *62*, 33-45.
3 Bechtold, R. A.; Kaczmarczyk, A. *J. Med. Chem.* **1975**, *18*, 371-376.
4 Patent: EP 0068584.
5 Lee, M. W.; Shelly, K.; Kane, R. R.; Hawthorne, M. F. *Advances in Neutron Capture Therapy 2006, Proceedings of ICNCT-12, Chemistry and Pharmacy*, (Eds. Y. Nakagawa, T. Kobayashi, H. Fukuda), **2006**, 234-236.
6 Tjarks, W.; Barth, R. F.; Rotaru, J. H.; Adams, D. M.; Yang, W.; Kultyshev, R. G.; Forrester, J. ; Barnum, B. A.; Soloway, A. H.; Shore, S. G. *Anticancer Res.,* **2001**, *21*, 841-846.
7 Stadlbauer, S.; Hey-Hawkins, E. WO2009021978 (A2).
8 Reubi, J. C.; Gugger, M.; Waser, B.; Schaer, J. C. *Cancer Res.* **2001**, *61*, 4636-4641.
9 Söll, R. M.; Dinger, M. C.; Lundell, I.; Larhammer, D.; Beck-Sickinger, A. G. *Eur J Biochem*. **2001**, 268, 2828-2837.
10 Stadlbauer, S.; Welzel, P.; Hey-Hawkins, E. *Inorg. Chem.* **2009**, *48*, 5005-5010.
11 Stadlbauer, S.; Frank, R.; Maulana, I.; Lönnecke, P.; Kirchner, B.; Hey-Hawkins, E. *Inorg. Chem.* **2009**, *48*, 6072-6082.
12 Stadlbauer, S.; Lönnecke, P.; Welzel, P.; Hey-Hawkins, E. *Eur. J. Org. Chem.* in press.
13 Stadlbauer, S.; Lönnecke, P.; Welzel, P.; Hey-Hawkins, E. *J. Org. Chem.* manuscript in preparation.

COMPARATIVE STUDY OF CATALYTIC ACTIVITY OF METALLOPHTHALOCYANINES AND METALLOTETRAPYRAZINOPORPHYRAZINES

Sergei V. Makarov

Department of Food Chemistry, University of Chemistry and Technology.
153000 Ivanovo, Engels str, 7, Russia

** E-mail : makarov@isuct.ru*

ABSTRACT

A comparative study of the reduction of nitrite by L-ascorbic acid and thiourea dioxide in water catalyzed by the octasulfophenyltetrapyrazinoporphyrazine and tetrasulfophthalocyanine complexes of Co(II) was carried out. Kinetic parameters of the different reaction steps of the catalytic process were determined. The final products of the nitrite reduction were found to be nitrous oxide or ammonia.

Key words: porphyrazine, phthalocyanine, cobalt complexes, nitrite, L-ascorbic acid, thiourea dioxide.

INTRODUCTION

Tetrapyrazinoporphyrazines (PyzPz) are aza analogues of phthalocyanines (Pc). Metallophthalocyanines are used in different fields especially as colorants[1], gas sensors[2], field effect transistors[3] and photovoltaic cells.[4] In contrast to the phthalocyanines, tetrapyrazinoporphyrazines are much less studied, although they are receiving rapidly increasing attention due to their effectiveness as photosensitizers in photodynamic therapy of cancer[5], as molecular scaffolds[6]and as highly conducting films.[7] Properties of tetrapyrazinoporphyrazines and their metal complexes are quite different from their phthalocyanine analogues due to the presence of strong electron-acceptor groups in the macrocyclic ligands. Earlier we have found that cobalt (II) octasulfophenyltetrapyrazinoporphyrazine $[Co^{II}\{PyzPz(PhSO_3)_8\}(H_2O]^{8-}$ is an effective catalyst of oxidation of N,N'-dimethylthiourea and thiourea by dioxygen in water under mild conditions.[8] The reaction is shown to include the formation of an intermediate anionic five-coordinate complex followed by a two-electron oxidation to produce the corresponding urea and elemental sulfur. Kinetic and thermodynamic parameters for the different reaction steps of the process were determined. Drastic differences in catalytic activity of cobalt and iron octasulfophenyltetrapyrazinoporphyrazines were observed (catalytic oxidation of thiourea in the presence of iron complex does not proceed with measurable rates at room temperature and pH 5-9).

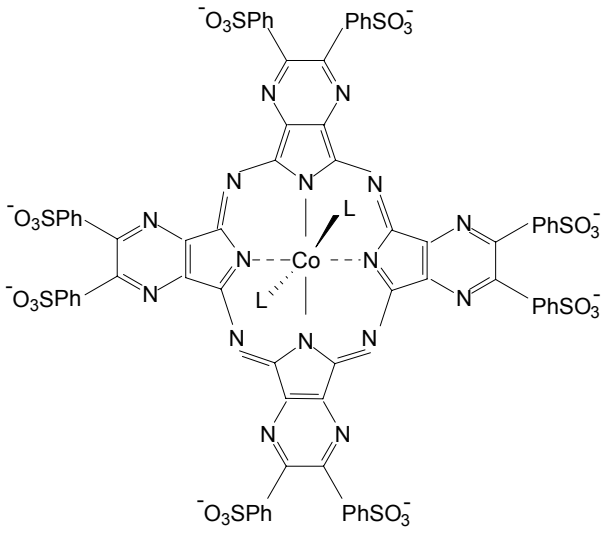
Earlier we used Co(II) tetrasulfophthalocyanine ($Co^{II}(TSPc)^{4-}$) as a catalyst of reduction of nitrite by dithionite.[9] Kinetic parameters for the different reaction steps in the catalytic reduction were determined. Here, kinetic data of the two steps of catalytic process – reduction of cobalt tetrasulfophthalocyanine or octasulfophenyltetrapyrazinoporphyrazine by thiourea dioxide (TDO) and L-ascorbic

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 11 - 20, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

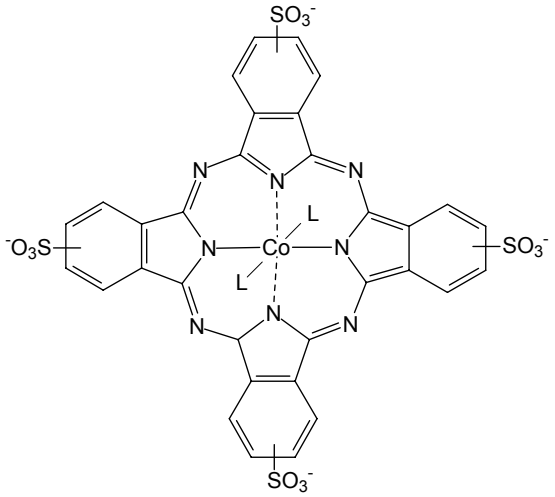
acid in aqueous solutions as well as data on reaction of reduced cobalt complexes with nitrite will be discussed.

MATERIALS AND METHODS

All chemicals were of p.a. grade and used as received. Cobalt(II) octasulfophenyltetrapyrazinoporphyrazine (**I**) was prepared and purified as reported in the literature.[8] Cobalt tetrasulfophthalocyanine (**II**) was prepared and purified following the known procedure.[10] TRIS and acetate buffers were purchased from Aldrich and used to control the pH. $Na^{15}NO_2$ (95 % ^{15}N grade) was used for ^{15}N NMR analysis. All solutions were prepared under strict exclusion of air.



I



II

Conventional kinetic experiments were performed on a Cary 5 UV-vis spectrophotometer under anaerobic conditions. Concentrated buffer solutions (0.1 M) were used for control of ionic strength and pH. A thermostated (± 0.1 ^{0}C) Applied Photophysics SX 18MV stopped-flow spectrophotometer was used to follow the fast reactions. The data were analyzed using Origin 7.5 and SPECFIT software.

The concentration of NO formed in the reaction between the reduced Co(II) complex and $NaNO_2$ at pH 7 was determined with an ISO-NOP electrode connected to an ISO-NO Mark II NO sensor from Word Precision Instruments.[11] The NO electrode was calibrated daily with fresh solutions of sodium nitrite and potassium iodide according to the method suggested by the manufacturer. The calibration factor nA/μM was determined with a linear fit program.

^{15}N NMR measurements were performed using a Bruker Avance DRX 400 WB spectrometer equipped with a superconducting BS-94/89 magnet system at 40.56 MHz for ^{15}N and 400.13 MHz for ^1H. ^{15}N chemical shifts were referenced externally to neat nitromethane. D_2O (99 %) was used as a solvent for the NMR measurements. Solubility N_2O in H_2O is $\sim 10^{-3}$ M that is reliable to fix it in case of use labeled compounds.[12]

All kinetic experiments were performed under pseudo-first-order conditions. The studied reactions exhibited pseudo-first-order behaviour for at least three half-lives. In all stopped-flow experiments, at least five kinetic runs were recorded under all conditions, and the reported rate constants are the mean values. The activation parameters and corresponding error limits were calculated from a weighted linear least-squares fit of the data.

RESULTS AND DISCUSSION

1. Reaction of L-Ascorbic acid with Sodium Nitrite in the presence of Cobalt(II) Octasulfophenyltetrapyrazinoporphyrazine

1a. Reaction of L-Ascorbic acid with Cobalt(II) Octasulfophenyltetrapyrazinoporphyrazine

After addition of L-ascorbic acid (H_2A) to the solution of **I**, the colour changes from green to red. Fig. 1 shows that in the presence of this reductant, the Q-band disappears almost completely, and a new absorption band appears at 510 nm.

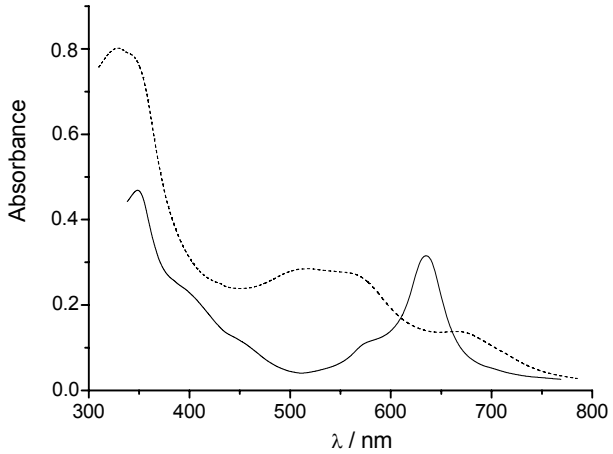


Fig. 1. Electronic absorption spectra of complex **I** after bubbling with argon (solid line) and after the reaction with L-ascorbic acid (broken line). Experimental conditions: [**I**] = 2.2×10^{-5} M, $[H_2A]_T$ = 0.28 mM, 0.1 M TRIS buffer (pH = 7.0) and room temperature.

Similar spectral changes were observed for the reaction with other reducing agents such as thiourea, N,N´-dimethylthiourea (DMTU), sodium dithionite, diethylamine and L-cysteine.[8,13] Therefore, it is reasonable to assume that in all cases the reduction of complex **I** leads to the formation of the same product. If the reduction of complex **I** would lead to formation of a Co^{I} complex, the appearance of a blue shifted band compared to 450 nm (as in $Co^{I}(TSPc)^{5-}$) is expected, since metalloporphyrazines always absorb at lower wavelengths than the corresponding metallophthalocyanines.[14] Furthermore, it should be noted that the UV-Vis spectrum of reduced complex **I** is similar to spectra of phthalocyanine anion-radicals. The intense CT-band at 500-560 nm and much less intense Q-band (near 680 nm) are typical for phthalocyanines anion-radicals.[15] On the basis of the data mentioned above it is reasonable to assume that reduction of complex **I** by ascorbic acid leads to formation of the porphyrazine anion-radical.

It is known that the pK_a values for L-ascorbic acid are 4.15 and 11.4 (for pK_1 and pK_2, respectively), such that there are three species, *viz*. H_2A, HA^- and A^{2-}, present as a function of pH, as summarized in eqs (1) and (2).

$$H_2A \overset{K_1}{\rightleftharpoons} HA^- + H^+ \qquad (1)$$

$$HA^- \overset{K_2}{\rightleftharpoons} A^{2-} + H^+ \qquad (2)$$

The reactivity increases in the order H_2A < HA^- << A^{2-}. Fig. 2 shows that the rate constant is independent of pH in the range of pH 5.0-6.5. This means that only one form of L-ascorbic acid (*viz*. HA^-) takes part in the reduction of complex **I** under these conditions. Furthermore, it should be kept in mind that the pK_a of coordinated water in complex **I** was previously found to be 12.4 ± 0.1,[8] which suggests that only the diaqua form of complex **I** will be reactive over the investigated pH range.

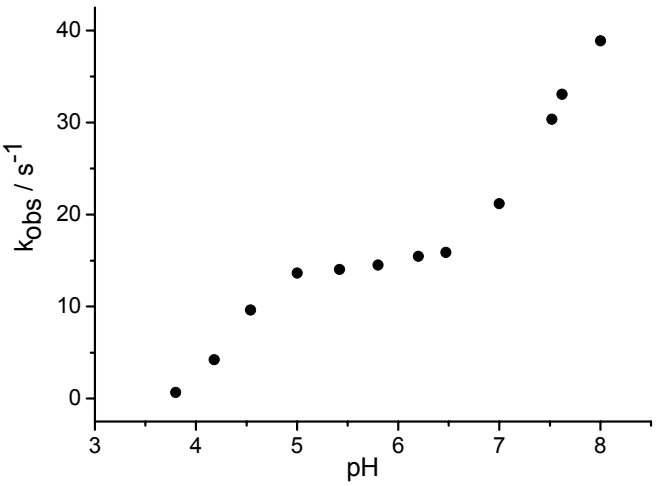
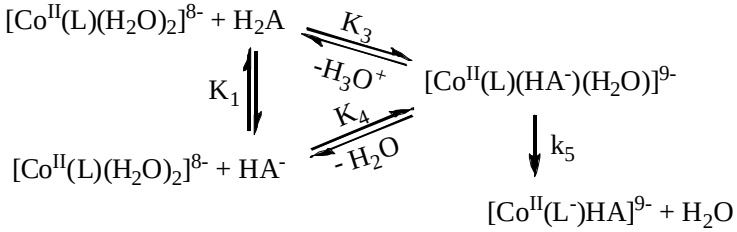


Fig. 2. Dependence of k_{obs} on pH for reduction of complex **I** by L-ascorbic acid under anaerobic conditions. Experimental conditions: [**I**] = 2×10^{-5} M, $[H_2A]_T$ = 0.5 mM, 0.1 M acetate buffer (pH 3.8 - 5.8) and 0.1 M TRIS buffer (pH 7.0 – 8.0) at 25 ^{0}C.

Fig. 3 reports the dependence of the observed rate constants on the total L-ascorbic acid concentration as a function of temperature at pH 5.8. The observed dependences are nonlinear, but can be linearized by plotting $1/k_{obs}$ versus $1/[H_2A]_T$, i.e. Michaelis-Menten kinetics is observed.

This is characteristic for an intramolecular electron transfer reaction that is preceded by a rapid pre-equilibrium in which L-ascorbic acid/ascorbate enters the coordination sphere of the Co(II) complex.

At pH < 5 the dependence of the observed rate constant on the total L-ascorbic acid concentration is similar to that at pH 5.8. At these pH values two ascorbic acid species, *viz.* H_2A and HA^-, are present in solution. Thus, the reduction of complex **I** can occur via the two parallel pathways as shown in Scheme 1.



Scheme 1 Reduction of complex **I** at pH 5.8.

The corresponding rate law is given in eq. (3).

$$k_{obs} = \frac{k_5K_3[H_2A]}{1 + K_3[H_2A]} + \frac{k_5K_4[HA^-]}{1 + K_4[HA^-]} \qquad (3)$$

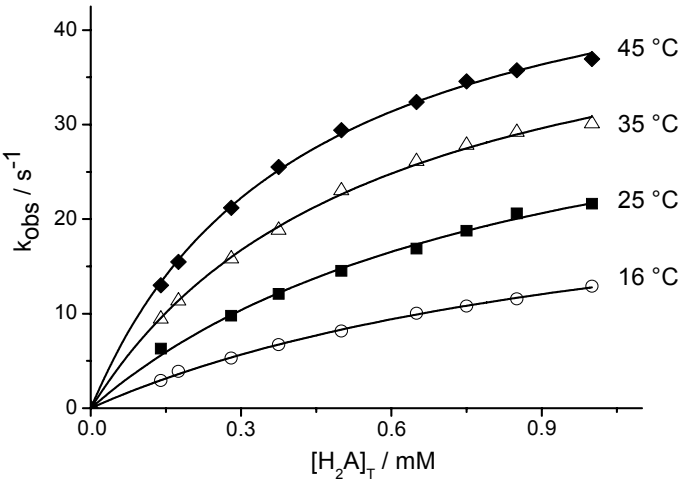


Fig. 3. Plots of k_{obs} vs. $[H_2A]_T$ for the reduction of complex **I** under anaerobic conditions. Experimental conditions: [**I**] = 2×10^{-5} M, $[H_2A]_T$ = (0.14–1.0) mM, 0.1 M acetate buffer at pH = 5.8.

Since H_2A is present at low concentrations in the selected pH range (between 5 and 6) and will coordinate less efficient than HA^- to complex **I**, i.e. $K_3 << K_4$, it follows that the reaction path involving HA^- presents the main contribution under such conditions and eq. (3) simplifies to (4).

The kinetic parameters for the reduction of complex **I** by the ascorbate anion (HA^-) at pH 5.8 are given in Table 1 as a function of temperature. K_4 and k_5 were determined from a non-linear fit of the data in Fig. 3 according to eq. (4).

$$k_{obs} = \frac{k_5 K_4 [HA^-]}{1 + K_4 [HA^-]} \qquad (4)$$

Table 1. Kinetic and thermodynamic parameters for the reduction of complex **I** by ascorbate at pH 5.8.

T / °C	K_4 / M^{-1}	k_5 / s^{-1}
45	2392 ± 85	53 ± 1
35	1769 ± 88	48 ± 1
25	1140 ± 112	41 ± 2
16	857 ± 50	28 ± 1
$\Delta H°(K_4)$, $\Delta H^{\neq}(k_5)$ / kJ mol^{-1}	+28 ± 1	+25 ± 1
$\Delta S°(K_4)$, $\Delta S^{\neq}(k_5)$ / J K^{-1} mol^{-1}	-45 ± 1	-102 ± 3

The second-order rate constant (k_5K_4) was determined at low ascorbate concentration, i.e. in the range of the linear dependence of k_{obs} on $[H_2A]_T$, and at different temperatures. A comparison of the values of K_4 at the investigated temperatures shows that at high temperatures coordination of the ascorbate anion (HA^-) to the cobalt(II) centre in complex **I** is more effective. Thus K_4 represents an endothermic equilibrium which further means that the water molecules in the axial

coordination sites on the cobalt(II) centre are more labile at higher temperatures and can be more easily replaced by HA^-. The $Co^{II}(L) \rightarrow Co^{II}(L^-)$ reduction step is less temperature dependent since a temperature increase of 30 °C only doubles the value of k_5. The activation parameters for the rate determining step (k_5) in Table 1 were determined from an Eyring plot. The activation enthalpy is indeed very low, whereas the activation entropy is significantly negative and could result from the formation of a highly ordered transition state.

1b. Reaction of Reduced Cobalt Octasulfophenyltetrapyrazinoporphyrazine with Nitrite

The reaction was studied under anaerobic conditions at pH 7 and 510 nm, which corresponds to the absorption of the reduced form of the catalyst. $NaNO_2$ was used in excess for pseudo-first-order conditions. The decrease in concentration of the reduced complex ($\mathbf{I}^{red}$) with time following the apparent induction period is well described by a single exponential function. Plots of k_{obs1} vs. [$NaNO_2$] are linear over the selected nitrite concentration range at different temperatures (Fig. 4).

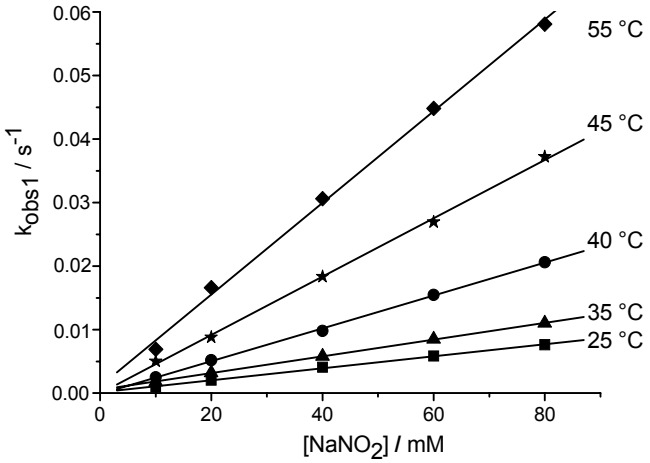


Fig. 4. Plots of k_{obs1} vs. $NaNO_2$ concentration for the reaction between $\mathbf{I}^{red}$ and nitrite as a function of temperature. Experimental conditions: [$\mathbf{I}^{red}$] = 2×10^{-5} M, $[H_2A]_T$ = 0.14 mM, [$NaNO_2$] = (10-80) mM, 0.1 M TRIS buffer (pH = 7.0).

The overall second-order rate constant is 0.10 ± 0.01 M^{-1} s^{-1} at 25 °C (calculated from the data at 25 °C in Fig. 4). The following activation parameters were determined from an Eyring plot, *viz*. $\Delta H^{\neq}$ = 51 ± 2 kJ mol^{-1} and $\Delta S^{\neq}$ = -94 ± 5 J K^{-1} mol^{-1}. The oxidation process is suggested to follow an inner-sphere electron transfer mechanism. The formation of NO (see reactions 5-7) as reaction product could be observed with the use of an NO selective electrode.

$$[Co^{II}(L^-)(HA)]^{9-} + NO_2^- \longrightarrow [Co^{II}(L^-)(HA^+)]^{8-} + NO_2^{2-} \quad (5)$$

$$[Co^{II}(L^-)(HA^+)]^{8-} \longrightarrow [Co^{II}(L)]^{8-} + HA \quad (6)$$

$$NO_2^{2-} + H_2O \longrightarrow NO + 2OH^- \quad (7)$$

It should, however, be noted that the presence of NO was not observed in the ^{15}N-NMR spectra. In the NMR spectrum three signals were observed at 228.4, -147.7 and -231 ppm. The first signal corresponds to nitrite, the second and third signals

correspond to N_2O (nitrous oxide has signals at -147.3 and -237.0 ppm in the gas phase).[16] The cobalt complex does not give the signal in the interval 300 to –300 ppm. Thus, NO is an intermediate in the reaction between L-ascorbic acid and nitrite in the presence of the reduced cobalt complex, and N_2O is the final product of the studied reaction under these conditions.

2. Reaction of Thiourea Dioxide with Sodium Nitrite in the presence of Cobalt(II) Octasulfophenyltetrapyrazinoporphyrazine

2a. Reaction of Thiourea Dioxide with Cobalt(II) Octasulfophenyltetrapyrazinoporphyrazine

Reduction of complex **I** solution with TDO at pH 8.3-9.7 is accompanied by similar spectral changes observed for the reactions with other reducing agents. Therefore it is reasonable to assume that reduction of complex **I** with TDO leads to the formation of porphyrazine anion-radical. The dependence of the observed rate constants on the TDO concentration is nonlinear but can be linearized by plotting $1/k_{obs}$ versus 1/[TDO], i.e. Michaelis-Menten kinetics is observed. This is characteristic for an intramolecular electron transfer reaction that is preceded by a rapid pre-equilibrium in which TDO enters the coordination sphere of Co^{II} complex:
$[Co^{II}(L)]^{8-} + NH_2NHCSO_2^- \leftrightarrow [Co^{II}(L)^{8-}\cdot NH_2NHCSO_2^-] \rightarrow [Co^{II}(L)^{9-\cdot} NH_2NHCSO_2]$ (8), where L = $PyzPz(PhSO_3^-)_8$

For reaction the following thermal activation parameters were determined from the corresponding Eyring plots: $\Delta H^{\neq}$ = 40 ± 2 kJ/mol, $\Delta S^{\neq}$ = - 116 ± 7 J/(K*mol).

2b. Reaction of Reduced Compex I with Sodium Nitrite

The reaction was studied at pH 9.7. The dependence of the observed rate constants on the nitrite concentration is nonlinear but can be linearized by plotting $1/k_{obs}$ versus $1/[NO_2^-]$, i.e. Michaelis-Menten kinetics is observed. This is characteristic for an intramolecular electron transfer reaction that is preceded by a rapid pre-equilibrium in which NO_2^- enters the coordination sphere of reduced complex:
$[Co^{II}(L)^{9-\cdot} NH_2NHCSO_2] + NO_2^- \leftrightarrow [Co^{II}(L)^{9-}\cdot NH_2NHCSO_2(NO_2)^-] \rightarrow$ products (9).

For reaction the following thermal activation parameters were determined from the corresponding Eyring plots: $\Delta H^{\neq}$ = 17 ± 1 kJ/mol, $\Delta S^{\neq}$ = - 219 ± 20 J/(K*mol).

The final product of nitrite reduction was found to be ammonia (signals at -283 and -382 ppm in ^{15}N NMR spectrum).

It should be noted that oxidation of reduced complex by nitrite does not proceed with measurable rates in strongly alkaline solutions, i.e. this complex is inert in the redox process.

3. Reaction of L-Ascorbic Acid with Sodium Nitrite in the presence of Cobalt(II) Tetrasulfophthalocyanine

The reaction does not proceed with measurable rates. L-ascorbic acid can reduce $(Co^{II}(TSPc)^{4-}$ only in strong alkaline solutions but in this media nitrite does not react with reduced cobalt complex.

4. Reaction of Thiourea Dioxide with Sodium Nitrite in the presence of Cobalt(II) Tetrasulfophthalocyanine

4a. Reaction of Thiourea Dioxide with Cobalt(II) Tetrasulfophthalocyanine

Reduction of a Co^{II}TSPc solution by excess TDO is accompanied by a color change from blue to yellow. An intensive absorption maximum appears at 450 nm in

the UV-vis spectrum, and simultaneously the Q-band is shifted to the red region (Fig. 5). This spectrum is essentially identical to that reported for $Co^{I}(TSPc)^{5-}$.[9,17,18] TDO decomposition was found to be the rate-determining step.

$NH_2NHCSO_2^- + H_2O \rightarrow SO_2H^- + (NH_2)_2CO$ (10)

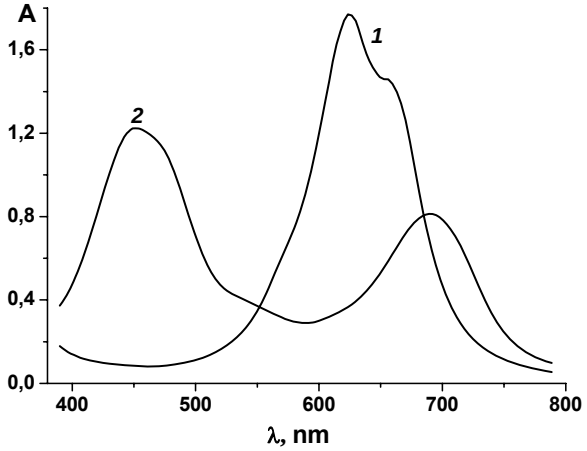


Fig. 5. Spectral changes observed during the reduction of $Co^{II}(TSPc)^{4-}$ by TDO, [TDO] = 1×10^{-3} M, [$Co^{II}(TSPc)^{4-}$] = 3.05×10^{-5} M, pH = 10, T = 298 K, 1 – $Co^{II}(TSPc)^{4-}$, 2 – $Co^{I}(TSPc)^{5-}$.

Further reduction of a $Co^{I}(TSPc)^{5-}$ by TDO is accompanied by a slow decreasing of absorption intensity in the all regions of the UV-vis spectrum. The reaction rate was found to be described by the equation $r = k[Co^{I}(TSPc)^{5-}][TDO]$. For reduction of a $Co^{I}(TSPc)^{5-}$ by TDO the following thermal activation parameters at pH 10.6 were determined from the corresponding Eyring plots: $\Delta H^{\neq}$ = 58 ± 2 kJ/mol, $\Delta S^{\neq}$ = - 140 ± 5 J/(K*mol). Importantly, the rate does not depend on the use of either fresh or aged (sulfoxylate, SO_2^{2-}) TDO, i.e. the rate-determining step is a reaction between Co^{I} and sulfoxylate.

$Co^{I}(TSPc)^{5-} + SO_2H^- \leftrightarrow [Co^{I}(TSPc)^{5-}\cdot SO_2H^-] \rightarrow$ products (11)

4b. Reaction of Reduced Cobalt Tetrasulfophthalocyanine with Sodium Nitrite

The kinetics of interaction between $Co^{I}(TSPc)^{5-}$ and nitrite was studied under anaerobic conditions at pH 10 and 298 K. Changes in absorbance at 450 nm were used for the kinetic measurements. Nitrite was introduced into a solution of the reduced phthalocyanine immediately after the highest optical density at 450 nm was received, that is, before the beginning of the second step of metal complex reduction. Fresh solutions of TDO were used. A typical kinetic curve is shown in Fig. 6. Kinetic traces show induction period that depends on the reductant concentration at fixed concentration of oxidant and *vice versa*. The kinetic curves are similar in shape to those obtained in the reduction of nitrite with sodium dithionite in the presence of cobalt phthalocyanine.[9] The presence of an induction period is explained by the redox cycle of the metal complex. When reducing agent is used up, only the oxidation of $Co^{I}(TSPc)^{5-}$ to $Co^{II}(TSPc)^{4-}$ occurs, and a decrease in the concentration of the reduced form with time is well described by the exponential law.

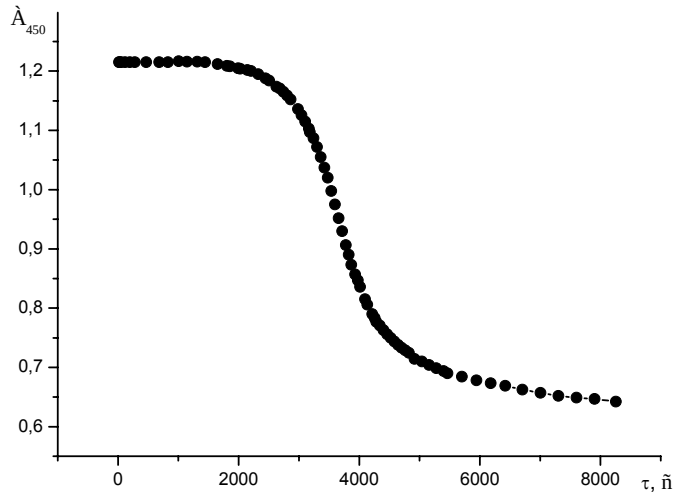


Fig. 6. Kinetic curve of the reduction of nitrite with thiourea dioxide in the presence of $Co^{I}(TSPc)^{5-}$; [TDO] = 1×10^{-3} M, [$NaNO_2$] = 0.03 M, [$Co^{I}(TSPc)^{5-}$] = 3.05×10^{-5} M, T = 298 K, pH 10.0.

The dependence of the observed rate constants on the nitrite concentration is nonlinear but can be linearized by plotting $1/k_{obs}$ versus $1/[NO_2^-]$, i.e. Michaelis-Menten kinetics is observed. This is characteristic for an intramolecular electron transfer reaction that is preceded by a rapid pre-equilibrium in which nitrite enters the coordination sphere of Co^{I} complex:

$Co^{I}(TSPc)^{5-} + NO_2^- \leftrightarrow [Co^{I}(TSPc)^{5-}\cdot NO_2^-] \rightarrow Co^{II}(TSPc)^{4-}$ + nitrite reduction products (12)

For reaction the following thermal activation parameters were determined from the corresponding Eyring plots: $\Delta H^{\neq}$ = 49 ± 1 kJ/mol, $\Delta S^{\neq}$ = - 146 ± 7 J/(K*mol). The final product of nitrite reduction was found to be ammonia (signals at -283 and -382 ppm in ^{15}N NMR spectrum).

Conclusions

Our study has shown that cobalt octasulfophenyltetrapyrazinoporphyrazine can effectively assist the oxidation of L-ascorbic acid and thiourea dioxide by nitrite. Cobalt tetrasulfophthalocyanine catalyzes only reaction between thiourea dioxide and nitrite. The catalytic cycle includes reversible reduction-oxidation of the cobalt complex via oxidation of coordinated reductant. But catalytic activity of studied complexes strongly depends on pH. Due to extremely strong electron accepting properties, cobalt octasulfophenyltetrapyrazinoporphyrazine easily accepts an electron from ascorbic acid even at neutral pH. After that the complex formed is re-oxidized by nitrite. However, in contrast to tetrasulfophthalocyanine complex, cobalt octasulfophenyltetrapyrazinoporphyrazine is inert as a catalyst in redox reactions in strongly alkaline solutions.

Acknowledgements

Author gratefully acknowledges financial support from the Russian Foundation for Basic Research.

REFERENCES

1. Gregory P. – J. Porphyrins Phthalocyanines, 2000, 4, 432.
2. Kuraoka K., Ueda T., Fujiwara M., Sato M. – J. Membrane Science, 2006, 286, 12.
3. Thami T, Simon J., Jaffrezic N., Maillard A., Sprikovitch S. – Bull. Chem. Soc. Fr., 1996, 133, 759.
4. Tang C.W. – Appl. Phys. Lett., 1986, 48, 183.
5. Sakamoto K., Kato T., Kawaguchi T., Ohno-Okumura E., Urano T., Yamaoka T., Suzuki S., Cook M.J. – J. Photochem. Photobiol. A: Chem., 2002, 153, 245.
6. Zhao M., Stern C., Barrett A.G.M., Hoffman B.M. – Angew. Chem., Int. Ed., 2003, 42, 462.
7. Toyama M.M., Demets G.J.F., Araki K., Toma H.E. – Electrochem. Commun., 2000, 2, 749.
8. Kudrik E.V., Theodoridis A., van Eldik R., Makarov S.V. – Dalton Trans., 2005, 1117.
9. Kudrik E.V., Makarov S.V., Zahl A., van Eldik R. – Inorg. Chem., 2003, 42, 618.
10. Weber J.N., Busch D.H. – Inorg. Chem., 1965, 4, 469.
11. Kudo S., Bourassa J.S.E., Sato Y., Ford P.C. – Anal Biochem., 1997, 247, 193.
12. Meng-Hui L., Ming-Der L. – J. Chem. Eng. Data, 1995, 40, 486.
13. Shishkin V.N., Kudrik E.V., Makarov S.V., Shaposhnikov G.P. – Kinetika i Kataliz, 2007, 48, 660.
14. The Phthalocyanines: Properties and Applications, ed. C.C. Leznoff and A.B. P. Lever, VCH Publ., New York, 1989–96, V. I-IV.
15. Keizer S.P., Mack J., Bench B. A., Gorun S.M., Stillman M.J. – J. Am. Chem. Soc., 2003, 125, 7067.
16. Kytzia A., Korth H-G., Sustmann R., de Groot H., Kirsch M. – Chem. Eur. J., 2006, 12, 8786.
17. Grodkowski J., Dhanasekaran T., Neta P. et al. – J. Phys. Chem. A, 2000, 104, 11332.
18. Nevin W.A., Liu W., Melnik M., Lever A.B.P. – J. Electroanal. Chem.,1986, 213, 217.

MISCANTHUS SINENSIS GIGANTHEUS' BEHAVIOUR ON SOILS POLLUTED WITH HEAVY METALS

Constantin-Horia BARBU*, Bianca-Petronela PAVEL, Camelia SAND, Mihai-Radu POP

"Lucian Blaga" University of Sibiu, Faculty of Agricultural Sciences, Food Industry and Environmental Protection, Ro-550024 Sibiu, 10 Victoriei Bv.

* E-mail : horiab@rdslink.com

ABSTRACT

Heavy metals in soils pose a great environmental and health problem to those consuming plants cultivated on these soils. Until now no feasible (cheap and quick) method for large areas remediation has been discovered, and farming on these lands could be regarded as dangerous.

The paper presents our results in cultivating a new plant, Miscanthus sinensis x giganteus, with great economic value and future, and with very few uptake of the heavy metals existing in the soils of Copsa Mica region (Pb, Cd).

Key words*: Miscanthus sinensis x giganteus*, energy crops, low heavy metal uptake

INTRODUCTION

Soils polluted with heavy metals (Pb, Cd) pose a great environmental risk, but when these soils are cultivated with edible/forage plants, as the case of Copsa Mica region in Romania, the risks are much greater. Even though the situation is well documented (Dumitru 2004, Lacatusu 2008, Constantinescu 2008), and there are some attempts in remediation of these soils (Barbu et al. 2006, Dumitru et al 2002, Dumitru 2004), until now no feasible solution for the decontamination of this large region has been found (Figure 1).

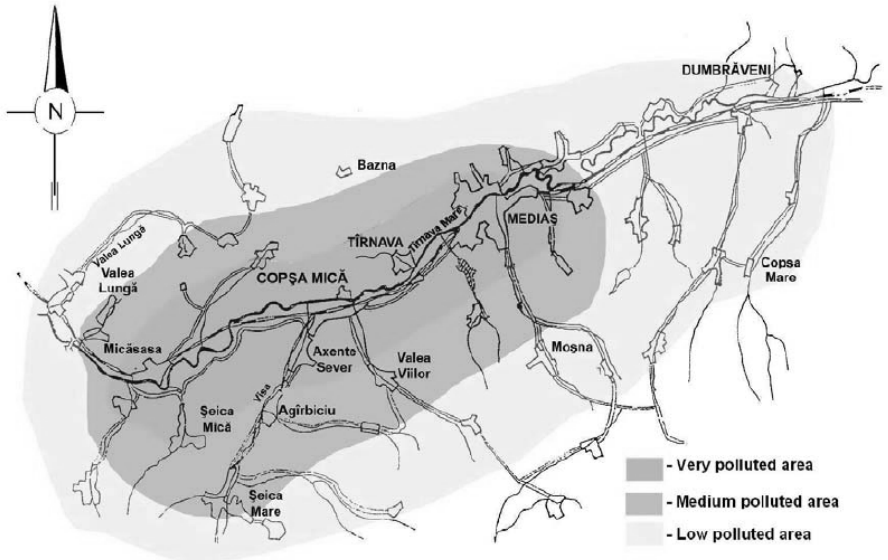


Figure 1. Degree of soil pollution within the Copsa Mica area (source APM Sibiu)

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 21 - 24, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

In our works we have started from the following assumptions: heavy metals already existing in soil are difficult to be removed, due to the very high costs and/or long time to perform any known remediation method; because groundwater level is quite deep (3 -10 m), it is unlikely to be contaminated; until a feasible method will be used, farmers in the area depend on their land, and cultivation of non-edible plants, with market value, could be a solution for their well-being.

After screening many species of non-edible plants, we have chosen for our research a new hybrid, *Miscanthus sinensis x giganteus* (Poaceae), trying to determinate if it can be cultivated on these lands (considering the climate and the pollution degree) and what is the amount of heavy metals in its useful parts.

MATERIALS AND METHODS

Miscanthus sinensis x giganteus Greef et Deu is a triploid hybrid between *M. sinensis* Anderss. (a diploid species) and *M. sacchariflorus* Hack (a tetraploid species), which is unable to produce viable seed, thus reducing the risk to become invasive (Jones and Walsh, 2001). Giant miscanthus is a perennial warm-season grass, with a special type of photosynthesis (C4), which implies the return of the nutrients in the rhyzome during the cold season (Fruhwirt and Liebhard, 2004). As temperatures cool in the fall, the dark green foliage fades to buff and drops, leaving stems (and sometimes sterile flowers at their terminus). Stems are the most commercially important portions of giant miscanthus, and harvesting the dried stems takes place during late winter or early spring (March-April).

Propagation is made mainly by rhyzomes and, in the third year of cultivation plants reach a height of 3-4 m, with a yield of 20 tons per hectare (at 15% humidity). Its current use is for energy, but research are performed for other industrial uses, as pulp and paper, additive in concrete walls, plastics replacement (Pyter et al. 2007, Ion and Ioana, 2006).

For our experiments we have chosen a piece of land (5000 m^2) within the town of Copsa Mica, situated one km westwards from the pollution source (SOMETRA SA). Land characteristics were determined according to the current Romanian standards, the average sample consisting on 10 sub-samples taken from different places. Soil loading with heavy metals was determined in solid state, using a High-resolution continuum source atomic absorption spectrometer ContrAA 700, produced by Jena Analitik (Welz et al. 2005). Details on this new technique are presented in the paper „Direct determination of heavy metals in plant solid samples using HD-CS-AAS”.

On this land, after soil preparation, we have planted manually, in May 2008, Miscanthus rhizomes (from Fa. Schweighoffer GmbH, Austria), in rows (1 m between them, 1 m between the plants), at a depth of 10-12 cm. No pesticides and fertilizers were used.

In April 2009, after a mild winter that didn’t pose freezing problems, we have cropped the stems and the remaining leaves (the great majority have fallen on earth, creating a mulch), and analyzed them, as well as rhizomes and leaves.

For the analysis we did not wash stems and leaves (because they will be used as they are), only rhizomes, with distilled water. Because there is only one or two leaves per stem, their mass fraction in the upper parts is below 7%. Soil and vegetal samples were oven dried at 105^{o}C, for two hours, then cut into small pieces and grinded under 10μm (Fritch – Pulverisette 0).

From each analysis there were taken at least four samples. In the AAS graphite furnace there were introduced amounts of 1.0000 mg at a time.

RESULTS AND DISCUSSION

The piece of land where we have performed our research has the following agrochemical characteristics, as determined in April 2009, at harvest (Table 1).

Table 1. Agrochemical and pedological characteristics of the land.

Depth (cm)	pH	Humus (%)	P_2O_5 (mg/kg d.w.)	K_2O (mg/kg d.w.)	Texture	Pb (mg/kg d.w.)	Cd (mg/kg d.w.)
0-20	5.2	2.03	110	460	Sandy clay	682.50	13.47
20-40	5.3	1.87	73	361	Sandy clay	492.00	10.23
40-60	6.2	0.31	88	301	Sandy loam	67.50	4.67

As it may be seen, the soil is very polluted, very acidic and has low amounts of nutrients.

Despite this, the first agronomical results are encouraging: the plants have over-wintered well, with a survival rate of more than 96%, and from rhizomes have developed small roots until the depth of 30 cm, where the pollution degree is lower, this demonstrating that *Miscanthus sinensis x giganteus* can be successfully cultivated in Romania, even on poor, acidic soils, even polluted with Pb and Cd.

In order to determine if, beside nutrients, heavy metals are also transported from the upper parts into rhizomes in wintertime, we have determined the amount of Pb and Cd in plant samples taken in September 2008 and in April 2009.

The results of the determinations are presented in table 2.

Table 2. Amount of heavy metals in plant samples (mg/kg, d.w.)

Period of vegetation	Plant part	Heavy metal amount	
		Pb	Cd
September 2008	Leaves	10.54±4.55	1.22±0.34
	Stem	***4.38±2.67***	***2.07±0.77***
	Rhizome	382.7±111.0	4.90±2.27
April 2009	Leaves	7.33±3.09	0.75±0.12
	Stem	***2.62±0.82***	***1.62±0.56***
	Rhizome	452.7±145.5	5.62±2.34

As it may be seen from the above table, the amount of heavy metals uptaken by Miscanthus is extremely low, this making the plant unsuitable for phytoextraction (only around 35 – 55 g heavy metals per hectare and year), but allowing it to be used as green energy or in various other fiels, without any danger. The amount of heavy metals being significantly greater when green, we do not recomend its use unless cropped in spring.

We expect this amount of heavy metals to decrease further, when root system will go deeper, in less polluted soil layers (roots can reach 2-3 m in depth).

CONCLUSIONS

Even if at its beginning, our research allowed us to draw the following major conclusions:

1. *Miscanthus sinensis x giganteus*, a valuable energy plant, can be successfully cultivated in Romania, even on soils polluted with Pb and Cd.
2. The amount of Pb and Cd in the upper parts of the plants, even cultivated on soils heavily polluted with Pb and Cd, is very small, this allowing its unrestricted industrial use.

ACKNOWLEDGEMENTS

This research was performed within the Romanian R&D Grant Scheme „Parteneriate", Contract no. 31-022, and the analysis were done with the kind help of Mrs. Michaela Portala and Mr. Oliver Buettel, from Analytik Jena GmbH, Germany.

REFERENCES

1. Barbu C.H., C. Sand C., Oprean L. - *Use of zeolites in the remediation of metal contaminated soils*, in Phytoremediation of metal-contaminated sites, Editors: Jean-Louis Morel, G. Echevarria, Ed. Springer, Dordrecht, Olanda, 2006,. 152-159.
2. Constantinescu L. - *Soil pollution with heavy metals in critical areas*, in Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Agriculture, Vol 65, No 2, 2008.
3. Dumitru, M., Vrinceanu, N., Motelica, M. - *Aspects concerning rehabilitation of soils polluted with heavy metals in Copsa mica area*, in Proceedings of the COST837 Meeting, Bordeaux, Franţa, 2002, http:// lbewww.epfl.ch/COST837/ bordeaux/S3.pdf.
4. Dumitru M., *Integrated approach for the remediation of contaminated sites – Country Report*, http://www.ics.trieste.it/Portal/ActivityDocument.aspx?id=378, 2004.
5. Fruhwirth, P., Liebhard, P. - *Miscanthus sinensis Giganteus. Produktion, Inhaltsstoffe und Verwertung,* In.*Miscanthus sinensis Giganteus. Chinaschilf als nachwachsender Rohstoff*, Landliches Fortbildungsinstitut und Landwirtschaftskammer Osterreich, 2004, 34-38.
6. Ion, V., Ioana D. - *Energie din Biomasă*, Tehnica instalaţiilor nr. 7 (38), 2006, 14-30.
7. Jones M.B., Walsh M. – *Miscanthus for energy and fibre - Origins and Taxonomy of Miscanthus,* James & James Publishers, p. 2-9, London 2001.
8. Lacatusu R., Lacatusu A.R. - *Vegetable and fruits quality within heavy metals polluted areas in Romania*, Carpth. J. of Earth and Environmental Sciences, 2008, Vol. 3, No. 2, 115-129.
9. Pyter R., Voigt T., Heaton E, Dohleman F., Long S. - *Giant Miscanthus: Biomass Crop for Illinois*, in Issues in new crops and new uses. J. Janick and A. Whipkey, (eds.) ASHS Press, Alexandria, VA. 2007, 234-238.
10. Weltz B., Becker-Ross H., Florek S., Heitmann U.- *High-Resolution continuum Source AAS, The Better Way to Do Atomic Absorption Spectrometry*, Weinheim, 2005.

PROPERTIES AND FUNCTION OF [2Fe-2S] CENTERS IN FERROCHELATASES

Angela-Nadia Albetel[1*], Tamara A. Dailey[2], Harry A. Dailey[2], Michael K. Johnson[1]

[1]Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602, [2]Biomedical and Health Sciences Institute and Department of Microbiology, University of Georgia, Athens, Georgia 30602, USA

[*]E-mail: angelas@uga.edu

ABSTRACT

Ferrochelatase (EC 4.99.1.1), the terminal enzyme of the heme biosynthetic pathway, catalyzes the insertion of ferrous iron into protoporphyrin IX. Deficiencies in the enzymes of heme biosynthesis in man result in clinical conditions called porphyrias. The enzymes exist as monomers and dimers, both with and without [2Fe-2S] clusters. The role of the [2Fe-2S] centers in ferrochelatases is unknown, but previously reported data show that the [2Fe-2S] cluster present in human ferrochelatase is destroyed by nitric oxide. Ongoing work includes the characterization and the elucidation of the role of [2Fe-2S] centers in ferrochelatases.

Key words: *ferrochelatase, heme, iron-sulfur cluster, porphyria*

Ferrochelatase (EC 4.99.1.1), is the terminal enzyme of the heme biosynthetic pathway, catalyzing the insertion of ferrous iron into protoporphyrin IX macrocycle to yield protoheme IX (*1-3*). The enzyme is present in all prokaryotes and eukaryotes with the exception of a few obligate pathogens and some anaerobic prokaryotes (*3;4*).

Deficiencies in the enzymes of heme biosynthesis in humans result in clinical conditions called porphyrias. In particular, point mutations and/or deletion in the human gene that encodes ferrochelatase can result in the disease erythropoeitic protoporphyria (EPP), which manifests as an accumulation of protoporphyrin IX in the skin and liver. Protoporphyrin IX is a photosensitive pigment that may form free radicals upon exposure to sunlight, causing blistering in cutaneous tissues. However, although this condition can be very painful, it is rarely fatal, but in some cases can lead to fatal liver damage (*5-7*).

In most organisms, ferrochelatase is a membrane-associated protein, but a few bacterial organisms, e.g. *Bacillus subtilis* (3;8) and *Mycobacterium tuberculosis* (4), contain soluble ferrochelatases. The overall sequence homology among currently known ferrochelatases is less than 10%, with only approximately 20 amino acid residues being invariant. With few exceptions, most of the conserved residues are located in the active site pocket. A comparison of all currently known ferrochelatase sequences reveals the presence of three distinct domains (8): the first domain (I) is an amino-terminal organelle-targeting motif and is present in all eukaryotes, the second

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 25 - 29, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

domain (II) represents the core 330 amino acid residues of the enzyme and is present in all ferrochelatases, the third domain (III) is a 30- to 50-amino-acid-residue extension at the C-terminus that is not present in most bacterial ferrochelatases (4). In animal ferrochelatases, the carboxyl-terminal extension is involved in the dimerization motif for these enzymes and contains three of the four cysteines that ligate a [2Fe-2S] cluster, the fourth cysteine ligand being present in domain II (9-15). *Saccharomyces cerevisiae* and plant ferrochelatases also contain domain III, which is approximately 50 residues in length, but do not contain the cluster-ligating cysteinyl residues and do not possess a [2Fe-2S] center (4). The function of the carboxyl-terminal extension is unknown, yet its removal from *S. cerevisiae* or animal ferrochelatases results in loss of enzyme activity (10).

To date the structures of two ferrochelatases have been solved and refined to approximately 2.0 Å resolution. The first to be solved was the structure of ferrochelatase from bacterium *Bacillus subtilis* (*16*). This protein is a water-soluble, monomeric protein that possesses two structurally related domains. The second ferrochelatase structure solved was for the human protein, which is a mitochondrial membrane-associated, homodimeric protein containing one all cysteinyl ligated [2Fe-2S] cluster in each monomer (*15*). The current presentation will focus on the [2Fe-2S] centers that are present in all vertebrate and some yeast and bacterial ferrochelatases. Although mutagenesis studies show that the [2Fe-2S] clusters are required for ferrochelatase activity, the function of these clusters has yet to be elucidated.

The spectroscopic properties of human ferrochelatase [2Fe-2S] cluster are consistent with coordination of the cluster via cysteinyl residues (*11*). Site-directed mutagenesis and spectroscopic studies suggested an unusual NH_2-Cys-X_{206}-Cys-X_2-Cys-X_4-Cys-COOH coordinating motif for the [2Fe-2S] cluster from the human ferrochelatase (*11;14;17*). Although the overall pattern of coordinating cysteines in human ferrochelatase is similar to that found in simple [2Fe-2S]-containing ferredoxins, ***e.g.*** NH_2-Cys-X_4-Cys-X_2-Cys-X_{29}-Cys-COOH in chloroplast ferredoxins (*18;19*), the remote cysteine is located closest to the NH_2 terminus in ferrochelatase and the spacing to the remote cysteine is larger (*14*). The [2Fe-2S] cluster present in the human ferrochelatase exhibits a number of interesting features. Resonance Raman and variable-temperature magnetic dichroism measurements on ferrochelatases indicate anomalous vibrational and electronic properties compared to other structurally characterized [2Fe-2S] centers (*11;17*), which are likely to reflect differences in cysteine dihedral angles and cluster environment. For example, unlike other [2Fe-2S] centers, the cluster in human ferrochelatase has two Fe-S-C-C dihedral angles close to 180º and has no H-bonding interactions involving cysteinyl or inorganic S atoms (*15*).

Although the initial published bacterial ferrochelatase sequences did not contain the carboxyl-terminal domain (*8*) and their spectral properties revealed no Fe-S cluster, lately it has been discovered that some bacterial ferrochelatases, including *Caulobacter crescentus* and *Mycobacterium tuberculosis* (*4*) also possess the C-terminal extension of a length similar to that of animal ferrochelatases, and that this region contains three cluster-ligating cysteine residues. However, the spacing between these residues (C-X_6-C-X-C and C-X_8-C-X_4-C for *C. crescentus* and *M. tuberculosis*, respectively) is unlike that found in animal and *S. pombe* ferrochelatases (C-X_2-C-X_4-C) (*4;8;10;12;20;21*). More significant was the discovery that the

amino-proximal cluster-ligating cysteine residue of animal (C196 in human ferrochelatase) and *S. pombe* ferrochelatase (C162) was absent in *C. crescentus*. The identity of the four amino-acid residues ligating the [2Fe-2S] cluster in *C. crescentus* ferrochelatase was revealed by site-directed mutagenesis studies to be C158, C332, C339, and C341 (*4*). This results in a four-cysteinyl ligating motif for the cluster, with the spacing between ligands (NH_2-C-X_{170}-C-X_6-C-X-C-COOH) being unique among all [2Fe-2S] cluster-containing proteins currently known. In *M. tuberculosis* ferrochelatase the putative amino-proximal cysteine ligand is located two or three amino acid residues away from the corresponding cysteine of animal and *S. pombe* ferrochelatases (*4*). The recent discovery of a [2Fe-2S] center in *M. xanthus* ferrochelatase that does not have a C-terminal extension adds additional diversity to the [2Fe-2S] centers in ferrochelatases (*22*). Mutagenesis results indicate that the cluster is ligated by four cysteines in a centrally located NH_2-C-X_5-C-C-X_9-C-COOH motif and sequence alignments indicate that similar clusters are present in ferrochelatases from several other bacterial organisms, *e.g. Azotobacter vinelandii*, *Bdellovibrio bacteriovorus, and Pseudomonas syringae (22)*.

The question that needs to be addressed is the function of the [2Fe-2S] cluster in ferrochelatases. Research over the last decade has served to demonstrate the functional diversity of biological Fe-S clusters (*23*). Although the majority of biological Fe-S clusters are involved in electron transport, it is now evident that several redox enzymes (*e.g.*, hydrogenases, nitrogenases, and CO dehydrogenases) and nonredox enzymes (*e.g.*, aconitase, (de)hydratases) utilize homometallic or heterometallic Fe-S clusters for substrate binding and activation.. In addition, regulatory roles have been proposed in the iron-responsive element binding protein, IRP-1, (Fe-sensing), the fumarate-nitrate reduction protein, FNR (O_2 sensing), SoxR (reactive oxygen species (ROS) sensing), and IscR (Fe-S cluster sensing) (*24*). The key role of Fe-S clusters in determining the protein structure makes them important regulatory targets for controlling enzyme activity or gene expression in response to external stimuli such as O_2, NO, ROS, Fe, or Fe-S cluster concentrations. Regulatory control can be accomplished via cluster interconversion (*e.g.* the [4Fe-4S]/[2Fe-2S] cluster interconversion in FNR), cluster assembly/degradation (*e.g.* IRP-1 and IscR), or reversible oxidation/reduction (*e.g.* the $[2Fe\text{-}2S]^{2+,+}$ redox couple in SoxR) (*24)*

Currently the only answers concerning the role of the Fe-S cluster in ferrochelatases are negative ones. Since many organisms possess a ferrochelatase that lacks the cluster and still has enzymatic activity, it is clear that the [2Fe-2S] cluster does not play a direct role in catalysis. Likewise, there is no direct evidence that it could be involved in ferric iron reduction since all ferrochelatases assays always have ferrous iron and removal of the cluster destroys the enzyme activity. The sensitivity of mammalian [2Fe-2S] cluster to NO led to the suggestion that it may play a role in local immune response preventing bacteria from using heme synthesized by the host organism (*20*), but the subsequent finding of clusters in *Drosophila* (*14*), yeast *Schizosaccharomyces pombe* (*4*), and bacterial (*4*) ferrochelatases diminishes support for this hypothesis. The cluster may play some structural role, but the minor contribution of the [2Fe-2S] center to the enzyme active-site structure suggests that other hypotheses need to be considered.

The above discussion serves to illustrate the need for further investigations into the role and properties of the [2Fe-2S] clusters in a wide range of ferrochelatases. To this end we have initiated detailed structural, redox, electronic, magnetic, and vibrational characterization of the clusters in a wide range of ferrochelatases. In addition, the NO sensitivity of [2Fe-2S] clusters in different ferrochelatases needs to be addressed to assess the possibility that the [2Fe-2S] clusters in vertebrate ferrochelatases play a specific role in NO sensing related to the immune response. Alternatively the recent realization of biological trafficking in [2Fe-2S] clusters, raises the intriguing possibility that the clusters in ferrochelatases may play a role as sensor of the cellular iron-sulfur cluster status, thereby regulating the use of Fe for heme or Fe-S cluster biosynthesis in vertebrate and some bacterial and yeast organisms. Both functional possibilities are under active consideration in our laboratories.

REFERENCES

1. Dailey, H. A. (1990) Comparison of Coproporphyrinogen to Protoheme in Higher Eukaryotes and Bacteria: Terminal Three Enzymes, in *Biosynthesis of Heme and Chlorophylls* (Dailey, H. A., Ed.) pp 123-161, McGraw-Hill, New-York.
2. Dailey, H. A. (1996) Ferrochelatase, in *Mechanisms of Metallocenter Assembly* (Hausinger, R. P., Eichorn, G. L., and Marzilli, L. G., Eds.) pp 77-98, VCH Publishers, Inc., New York, N.Y.
3. Dailey, H. A. and Dailey, T. A. (2003) Ferrochelatase, in *The Porphyrin Handbook* (Kadish, K. M., Smith, K. M., and Guilard, R., Eds.) pp 93-121, Elsevier Science (USA).
4. Dailey, T. A. and Dailey, H. A. (2002) Identification of [2Fe-2S] Clusters in Microbial Ferrochelatases, *J. Bacteriol. 184*, 2460-2464.
5. Nordman, Y. and Deybach, J. C. (1990) Human hereditary porphyrias, in *Biosynthesis of Heme and Chlorophylls* (Dailey, H. A., Ed.) pp 491-542, McGraw-Hill, New-York.
6. Moore, M. R., McColl, K. E., Rimington, C., and Goldberg, A. (1987) *Disorders in Porphyrin Metabolism* Plenum, New-York.
7. Najahi-Missaoui, W. and Dailey, H. A. (2005) Production and Characterization of Erythropoietic Protoporphyric Heterodimeric Ferrochelatases, *Blood 106*, 1098-1104.
8. Dailey, H. A., Dailey, T. A., Wu, C.-K., Medlock, A. E., Wang, K.-F., Rose, J. P., and Wang, B.-C. (2000) Ferrochelatase at the Millennium: Structures, Mechanisms and [2Fe-2S] Clusters, *Cell. Mol. Life Sci. 57*, 1909-1926.
9. Burden, A. E., Wu, C.-K., Dailey, T. A., Busch, J. L. H., Dhawan, I. K., Rose, J. P., Wang, B.-C., and Dailey, H. A. (1999) Human ferrochelatase: crystallization, characterization of the [2Fe-2S] cluster and determination that the enzyme is a homodimer, *Biochimica et Biophysica Acta 1435*, 191-197.
10. Dailey, H. A., Sellers, V. M., and Dailey, T. A. (1994) Mammalian Ferrochelatase, *J. Biol. Chem. 269*, 390-395.
11. Dailey, H. A., Finnegan, M. G., and Johnson, M. K. (1994) Human Ferrochelatase Is an Iron-Sulfur Protein, *Biochemistry 33*, 403-407.
12. Day, A. L., Parsons, B. M., and Dailey, H. A. (1998) Cloning and Characterization of *Gallus* and *Xenopus* Ferrochelatases: Presence of the

[2Fe-2S] Cluster in Nonmammalian Ferrochelatase, *Arch. Biochem. Biophys. 359*, 160-169.
13. Ferreira, G. C., Franco, R., Lloyd, S. G., Pereira, A. S., Moura, I., Moura, J. J., and Huynh, B. H. (1994) Mammalian ferrochelatase, a new addition to the metalloenzyme family, 269 ed., pp 7062-7065.
14. Sellers, V. M., Wang, K. F., Johnson, M. K., and Dailey, H. A. (1998) Evidence That the Fourth Ligand to the [2Fe-2S] Cluster in Animal Ferrochelatase Is a Cysteine, *J. Biol. Chem. 273*, 22311-22316.

15. Wu, C.-K., Dailey, H. A., Rose, J. P., Burden, A., Sellers, V. M., and Wang, B.-C. (2001) The 2.0 Å Structure of Human Ferrochelatase, the Terminal Enzyme of Heme Biosynthesis, *Nat. Struct. Biol. 8*, 156-160.
16. Al-Karadaghi, S., Hansson, M., Nikonov, S., Jönsson, B., and Hederstedt, L. (1997) Crystal Structure of Ferrochelatase: The Terminal Enzyme in Heme Biosynthesis, *Structure 5*, 1501-1510.
17. Crouse, B. R., Sellers, V. M., Finnegan, M. G., Dailey, H. A., and Johnson, M. K. (1996) Site-Directed Mutagenesis and Spectroscopic Characterization of Human Ferrochelatase: Identification of Residues Coordinating the [2Fe-2S] Cluster, *Biochem. 35*, 16222-16229.
18. Matsubara, H. and Saeki, K. (1992) Structural and Functional Diversity of Ferredoxins and Related Proteins, *Adv. Inorg. Chem. 38*, 223-280.
19. Johnson, M. K. (1994) Iron-Sulfur Proteins, in *Encyclopedia of Inorganic Chemistry* (King, R. B., Ed.) 2 ed., pp 1896-1915, John Wiley & Sons, New-York.
20. Sellers, V. M., Johnson, M. K., and Dailey, H. A. (1996) Function of the [2Fe-2S] Cluster in Mammalian Ferrochelatase: a Possible Role as a Nitric Oxide Sensor, *Biochem. 35*, 2699-2704.
21. Medlock, A. E. and Dailey, H. A. (2000) Examination of the Activity of Carboxyl-Terminal Chimeric Constructs of Human and Yeast Ferrochelatases, *Biochem. 39*, 7461-7467.
22. Shepherd, M., Dailey, H. A., and Dailey, H. A. (2006) A New Class of [2Fe-2S]-Cluster-Containing Protoporphyrin (IX) Ferrochelatases, *Biochem. J. 397*, 47-52.
23. Johnson, M. K. and Smith, A. D. (2005) Iron-Sulfur Proteins, in *Encyclopedia of Inorganic Chemistry* (King, R. B., Ed.) 2 ed., pp 9612-9624, John Wiley & Sons, New-York.
24. Kiley, P. J. and Beinert, H. (2003) The Role of Fe-S Proteins in Sensing and Regulation in Bacteria, *Curr. Opin. Microbiol. 6*, 181-185.

MINERAL INGREDIENTS IN FOOD SUPPLEMENTS USED IN THE EUROPEAN UNION – SOME CURRENT ASPECTS

Gabriela GARBAN[1*], Zeno GARBAN[2], George–Daniel GHIBU[3], [4] Mihaela-Elisabeta MITROI

1 Laboratory of Environment and Nutrition, Institute of Public Health, Blvd. Dr. V.Babeş Nr.16, RO-300 226 Timişoara, Romania; 2. Department of Biochemistry and Molecular Biology, Faculty of Food Products Technology, University of Agricultural Sciences and Veterinary Medicine Timişoara, Romania; 3. Medical Department, S.C. CaliVita International, Timişoara, Romania; [4] Sanitary Veterinary and Food Safety Direction, Timiş, Timişoara, Romania;*
** E-mail: garban_g@yahoo.com*

ABSTRACT

Mineral ingredients in food supplements composition represent an important source of micronutrients for human diet. These micronutrients are organic or inorganic compounds including elements of cationic nature (metals)or anionic nature (non-metals). The mentioned substances are present in food supplements in diverse combinations and different concentrations, their role is to supplement the normal diet and they have nutritional or physiological effects.
Setting safety limits for minerals is of great importance because intakes above a certain level of some minerals can lead to undesirable or adverse effects on health. Establishing of maximum values for minerals should be set only on the basis of scientific risk assessment or based on nutritional needs and be set at 100% of the Recommended Daily Allowance (RDA) level or low multiples thereof. In high doses the minerals may have therapeutic effects and the product will be a drug and not food supplement.
Considered as food, in European Union discussions on food supplements approach aspects concerning their effects on human metabolism (sinergistic and antagonistic) and problems related to their manufacture, traceability, distribution and consumption. Last but not least the juristic bckground governing food supplements is of great importance for their safety.

Key words: *food supplements - mineral ingredients*

INTRODUCTION

Minerals are essential nutrients to life and are needed in small amounts and beside vitamins they constitute the class of micronutrients.

These nutrients participate in numerous catabolic and anabolic reactions in various biochemical pathways. Beside other nutrients the mineral compounds participate in physiological processes which assure the health maintenance. For the human organism there are essential about twenty eight elements, e.g. Ca, Mg, Na, K, Zn, Fe, Cu, Se, I etc.

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 30 - 37, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

Deficiency of minerals and especially of trace elements in food are actually more likely to occur than is vitamin deficiency. Because of differing geologic conditions minerals and trace elements may scare in the soil of certain region and rich in those of other regions (O`Dell and Sunde, 1997; Garban, 2008). The insufficiency or lack of one or more minerals leads to dysmineraloses.

Various diseases reduce also the mineral nutrients intake and especially those that interfere with the ingestion, digestion, absorption and requirement of nutrients: celiac disease, Crohn disease, irritable bowel syndrome, lactose intolerance, bacterial, viral ands parasitic infections.

Evidence from recent studies reveal that mineral trace element supplementation may help to prevent various forms of cancer, heart disease and some other degenerative processes.

1. THE GENERAL CHARACTERISTICS OF NUTRIENTS

Nutrients are defined as chemical constituents present in foods which are metabolized by the organisms assuring their morphophysiological status and the energy necessary to all living processes (Brody, 1994; Ganong, 1995). Nutrients are usually divided in three classes: 1) macronutrients which include: a) carbohydrates, b) lipids, c) proteins; 2) micronutrients including: a) vitamins, b) minerals (compounds with anionic or cationic nature); 3) other nutrients comprising: a) water, b) alimentary fibers, c) bioactive substances (Garban and Garban, 2004).

In the last decades the pollution of soil, water and air had as a secondary effect the lowering of the levels and quality of the nutrients from foods. While macronutrients are less affected by this phenomenon, micronutrients are more sensible to pollution and other environment stress factors (Champe and Harvey, 1987; Shrimpton, 1997). In the same time, due to the increased pollution of the environment and the deliberate contamination of foods (use of food additives) the need of human organism for minerals and vitamins is increasing. Also stress factors often lead to the decrease of nutrient absorption, therefore it appears the necessity to supplement the diet, especially, with micronutrients.

Food supplements, also known as “dietary supplements” or “nutritional supplement”, are concentrated sources of nutrients (vitamins and minerals) with the aim to complete the diet of a person if those nutrients are missing or not consumed in sufficient quantity (Ellis and Salt, 2003; Garban et al., 2009).

Having in view the continuous extension of food supplements consumption and the desire of manufacturers to use various nutrients in food supplements, e.g. minerals, vitamins, biologically active substances, make necessary to perform further experimental studies on laboratory animals in order to reveal the effects of food supplements ingredients on biochemical homeostasis, their role in morphogenesis and energy generation. Also, their effects can be followed in degenerative or chronic diseases etc.

An other important aspect related to food supplements is that of the setting of safety limits of each nutrient in its composition. If it is based on nutritional needs one must take account on the Recommended Daily Allowances (RDAs) of nutrients. In the framework of this issue one must mention that high doses of micronutrients in a food supplements may have therapeutic effect thus transforming it in a drug.

The following paper focuses only on the mineral micronutrients, their importance for the organisms and the problems raised by the establishing of their maximum safe limits and their Recommended Daily Allowance.

2. MINERAL NUTRIENTS

The mineral nutrients play an important role in the biological functions of all organisms. From nutritional point of view the minerals (beside vitamins) are included in the group of micronutrients. They are often found as cofactors in enzymes, e.g.: Zn^{2+} in alcohol dehyrogenase; Mn^{2+} in phosphotransferase; Fe^{2+} in cytochromes etc. (Bugg, 1997). Minerals can be present also in the composition of some hormones, e.g.: iodine in thyroid hormones or of certain amino acids, e.g.: selenomethionine. Mineral micronutrients are generally divided in two categories accordingly to their chemical characteristics: the micronutrients with cationic nature (metals) and micronutrients with anionic nature (non-metals). Also, taking in account the amount of this elements that is found in the organism, the mineral nutrients can be classified in macroelements (Na, K, Ca, Mg, P, Cl etc) and trace elements (Zn, Fe, Cu, I, Se).

The deficiency of mineral nutrients can lead to various symptoms and illnesses therefore making food supplementation a very important tool in avoiding this situation. Also one must consider that also the excess of mineral micronutrients may have as income unwanted effects. Therefore is necessary for the scientific community, regulatory authorities and industry to work together to ensure that levels of micronutrients in the total diet are safe, and that the sum of intakes from all the sources does not lead to excessive intakes and any adverse effects (Bernadier, 1998; Garban, 2006).

In the case of food supplements, in order to avoid excess intake a maximum safe level (MSL) has been established for each mineral micronutrients (Richardson, 2007). The MSL can be determined only if the tolerable upper intake level (UL) of the micronutrient is known. Tolerable upper intake level means the highest intake (MHI) and the intake from water (IW). Maximum safe level can be calculated by using the following formula: MSL = UL – [(MHI x 110%) + IW] – Richardson (2007).

It is important to know not only the cationic or anionic nature of the allowed substances but also the association of mineral compounds because in metabolic processes there are antagonistic and synergistic effects.

2.1. Micronutrients with cationic nature

According to the European Commission (2009), the micronutrients with cationic nature (i.e. metals) which may be used in the manufacture of food supplements are: sodium, potassium, calcium, magnesium, iron, copper, zinc, manganese, chromium and molybdenum.

Various organisms such as the Scientific Committee on Food (SCF), the European Food Safety Authority (EFSA), European Responsible Nutrition Alliance (ERNA) a.o. are involved in the establishment of the tolerable upper intake of minerals.

According to SCF and EFSA the tolerable upper intake levels for some of the cationic minerals are : Ca – 2500 mg, Mg – 250 mg, Cu – 5 mg, Zn – 25 mg, Mo – 600 μg (European Commission, 2007).

In table 1 comparative values of the maximum safe levels (MSLs) for food supplements per day are given: values used as industry guidelines based on risk assessments of the individual nutrients carried out by Shrimpton (1997) and values mentioned in ERNA Report (2004).

Table 1. Maximum safe levels of some micronutrients with cationic nature in food supplements

Micronutrient	Measure unit	MSLs / day (Shrimpton, 1997)	MSLs / day (ERNA Report, 2004)
Potassium	mg	—	1500
Calcium	mg	1500	1000–1500
Magnesium	mg	350	250
Iron	mg	15	14–20
Zinc	mg	15	10–15
Manganese	mg	15	2
Copper	mg	5	1–2
Chromium	mg	0.2	—
Molybdenum	μg	200	350

2.2. Micronutrients with anionic nature

The micronutrients with anion specificity (i.e. non-metals) which may be used in the manufacture of food supplements are, according to the European Commission (2009): iodine, selenium, fluoride, chloride, phosphorus, boron, silicon.

In the case of cationic minerals the tolerable upper intake levels for some of them, according to Scientific Committee on Food (SCF) and the European Food Safety Authority (EFSA) are: F – 600mg, I - 600 μg, Se – 300 μg.

Maximum safe levels of some anionic micronutrients in food supplements according to Shrimpton (1997) and to ERNA Report (2004) are given in table 2.

Table 2. Maximum safe levels of some anionic micronutrients in food supplements

Micronutrient	Measure unit	MSLs / day (Shrimpton, 1997)	MSLs / day (ERNA Report, 2004)
Phosphorus	mg	1500	1250
Fluoride	mg	—	3.5
Iodine	μg	500	150–200
Selenium	μg	200	200

Another way to increase the mineral micronutrient levels from diet is food fortification by adding such nutrients during the food processing. Therefore for an optimal supplementation one must take into account not only the food normal levels of mineral micronutrients but also the possible fortification of food which can present high variation from one country to another. So as the market (characteristic for every region), and hence the levels of micronutrients, varies over time, risk managers will need to monitor changing patterns of addition of nutrients to different foods and the use of food supplements in order to review the risk management options.

3. CHEMICAL FORMS OF MINERALS USED IN FOOD SUPPLEMENTS

Mineral micronutrients can be found in various chemical forms with different levels of absorption at the level of gastrointestinal tract and with various degrees of bioavailability. Therefore the regulatory institutions must establish which forms are

safer and have a higher bioavailability. Recently the European Commission had issued a draft commission Regulation amending Directive 2002/46/EC and Regulation 1925/2006/EC regarding the lists of vitamins and minerals and their forms that can be added to foods, including food supplements (European Commission, 2009).

The chemical forms of cationic and anionic minerals which can be used in food supplements that will be presented below are accordingly to this draft commission regulation.

In the future in the Member States of the European Union the problem of food supplements must be further harmonized taking into account the complex scientific, technologic and juristic aspects related the manufacture, placing on the market, consumption a.o. All these aspects have as aim to assure the safety use of food supplements thus protecting the consumers.

3.1. Cationic compounds

Among cationic minerals the most used in food supplements are: calcium, magnesium, copper and zinc.

Sodium and potassium supplementation is rarely needed. The accepted chemical forms for sodium are: bicarbonate, carbonate, chloride, citrate, gluconate, sodium lactate, hydroxide and salts of orthophosphoric acid.

Potassium is admitted in the following chemical forms: bicarbonate, carbonate, chloride, citrate, gluconate, glycerophosphate, lactate, hydroxide, L-pidolate, malate and salts of orthophosphoric acid.

In the case of calcium, the chemical forms admitted in food supplements are: acetate, L-ascorbate, bisglycinate, carbonate, chloride, citrate, malate, calcium salts of citric acid, gluconate, glycerophosphate, lactate, pyruvate, salts of orthophosphoric acid, succinate, hydroxide, L-lysinate, malate, oxide, L-pidolate, L-threonate and sulphate.

Magnesium can be used in food supplements in the following chemical forms: acetate, L-ascorbate, bisglycinate, carbonate, chloride, salts of citric acid, gluconate, glycerophosphate, salts of orthophosphoric acid, lactate, L-lysinate, hydroxide, malate, oxide, L-pidolate, potassium citrate, pyruvate, succinate, sulphate, taurate and acetyl taurate.

Iron is used in food supplement as ferrous (Fe^{2+}) or ferric ions (Fe^{3+}). Usually these forms are called in nutrition iron (II) respectively iron (III). Their derivatives are: a) ferrous compounds: carbonate, citrate, ferrous gluconate, ferrous fumarate, ferrous lactate, ferrous sulphate, ferrous bisglycinate, ferrous L-pidolate, ferrous phosphate and ferrous taurate; b) ferric compounds: ferric ammonium citrate, ferric sodium diphosphate, ferric diphosphate (ferric pyrophosphate), ferric saccharate, elemental iron (carbonyl + electrolytic + hydrogen reduced),

Copper can be added in food supplements as copper (Cu^{+}) or cupric (Cu^{2+}) ions, usually mentioned as Cu(I), respectively Cu(II), in the following forms: cupric carbonate, cupric citrate, cupric gluconate, cupric sulphate, copper L-aspartate, copper bisglycinate, copper lysine complex and copper (II) oxide.

Zinc is admitted in the food supplements in the following chemical forms: acetate, L-ascorbate, L-aspartate, bisglycinate, chloride, citrate, gluconate, lactate, zinc L-lysinate, malate, mono-L-methionine sulphate, oxide, carbonate, L-pidolate, picolinate and sulphate.

Manganese chemical forms accepted in the food supplements are: ascorbate, L-aspartate bisglycinate, carbonate, chloride, citrate, gluconate, glycerophosphate, pidolate, and sulphate.

In the case of chromium (III), the chemical forms used in food supplements are: chloride, trihydrate, nitrate, picolinate and sulphate.

Another cationic mineral used, although rarely, in food supplements is molybdenum. Its accepted chemical forms are: ammonium molybdate (molybdenum VI), potassium molybdate (molybdenum VI) and sodium molybdate (molybdenum VI).

3.2. Anionic compounds

From the anionic minerals used in food supplements selenium is more often encountered in food supplements. The accepted chemical forms for selenium are: L-selenomethionine, selenious acid, sodium selenate, sodium hydrogen selenite and sodium selenite. Also it can be found as selenium enriched yeast.

Iodine is an anionic mineral used in food supplements but more often used in food fortification. The chemical forms in which iodine is allowed in the food supplements are: sodium iodide, sodium iodate, potassium iodide and potassium iodate.

Another anionic mineral which is found in used supplements and more often found in food fortification is fluoride. The accepted chemical forms for the use of fluoride in supplements are: calcium fluoride, potassium fluoride, sodium fluoride and sodium monofluorophosphate.

Recently there were established the new values of the Recommended Daily Allowances (RDAs)for vitamins and minerals. In Table 3 are presented the values for minerals.

Table 3. Recommended Daily Allowances (RDAs) for minerals (according to Commission Directive 2008/100/EC)

Elements	RDAs
Metals	
Calcium (mg)	800
Magnesium (mg)	375
Sodium (mg)	-
Potassium (mg)	2000
Iron (mg)	14
Zinc (mg)	10
Manganese (mg)	2
Copper (µg)	1
Chromium (µg)	40
Molybdenum (µg)	50
Non-metals	
Chloride (mg)	800
Phosphorus (mg)	700
Iodine (µg)	150
Selenium (µg)	55
Fluoride (mg)	3.5

Two of the less often used anionic minerals in food supplements are boron and silicon. Their chemical forms accepted for use in food supplements are: boric acid, sodium borate, choline-stabilised orthosilicic acid, silicon dioxide and silicic acid.

Directive 2002/46/EC on food supplements regulates compositional aspects (e.g. positive lists of vitamins and minerals) and provide for appropriate specific rules on labelling, presentation and advertising of food supplements.

Although food supplements can contain a variety of ingredients, as a first step the Directive lays down specific provisions only for products containing vitamins and minerals.

A proposal for a Regulation on the addition of vitamins and minerals and of certain other substances to foods is currently under discussion at the European Parliament in and Council and is expected to be adopted by the end of 2009.

CONCLUSIVE ASPECTS

1. In case of food supplements an important aspect is related to source compounds of minerals, namely the chemical forms which can be used in their manufacture.

2. A special interest arises by the combination of nutrients in the composition of each food supplement. It is of great importance in the context of antagonistic or synergistic effects they can have.

3. A general overview on food supplements at the EU level reveal the necessity of laws harmonization, mutual recognition, appropriate Good Manufacturing Practice to ensure wholesome and useful product (establishing and the maintaining of the identity, purity, potency and hygiene of the food supplement), implementing the community rules with regard their place on the market, traceability, distribution, consumption etc.

4. An other actual problem is related to the establisment of maximum values for minerals in order to avoid the use of high doses which can develop therapeutic effects like drugs.

REFERENCES

1. Berdanier C.D. - *Advanced Nutrition: Micronutrients*. CRC Press, Boca Raton, 1998.
2. Brody T. – *Nutritional biochemistry*, Academic Press, San Diego, 1994.
3. Bugg T. – *Introduction to Enzyme and Coenzyme Chemistry*, Blackwell Science, Oxford-Cambridge, Mass. (USA), 1997.
4. Champe P.C., Harvey R.A. – Nutrition, pp. 297-309, in *Illustrated Review Biochemistry* (Champe P.C., Harvey R.A., Eds.), J.B. Lippincott Comp, Philadelphia, 1987.
5. Ellis D.R., Salt D.E. - Plants, selenium and human health. *Curr. Opin. Plant Biol*., 2003, 6, 273-279.
6. Ganong W.F. – *Review of Medical Physiology*, 17th edition, Lange Medical Book, Norwall, CT, 1995.
7. Garban Gabriela – *Monitorizarea şi inspecţia suplimentelor alimentare în Romania* - Sinteza naţională, Ed. Eurobit Timişoara, 2006.
8. Garban Gabriela, Ghibu G.-D., Mitroi Elisabeta-Mihaela, Garban Z. – Some actual problems concerning water soluble vitamins in food supplements in the European Union, pp.1-4, in "*Proceedings of The 16th Symposium on Analytical and Environmental Problems*", Szeged, 2009.
9. Garban Z. – *Biochemistry: Comprehensive treatise,* Vol. I, 4th ed., Ed. Solness, Timisoara, 2008.
10. Garban Z., Garban Gabriela - *Nutriţia umană* – Vol.I , ed. 3, Ed. Orizonturi Universitare, Timişoara, 2003.
11. O'Dell B.L., Sunde R.A. (Eds.) - *Handbook of nutritionally essential mineral elements*. New York, Marcel Dekker Inc., 1997.

12. Richardson D.P. - Risk management of vitamins and minerals: a risk categorisation model for the setting of maximum levels in food supplements and fortified foods, *Food Science and Technology Bulletin: Functional Foods*, 2007, 4 (6), 51–66.
13. Shrimpton D. H.- *Vitamins and minerals: a scientific evaluation of the range of safe intakes*, Council for Responsible Nutrition, UK, 1997.
14. *** - Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements
15. *** - European Responsible Nutrition Alliance (ERNA) - *Vitamin and mineral supplements: a risk management model*, Brussels, Belgium, 2004.
16. *** - Ordin MSP Nr. 1069/2007 privind suplimentele alimentare
17. *** - European Commission - *Orientation paper on setting maximum and minimum amounts for vitamins and minerals in foodstuffs*, Brussels, Belgium, 2007.
18. *** - Commission Directive 2008/100/EC of 28 October 2008, *amending Council Directive 90/496/EEC on nutrition labelling for foodstuffs as regards recommended daily allowances, energy conversion factors and definitions*
19. *** - European Commission - *Draft Commission Regulation amending Directive 2002/46/EC and Regulation (EC) No 1925/2006 as regards the lists of vitamins and minerals and their forms that can be added to foods, including food supplements*, Brussels, Belgium, 2009.

ARSENIC POLLUTION IN SOME MAJOR ROADSIDE STATIONS IN EDİRNE CITY (TURKEY)

Yıldız KALEBAŞI AKTAŞ and Arıkan KOCABAŞ

Trakya University, Department of Chemistry, 22030, Edirne, TURKEY
E-mail : yilaktas@yahoo.com

ABSTRACT

In this study, eight roadside stations were selected within immediate vicinity of the city of Edirne to determine local background levels for arsenic in order to investigate degree of traffic-sourced soil pollutions, pollution extent and limit values. The pollution distributions in soils were evaluated in terms of soil depth and horizontal distance from road. Arsenic was determined in roadside soil samples collected from different locations in Edirne using Graphite-Atomic Absorption Spectrometer (GAAS). The concentration of arsenic in the surface soil was higher than in subsoil and deep subsoil.

Keywords: Soil, Arsenic, traffic, pollution, Edirne

INTRODUCTION

Arsenic is a naturally occurring element that is widely distributed in the Earth's crust. Arsenic is classified chemically as a metalloid, having both properties of a metal and a nonmetal; however, it is frequently referred to as a metal. Elemental arsenic (sometimes referred to as metallic arsenic) is a steel grey solid material. However, arsenic is usually found in the environment combined with other elements such as oxygen, chlorine, and sulfur. Arsenic combined with these elements is called inorganic arsenic. Arsenic combined with carbon and hydrogen is referred to as organic arsenic.

In our country, contamination of environment with metals has been intensively studied (Ozkan et al. 2005; Arslan 2001; Bereket 1990; Yaman 1995; Haktanır et all. 1995; Ornektekin 1997; Soylak 2000; Bakırdere and Yaman 2008;; Arslan and Gizir 2004,2006; Narin et al. 1997,1999). But, such a study has not been carried out until now in Edirne, Turkey. The purpose of this study is to determine the concentrations of arsenic at roadside soil in Edirne. This research has been done by us only in Edirne and it is important to be comprehensive.

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 38 - 42, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

MATERIALS AND METHODS

Soil samples were collected at distances 1, 5 and 10 meters from roadside and two parallel samples taken from the same place. Soil samples were taken at depths of 0-10 and 10-30 cm from surfaces at every distance. Roadside soil samples were collected from 8 sites, to include low and high-density traffic roads in Edirne and total 384 samples were taken in a year. All the samples were collected with a spatula and kept in PVC packages.

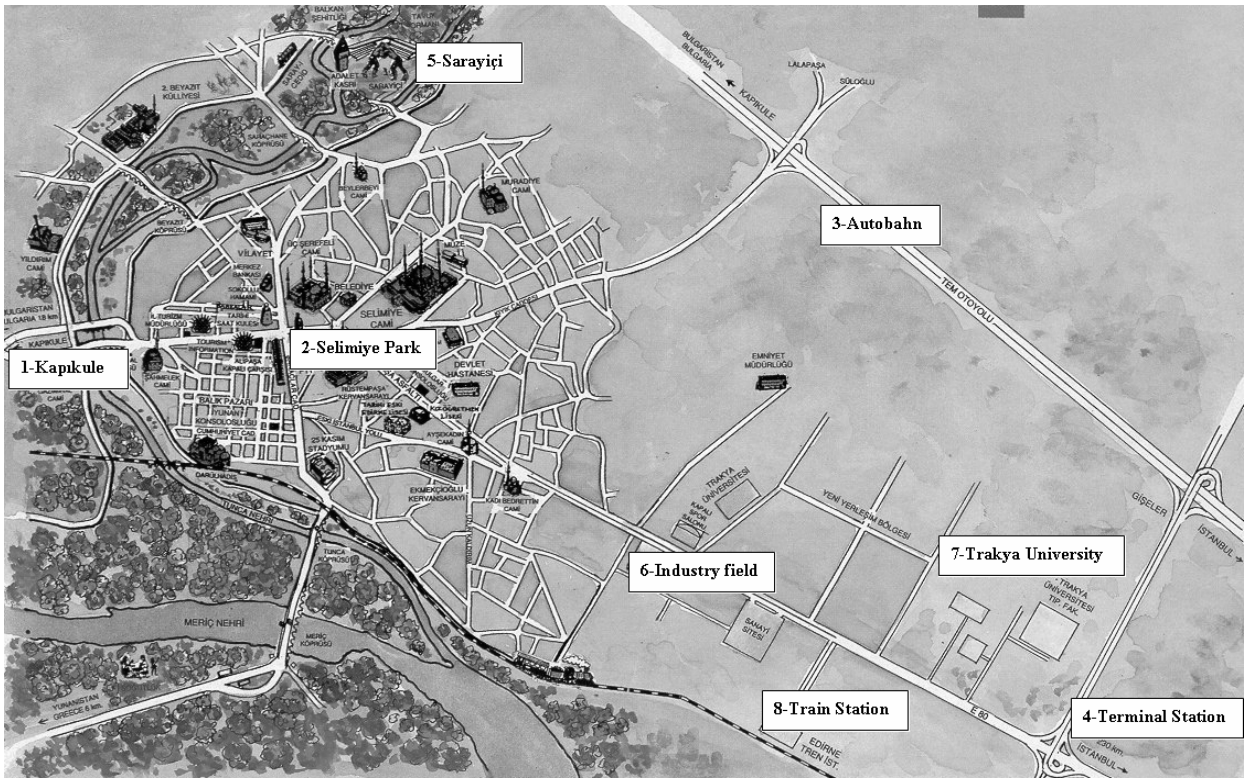


Figure 1. Sampling locations

The soil samples were dried 100-110 ºC for 2-3 hrs and for 3-4 hrs room temperatures, then ground to pass through a 200-mesh sieve, and homogenized for analysis. Microwave digestion oven (Antonpaar Multiwave 300) was used for preparing clear solutions. For this purpose, 0.4g of dried and fine powdered sample was weighed into vessel and 8 mL HNO_3 was added into vessel. And then, the samples were digested in a microwave oven (210 ºC and 180 psi). The samples were then cooled at room temperature and filtered with filtering paper without ashes (Whatman 42) before being diluted to a volume of 50 mL with water. The arsenic content of the samples was determined with a graphite-AAS (Varian AA280Z). Each sample was analyzed in duplicate.

RESULTS AND DISCUSSION

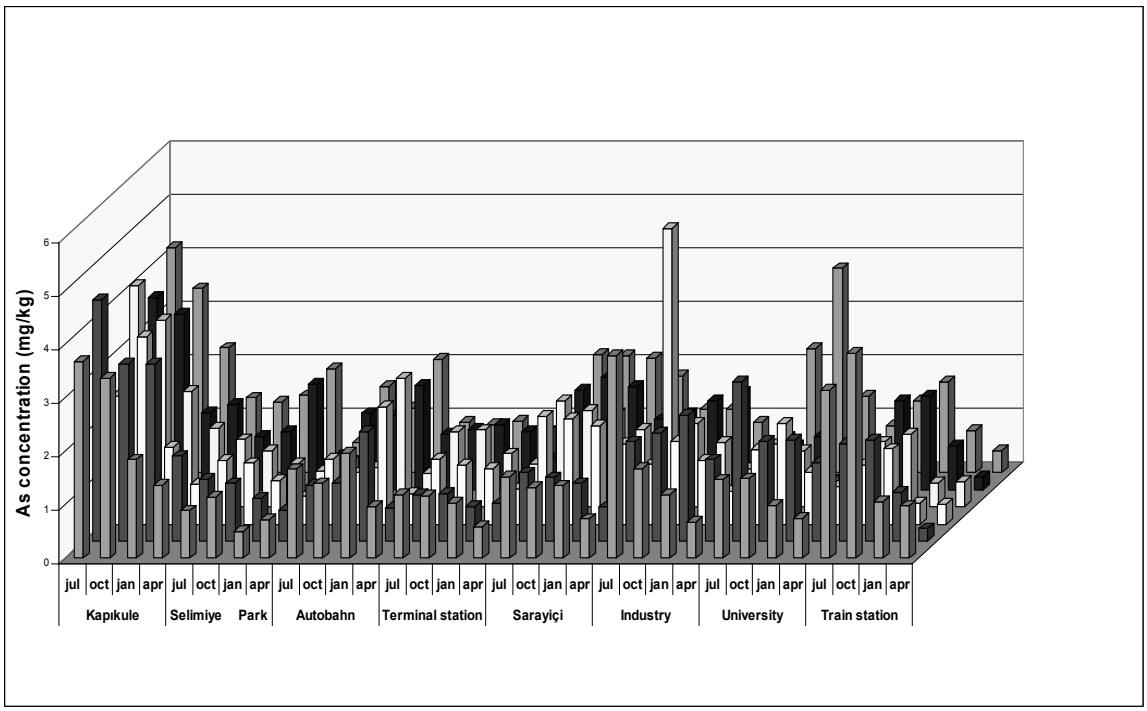


Figure 2. As concentrations (mg/kg) in soil samples for eight roads at different depths (0-10 and 10-30 cm) with distance (1, 5 and 10 m) from the road

SAMPLING LOCATIONS	As (mg/kg)
Kapıkule	2,651
Selimiye Park	1,012
Autobahn	1,336
Terminal	0,957
Sarayiçi	1,625
Industry	1,582
University	1,352
Train Station	1,221

Table 1. The means of As concentrations in Edirne roadside (mg/kg dry soil)

Arsenic pollution was low in Edirne since all soil samples along the eight roads was lowly or no polluted, followed by the order of Kapıkule > Sarayiçi >Industry Field> University>Autobahn> Train Station> Selimiye Park> Terminal Station (Table 1).

For the samples from the first 0-10 cm of depth As concentration was found between 0.236-3.833 mg/kg ; for the samples from 10-30 cm depth, this value was between 0.234-5.205 mg/kg. This indicates that As concentration in surface layers are generally higher.

Moreover, the arsenic concentrations from high traffic density areas are higher than those from low traffic density areas.

Table 2. As concentrations in Edirne roadside (mg/kg dry soil)

Sampling locations	Months	Distance from-1m		Distance from-5m		Distance from-10m	
		0-10cm	*10-30cm*	*0-10cm*	*10-30cm*	*0-10cm*	*10-30cm*
Kapıkule	July	3,685	4,507	2,398	4,149	3,59	4,206
	October	3,361	3,312	3,503	3,504	3,282	3,444
	January	1,845	3,315	1,438	2,158	1,44	2,335
	April	1,362	1,59	0,74	1,478	1,597	1,402
Selimiye Park	July	0,888	1,158	1,201	1,26	0,99	1,323
	October	1,14	1,08	1,138	1,046	1,09	1,443
	January	0,502	0,795	0,82	0,804	1,973	1,936
	April	0,727	0,585	0,513	0,669	0,644	0,572
Autobahn	July	1,667	1,049	1,219	0,842	1,432	1,607
	October	1,401	1,082	0,958	0,744	1,388	1,213
	January	1,974	2,048	2,19	2,413	1,945	2,123
	April	0,96	0,618	0,573	0,628	1,048	0,949
Terminal Station	July	1,188	0,876	1,21	1,404	1,132	0,897
	October	1,168	0,891	1,11	1,441	1,223	0,975
	January	1,031	0,639	1,043	0,994	1,083	0,758
	April	0,593	0,703	0,667	0,805	0,585	0,561
Sarayiçi	July	1,52	1,278	2,01	1,984	1,87	2,215
	October	1,33	1,19	1,974	1,809	2,099	2,188
	January	1,356	1,078	1,829	1,745	1,938	2,153
	April	0,733	0,635	1,499	1,442	1,319	1,819
Industry	July	3,786	1,858	1,115	5,205	1,418	1,183
	October	1,669	2,032	1,546	1,56	1,668	1,194
	January	1,195	2,358	1,194	1,208	1,829	0,951
	April	0,678	1,526	0,613	0,722	0,821	0,651
University	July	1,466	2,977	1,388	1,173	0,822	2,321
	October	1,488	1,871	1,882	1,059	0,998	3,824
	January	0,995	1,895	0,978	0,493	0,34	1,436
	April	0,749	1,473	0,708	0,666	0,57	0,886
Train station	July	3,15	1,816	1,107	1,219	1,667	1,342
	October	3,833	1,883	1,403	1,355	1,748	1,693

	January	1,058	0,918	0,39	0,445	0,811	0,78
	April	0,978	0,234	0,37	0,467	0,236	0,416

REFERENCES

1- Bereket, G. & Yücel, E. Monitoring of heavy metal pollution of traffic origin in Eskişehir. Turkish Journal of Chemistry, 1990,14, 266-271.

2- Yaman, S. Karayolu kenar topraklarında kurşun kirlenmesi, Ceyhan-Adana. Tr.J. of Engineering and Environmental Sciences, 1995,19, 303-306.

3-Ornektekin, S. A study of heavy metal pollution from motor vehicle emissions and its effect on soil in İskenderun, Nort-east Mediterranean. Turkish Journal of Engineering and Environmental Sciences, 1997,21, 45-49.

4-Soylak, M., Narin, I., Elçi, L. & Doğan, M. Lead concentrations of dust samples from Niğde city, Turkey. Fresenius Environmental Bulletin, 2000,9(1-2), 36-39.

5-Bakirdere, S. & Yaman, M. Determination of lead, cadmium and copper in roadside soil and plants in Elazıg, Turkey. Environmental Monitoring and Assessment, 2008,136, 401-410.

6-Özkan, M.H. Gürkan, R. Özkan, A. & Akçay, M. Dertermination of manganese and lead in roadside soil samples by FAAS with ultrasound assisted leaching. Journal of Analytical Chemistry, 2005,60(5), 469-474.

7-Arslan, H. Investigation of heavy metal pollution of traffic in Kemalpaşa-Turkey. Fresenius Environmental Bulletin, 2001,10(4), 405-408.

8-Arslan, H. & Gizir, A.M. Monitoring of heavy metal pollution of traffic origin in Adana. Fresenius Environmental Bulletin, 2004,13(4), 361-365.

9-Arslan, H. & Gizir, A.M. Heavy metal content of roadside soil in Mersin, Turkey, Fresenius Environmental Bulletin, 2006,15(1), 15-20.

10-Narin, I., Soylak, M. & Doğan, M. Traffic pollution in Niğde-Turkey: Investigation of trace element contents of soil samples. Fresenius Environmental Bulletin, 1997,6, 749-752.

11-Narin, I. & Soylak, M. Monitoring trace metal levels in Niğde, Turkey: nickel, copper, manganese, cadmium and cobalt contents of the street dust samples. Trace elements and Electrolytes, 1999,16(2), 99-103.

EFFECTS OF SODIUM NITRATE ADMINISTRATION IN DRINKING WATER OF LEPORIDES REGARDING ZINC CONCENTRATION IN ORGANS AND TISSUES

Zeno GARBAN[1], George-Daniel GHIBU[2], Adina AVACOVICI[3], Cornel BALTĂ[1], Gabriela GARBAN[4], Mihaela-Elisabeta MITROI[5]

1. Department of Biochemistry and Molecular Biology, Faculty of Food Products Technology, University of Agricultural Sciences and Veterinary Medicine Timişoara, Calea Aradului nr. 119, RO-300 645 Timişoara, Romania; 2. Medical Department, S.C. CaliVita International, Timişoara, Romania; 3. Faculty of Chemistry and Environmental Engineering, University Politehnica, Timişoara, Romania; 4. Laboratory of Environment and Nutrition, Institute of Public Health, Timişoara, Romania; 5. Sanitary Veterinary and Food Safety Direction, Timiş, Timişoara, Romania*

** E-mail: zgarban @yahoo.com*

ABSTRACT

Nitrates added to the drinking water of experimental animals (leporides) were administered "ad libitum" in order to determine the potential effects on the metals concentration. In this paper the effect of nitrates on the zinc concentration in some organs and tissues of leporides is discussed. The metallograms were obtained using the atomic absorption spectrophotometry.

The administered sodium nitrate solutions were in high concentrations as compared with the reference value representing the maximum contaminant level (MCL) in drinking water. The experiment investigated the effects of sodium nitrate administered on two groups of rabbits in concentrations of 20 x MCL (E_1) and 40 x MCL (E_2) compared with a control group (C). The analytical determinations underline the effects of sodium nitrate on the biochemical homeostasis of Zn by determination of its concentration in organs (liver, kidney), brain tissue and muscle tissue of rabbits.

Key words: sodium nitrate effects - zinc metallograms

INTRODUCTION

Nitrates present in the drinking water and foods are considered as chemical xenobiotics for the animal and human organism. Inorganic nitrogen occurs primarily as nitrates which are derived from the demineralization of the organic matter in soil. The excessive use of fertilizers based on nitrates in agriculture and the industrial pollution can lead to accumulation of high levels of nitrates in the environment.

From the environment nitrates accedes in plants and finally in the organism of animals and humans. Nitrates act as exogenous sources of nitric oxide which can lead to the formation of nitrosyl hemoglobin and dinitrosyl iron complexes with thiol groups of proteins (compounds which are stable enough to function as a depot of nitric oxide). Nitrate toxicity varies according to species and, generally, methemoglobinemia (Kaneko, 1989) and severe gastritis can be observed (Prakasa and Puttanna, 2002).

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 43 - 48, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009

Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

Another aspect of nitrate consumption is its ability to be converted in nitrites which are capable of inducing methemoglobinemia in various species (Zabulyte et al., 2007). Also, if certain conditions are met (i.e.: the presence of nitrites and amines) nitrates consumption may lead to carcinogenic nitrosamines (Vermeer et al., 1998; Garban, 2005).

Several types of cancers are suspected to be associated with the nitrate concentration in the drinking water, e.g.: Non-Hodgkin Lymphoma (Ward et al., 2006), gastrointestinal cancer (De Roos et al, 2003), brain cancer (Ward et al., 2005) and pancreatic cancer (Coss et al., 2004).

Nitrate administration is known to cause disturbances of kidney and liver function and also to alter the biochemical parameters of blood serum (urea, creatinine, uric acid). Another aspect which is often neglected in the scientific literature is the fact that nitrates, being salts of nitric acid, also have in their chemical structure metallic ions. These ions are released inside the organism and may interact with other bioconstituents and interfere in the absorption and biodisponibility of other biometallic ions.

MATERIALS AND METHODS

Experimental model. Sodium nitrate ($NaNO_3$) solutions were added in the drinking water of rabbits which was administrated “ad libitum”. The effect of nitrates consumption on the metabolism of Zn was studied. The reference value chosen for the preparation of nitrates solutions was the maximum contaminant level (MCL) admitted in drinking water. The MCL value was established by the Environment Protection Agency (EPA) from United States Department of Agriculture (USDA) to 10 mg/L nitrogen nitrate (usually noted as $N\text{-}NO_3$) and 1 mg/L nitrogen nitrite (usually noted $N\text{-}NO_2$).

In the present study the experiments were performed on 30 days old leporides with an average weight of 700 ± 25 g. The rabbits (Oryctolagus cuniculus) were divided in two experimental groups: E_1 and E_2 and one control group (C). All groups included 10 animals (5 males and 5 females). Animals were fed with VivaBio - a granulated fodder for rabbits produced by Freeman S.R.L (content: 14.94% protein, 2.86% fat and 8.51% cellulose and necessary minerals). Animals from control group received drinking water, while animals from E_1 group received drinking water with $NaNO_3$ solution - a concentration equivalent with 20 x MCL and E_2 group received drinking water with $NaNO_3$ solution - a concentration equivalent with 40 x MCL established for nitrate. The water used in the preparation of nitrates solutions was obtained from the same source as the one used for the control group.

Before the start of the experiment a quarantine period of 10 days was kept. During the quarantine period, the health status of rabbits was evaluated and the rabbits were accommodated with the experimental laboratory environment and with the diet made of granulated fodder. After the quarantine, the experiment has started and lasted for 20 days.

In order to obtain liver, kidney, brain and muscle samples. the animals were killed on day 20^{th} of the experiment. On this purpose ketamine was administered intravenously. The samples were than prelevated according to the techniques of laboratory animal’s necropsy and stored in 25 ml glass bottles which were placed in a refrigerator until the biochemical investigations were performed. Procedures respected all the recommendations regarding the use of experimental animals given by the national law (see O.G. 37/2002).

Biochemical investigations. Tissues were prelevated from animals of the control and experimental groups. Zinc concentration was determined in the "Laboratory of molecular and atomic spectroscopy" of the Faculty of Food Products Technology from Timişoara.

The samples were weighted and then calcined at a 700 ºC temperature for a 3 hours time and the resulted ash was mineralized with nitric acid (0,5 N) and brought in gradated flasks of 50 ml. The obtained solutions were then analyzed using a spectrophotometer with continuous atomic absorption (spectrophotometer type: Analytik Jena ContrAA 300). Every measurement was preceded by an adequate calibration of the spectrophotometer. Analytical data were expressed in μg/g wet weight.

Statistical analysis. Mean values (X) and standard deviation (SD) were determined for each parameter obtained. Using software Origin 6.0. the Student test was performed.

RESULTS AND DISCUSSIONS

Zinc is a ubiquitous component of plant, animal and human tissue. The high affinity of zinc for nitrogen-containing and sulfur-containing ligands is primary responsible for the occurrence of zinc in a wide variety of biological compounds, such as proteins, amino acids, nucleic acids, and porphyrins. It is estimated that 3000 of the hundreds of thousands of proteins in the human body contain zinc and a part of these proteins are enzymes which have an important role in the organism (e.g: carbonic anhydrase, carboxypeptidase, alcohol dehydrogenase, DNA polymerase, RNA polymerase, tyrosinase, Zn-Cu superoxide dismutase etc). Zinc plays also an important role in DNA biosynthesis, in transcription and transfer of polynucleotides and in transmission of the genetic information (Anke et al, 1993; Garban, 1994). Generally it is distributed in blood as follows: red blood cells (75-85%); in plasma (12-22%); in leucocytes (3%). In the organism zinc was found in higher quantities in liver, muscle, suprarenal gland, prostate. Zinc deficiency is related to daily food intake and was observed in certain diseases, e.g. cirrhosis, pancreatic failure, uremia, giardosis etc.

In order to investigate the influence of sodium nitrates ($NaNO_3$) solutions in two different concentrations on Zn the concentration in the studied organs (kidney, liver) and in tissues (brain, muscle) we compared the results from the E_1 and E_2 groups. The comparison showed different effects of $NaNO_3$ at 20xMCL and 40xMCL concentration (Ghibu, 2008a).

The results of nitrates influence on Zn concentration in the studied organs is shown in table 1.

Table 1. Zinc concentration in liver and kidney of rabbits after administration of $NaNO_3$ solutions of 20xMCL and 40xMCL

Studied organ	n	Group C (μg/g) $\overline{X} \pm DS$	Group E_1 (μg/g) $\overline{X} \pm DS$	ΔX_1	Group E_2 (μg/g) $\overline{X} \pm DS$	ΔX_2
Liver	10	24,60 ±4,39	23,37 ±1,93	- 1,23	22,44 ±1,46	- 2,16
Kidney	10	24,87 ±0.42	23,21 ±0,90*	- 1,66	22,90 ±0,89*	- 1,97

*p<0,01

One can see that the administration of sodium nitrate solutions decreased the concentration of zinc in the liver and kidney and that this decrease is directly

proportional with the concentration of sodium nitrate added in the drinking water. The values were statistically significant only in the case of zinc concentrations in the kidney. The main explanation consists in the diuretic properties of sodium nitrate which lead to the elimination of a higher quantity of minerals through urine.

Also the decrease of zinc concentration can be due to the presence of diverse xenobiotics in the environment, e.g. nitrates, nitrites, nitrosamines (Garban, 2008). The case of nitrates is revealed in the present paper.

Carbonic anhydrase is a zinc containing metallo-enzyme whose activity has been proved in laboratory experiments to be inhibited by the inorganic anions like nitrates, nitrites, sulfites etc (Franchi et al, 2003; Innocenti et al, 2004). Therefore the decrease of zinc after the consumption of drinking water with sodium nitrate can be also explained by this fact.

The decrease of zinc in kidney and liver can have important health consequences. Decreased zinc levels have been implicated in both acute and chronic liver disease states, and zinc deficiency has been implicated in the pathogenesis of some liver diseases as cirrhosis (Stamoulis et al., 2007). Also zinc is known to have a direct effect on the activity of glutathione transferase which is considered to be a chemical carcinogen-detoxifying enzyme which explains why carcinogenic effects of nitrosamines are enhanced in zinc-deficient rats (Lee and Fong, 1986).

Table 2. Zinc concentration in brain tissue and in muscle tissue of rabbits after administration of $NaNO_3$ solutions of 20xMCL and 40xMCL

Studied tissue	n	Group C (μg/g) $\overline{X} \pm DS$	Group E_1 (μg/g) $\overline{X} \pm DS$	ΔX_1	Group E_2 (μg/g) $\overline{X} \pm DS$	ΔX_2
Brain	10	12,90 ±0,84	11,51 ±2,37	- 1,39	11,19 ±0,97	- 1,71
Muscle	10	12,93 ±1,43	14,03 ±1,46	+ 1,10	15,05 ±1,12*	+ 2,12

*p<0,01

The obtained results revealed an increase of zinc concentration in muscle tissue. The increase is direct proportional with the increase of sodium nitrate concentration and is significant only in group E_2. Usually the depletion of zinc in most of the organs does not affect in the same way the muscular tissue. Experiments have shown that in zinc depleted animals there was no decrease in the zinc content of hair, skin, heart or three different skeletal muscles although the levels in plasma, liver, bone and testes were significantly reduced (Jackson et al., 1982).

Also as an experimental observation it must be noted that the rabbits have shown behavioral modifications and hyperkinesis. Therefore the increase of the zinc level in muscle can be correlated with the fact that zinc is a key component of some metallo-enzymes like lactate dehydrogenase which play an important role the muscular activity. Lactate dehydrogenase is found in higher concentration in muscle in the case of a prolonged effort and it has the role of convert pyruvate in lactic acid (Maltin et al., 1983).

Creatinine derives from the non-enzymatic dehydration of creatine in skeletal muscle or from or through spontaneous cyclization of phosphocreatine. The quantity of creatinine per body mass unit is constant, therefore the rate of creatinine production is constant (Devlin, 2002).

Serum and urinary creatinine levels depends with the subject muscle mass and shows little response to dietary changes in the case of healthy subjects. In the kidney

most of the serum creatinine undergoes a glomerular filtration process, though a small quantity is actively secreted. Due to this particularity the clearance of creatinine may be used to estimate the “glomerular filtration rate” (Mathews et al., 2000) and also it can be used as an indicator of renal physiological status (Perrone et al., 1992). The reference value for creatinine in healthy rabbits is 0.5 - 2.6 mg/dL (Kaneko, 1989).

Zinc is a cofactor in the sense of metabolic effector and intervenes in hormones like insulin (containing about 0.153% zinc), gonadotropic hormone secreted by the anterior pituitary gland and placenta favoring fecundation.

In case of zinc in muscle tissue is observed a direct proportional relation between its concentration and the quantum of sodium nitrate from drinking water.

In our investigations values of serum creatinine levels increase consecutively to nitrate solutions administration is revealed. The variation of creatinine concentration is directly proportional with the concentration of the sodium nitrate administered in drinking water (Ghibu et al., 2008b).

The increase of serum creatinine levels is correlated with the observation made on the behaviour of the laboratory animals which had shown a state of hyperkinesias (explained through the biochemical interactions regarding the interconversion between creatine and creatine-phosphate)

Similar data on biochemical homeostasis after to sodium nitrate administration have been found in rats by Zabulyte et al. (2007). In another study (Shour et al., 1999) approaching the problem of sodium nitrate administered to experimental groups of 5 rabbits revealed an increase of the serum cretinine values together with those of uric acid and urea.

CONCLUSIONS

1. Zinc homeostasis in the studied organs and tissues is modified in the case of sodium nitrate administration at different concentration (20xMCL and 40xMCL). The resulted dyshomeostasis may affect the health status with implications in time.
2. Administration of sodium nitrate in drinking water of leporides produces the depression of zinc concentration in liver and kidney. The depression of zinc concentration is inversely proportional with the concentration of the nitrate in drinking water. Renal function is affected in a larger extent, fact that is ascertained by significant values.
3. In case of brain tissue it has also been observed a decrease of the zinc concentration, the values being not statistically significant.
4. Zinc concentration in the muscular tissue increased after the sodium nitrate consumption. This increase was directly proportional with the sodium nitrate concentration added to the drinking water.

REFERENCES

1. Anke M., Meissner D., Mills C.F. - *Trace elements in man and animals*, Verlag Media Touristik, Gersdorf, 1993.
2. Coss A., Cantor K.P., Reif J.S., Lynch C.F., Ward M.H. - Pancreatic cancer and drinking water and dietary sources of nitrate and nitrite, *Am. J. Epidemiol.*, 2004, 159, 693-701.
3. De Roos A.J., Ward M.H., Lynch C.F., Cantor K P. - Nitrate in public water supplies and the risk of colon and rectum cancers, *Epidemiology*, 2003, 14, 640-649.
4. Devlin T.M. (ed) - Textbook of biochemistry with clinical correlations, 5th ed., Wiley-Liss, NewYork, 2002.

5. Franchi M., Vullo D., Gallori E., Antel J., Wurl M., Scozzafava A., Supuran C.T. - Carbonic anhydrase inhibitors: Inhibition of human and murine mitochondrial isozymes V with anions, *Bioorganic & Medicinal Chemistry Letters*, 2003, 13 (17), 2857-2861.
6. Garban Z. **-** Zinc and copper effects on deoxyribonucleic acid studied in vitro and vivo, pp.357-362, in *"New Perspectives in the research of Hardly Known Trace Elements" (*Ed. Pais I.) University Press of U.H.F.S. Budapest, 1994.
7. Garban Z. – *Xenobiochimie: Tratat comprehensiv*, Vol. IV, Editura Eurobit, Timişoara, 2005
8. Garban Z. – *Biochemistry: comprehensive treatise*, Vol. I, Publishing House Solness, Timişoara, 2008
9. Ghibu G.D., Baltă C., Gabriela Garban, Sayti L, Corina Falcă, Garban Z. - Peculiarities of hepatic metallograms in rabbits after nitrates administration, *Journal of Agroalimentary Processes and Technologies*, 2008a, 14, 361-366.
10. Ghibu G.-D., Garban Gabriela, Falcă Corina, Baltă C., Precob V., Garban Z. - Dyshomeostatic Effects of Nitrates from Drinking Water on Non-protein Nitrogen Metabolites in Leporides, *Chem. Bull. "Politehnica" Univ. (Timişoara),* 2008b, 53(67), 1-2, 230-233.
11. Innocenti A., Zimmerman S., Ferry J., Scozzafava A., Supuran C.T. - Carbonic anhydrase inhibitors. Inhibition of the beta-class enzyme from the methanoarchaeon Methanobacterium thermoautotrophicum (Cab) with anions, *Bioorganic & Medicinal Chemistry Letters*, 2004, 14 (17), 4563-4567.
12. Jackson M.J., Jones D.A., Edwards R.H.T. - Tissue zinc levels as an index of body zinc status, *Clinical Physiology and Functional Imaging*, 1982, 2 (4), 333-343.
13. Kaneko J.J. - *Clinical biochemistry of domestic animals*, Academic Press, New-York, 1989.
14. Lee J.S., Fong L.Y. - Decreased glutathione transferase activities in zinc-deficient rats, *Carcinogenesis*,1986, 7(7), 1111-1113.
15. Maltin C.A., Duncan L., Wilson A.B., Hesketh J.E.- Effect of zinc deficiency on muscle fibre type frequencies in the post-weanling rat, *Br .J. Nutr*, 1983, 50 (3), 597-604.
16. Mathews C.K., van Holde K.E., Ahern K.G. – *Biochemistry*, 3rd ed, Cummings, San Francisco, 2000.
17. Perrone R.D., Madias N.E., Levey A.S, - Serum creatinine as an index of renal function: new insights into old concepts, *Clin. Chem*., 1992, 38, 1933-1953.
18. Prakasa Rao E.V.S., Puttanna K. - Nitrates, agriculture and environment, *Current. Sci.*, 2002, 79*,* 1163-1168.
19. Shour A., El-Aziz I., Kerrit A., Al-Agha M. – Effects of oral administration of nitrate on serum glucose, some lipids, and non-protein nitrogen constituents, *Islamic University Journal*, 1999, 7(1), 1-13.
20. Stamoulis I., Kouraklis G., Theocharis S. - Zinc and the liver: an active interaction, *Dig. Dis. Sci.*, 2007, 52(7),1595-1612.
21. Vermeer I.T.M., Pachen D.M.F.A., Dallinga J.W., Kleinjans J.C.S., van Maanen J.M.S. - Volatile N-nitrosamines formation after intake of nitrate at the ADI level in combination with an amine-rich diet, *Environ. Health Perspectives*, 1998, 106, 459–463.
22. Zabulyte D., Uleckiene S., Kalibatas J., Paltanaviciene A., Jascaniniene N., Stosik M. – Experimental studies on effect of sodium fluoride and nitrate on biochemical parameters in rats, *Bull. Vet. Inst. Pulawy*, 2007, 51, 79-82.
23. Ward M.H., Heineman E.F., McComb R.D., Weisenburger D.D. - Drinking water and dietary sources of nitrate and nitrite and risk of glioma, *J. Occup. Environ. Med.,* 2005, 47, 1260-1267.
24. Ward M.H., Cerhan J.R., Colt J.S., Hartge P. - Risk of non-Hodgkin lymphoma and nitrate and nitrite from drinking water and diet, *Epidemiology*, 2006, 17, 375-382.
25. *** Ordonanţa de Guvern nr.37 din 30 ianuarie 2002 pentru protecţia animalelor folosite în scopuri ştiinţifice sau în alte scopuri experimentale.

DIRECT DETERMINATION OF HEAVY METALS IN PLANT SOLID SAMPLES USING HR-CS AAS

Bianca-Petronela PAVEL, Constantin-Horia BARBU*, Camelia SAND, Mihai-Radu POP

"Lucian Blaga" University of Sibiu, Faculty of Agricultural Sciences, Food Industry and Environmental Protection, Ro-550024 Sibiu, 10 Victoriei Bv.

** E-mail : horiab@rdslink.com*

ABSTRACT

One of the most useful analytical methods for the determination of heavy metals, atomic adsorption spectroscopy, has entered in a new phase, due to the possibility of analyzing directly solid samples, without any digestion.

The paper presents the application of this technique to the determination of Cd in stems of Miscanthus sinensis x giganteus*, a plant that could be cultivated on soils poolutyed with heavy metals*.

Keywords: high-resolution continuum source AAS, cadmium, Miscanthus, Copsa Mica

INTRODUCTION

As many know, atomic absorption spectrometry (AAS) is one of the widest used techniques for determination of heavy metals, its accuracy, reliability and simplicity being proven in laboratories all over the world. But, as also many can tell, classical AAS has several disadvantages, such as the necessity of changing the hollow cathode lamps according to the element(s) to be determined and the impossibility of analyzing samples but in liquid state, this meaning that any solid material should be brought in solution, which, in case of soil or plant samples requires digestion, a time-consuming and error-generating operation.

These limitations were exceeded by the specialists from Analytik Jena, whose equipment, ContrAA 700 represent a major breakthrough in the area, introducing the concept of High Resolution Continuum Source Atomic Absorption Spectrometry (HR-CS AAS).

The novelties of this modern device are: a high-intensity xenon short-arc lamp as continuum radiation source, a high-resolution double echelle monochromator as spectral sorting device, and a charge coupled device (CCD) array detector, providing a resolution of ~2 pm per pixel (Vale et al. 2001, Welz et al., 2007).

Among the major advantages of the system are:

- an improved signal-to-noise ratio due to the high intensity of the radiation source, resulting in improved photometric precision and detection limits;
- secondary lines can be used without compromises;
- new elements might be determined, for which no radiation source has been available;
- the entire spectral environment around the analytical line becomes 'visible', giving a lot more information than current AAS instruments;

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp..49 - 52, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

- the CCD array detector allows a truly simultaneous background correction close to the analytical line (Weltz et al 2003, Vale et al. 2006).

MATERIAL AND METHOD

Instrument

For determination of cadmium in solid state Miscanthus stems we have used a ContrAA700 Analytik Jena equipment (High Resolution Continuum Source Atomic Absorption Spectrometer) with SSA600 automatic solid sampler (single tray – 42 positions). The atomization cell was purged with argon. Starting from the data provided by the manufacturer, after several attempts, there were determined the best conditions for the analysis (method development), including the graphite furnace temperature program (Table 1). Wavelength was chosen for the secondary line of 326.1055 nm, not tthe 228nm principal one, in order to eliminate any interference and scattering effect, and a reading time of 5 seconds was set up.

Table 1. Graphite furnace temperature program

Step name	Temp. (°C)	Ramp (°C/s)	Hold (s)	Time (s)	Argon washing	Additional air injection
Drying	120	10	20	28.0	Max	Stop
Drying	150	10	20	23.0	Max	Stop
Drying	200	10	10	15.0	Max	Stop
Ash	350	25	45	51.0	Min	Max
Ash	430	15	45	50.3	Min	Max
Ash	600	15	20	31.3	Min	Max
Pyrolysis	600	0	10	10.0	Max	Stop
Pyrolysis	650	30	10	11.7	Max	Stop
AZ (auto zero)	650	0	5	5.0	Stop	Stop
Atomization	2100	1200	5	6.2	Stop	Stop (read)
Cleaning	2500	500	4	4.8	Stop	Stop

To reduce the organic matrix influence for cadmium analysis, ashing with addition of air (0.5 L/min) was used.

Reagents

The equipment was calibrated starting from a 1000±5 mg/L Cd CertiPUR solution from Merck, diluted in a 0.5% HNO_3 solution. These standard solutions were obtained from ultrapure concentrated Merck HNO_3 and ultrapure water (TKA Smart2Pure, 0.055µS/cm). To stabilize Cd analyte thermally in the pyrolysis stage and to make the other elements in the sample more volatile to facilitate separation Cd from the matrix it was used a 0.1% $Pd(NO)_3$ solution (Merck), as modifier (Resano et al, 2008). Class A volumetric glass flasks were soaked in a 10% HNO_3 solution for 24 h, then rinsed with ultrapure water and dried. Eppendorf adjustable pipettes (2-20 µL) were used.

Calibration

Calibration was made using 50,100 and 400 µg/L Cd standard solutions, of which 10-20 µL were added in the graphite platforms, respectively. In each platform there were added 10 µL of modifier - 1% $Pd(NO)_3$ solution. For zero-point it was used the 1% HNO_3 solution, with modifier. The calibration points, after addition of standard solutions have represented 1.0, 1.5, 2.0, 4.0 and 8.0 ng Cd, respectively.

Analytical procedure

Stems and leaves from a *Miscanthus sinensis x gigantheus* culture, established on a very polluted soil in Copsa Mica, were collected in April 2009, in HDPE bags. In the laboratory they were oven dried at 105^{o}C, for 2 h, and cut into small pieces, with a stainless steel lancet and then grinded under 10µm (Fritch – Pulverisette 0). Small stem pieces were brought into a graphite boat, then automatically weighted. After addition of 10 µL of modifier, every sample was introduced into the graphite furnace and analyzed.

The equipment performs three sets of measurements for each sample (weight and absorbance), automatically calculating the average values and the standard deviations. At the end, diagrams showing the absorbance and result tables are generated.

RESULTS AND DISCUSSION

The aim of our work was to determine the amount of heavy metals in plants cultivated on soils polluted with heavy metals, in order to give farmers a chance for the sustainable use of their land. For this reason, we have chosen a plant that is not yet cultivated in Romania, *Miscanthus sinensis x gigantheus,* with very large yields (20 tons per hectare and year), and multiple uses, mainly for green energy (Jones and Walsh, 2001). Because only stems are used, we have focused our attention on these.

The results of the analyzes, including Standard deviation (SD) and Relative standard deviation (RSD) are presented in Table 2.

Table 2. Cadmium concentration (µg/kg, d.w.) in Miscanthus stems.

	Concentration (µg/kg)	**Absorbance**	**Cd mass (pg)**	**SD**	**RSD**
Sample 1	1205	0.01042	2057	233.2	19.4
Sample 2	1625	0.01417	1812	502.4	30.9
Sample 3	2686	0.02258	5039	1060	39.5
Sample 4	1490	0.01303	3518	1059	71.1
Sample 5	2518	0.02152	2855	1285	51.0
Sample 6	2992	0.02528	4101	1054	35.2

As it may be seen, the amount of Cd in the analyzed stem is about 2.1 ppm, a very low value considering the soil load (14.5 ppm Cd), this enabling us to recommend the cultivation of Miscanthus on these type of soils.

Considering that the analysis, including calibration took less than 40 minutes, and the amount of Argon used was 25 L, we consider that for these kind of samples can be accurate and efficient analyzed using this method.

CONCLUSIONS

Even though we have started using the ContrAA 700 equipment only recently, we can conclude that high-resolution continuum source atomic absorption spectrometry is a fast, reliable and efficient method for direct analysis of solid samples.

ACKNOWLEDGEMENTS

This research was performed within the Romanian R&D Grant Scheme „Parteneriate", Contract no. 31-022, and the analysis were done with the kind help of Mrs. Michaela Portala and Mr. Oliver Buettel, from Analytik Jena GmbH, Germany.

REFERENCES

1. Jones M.B., Walsh M. – *Miscanthus for energy and fibre,* James & James Publishers, p. 23-49, London 2001.
2. Resano M., Vanhaecke F., de Loos-Vollebregt M. T. C. - *Electrothermal vaporization for sample introduction in atomic absorption, atomic emission and plasma mass spectrometry—a critical review with focus on solid sampling and slurry analysis*, J. Anal. At. Spectrom., 23, 1450–1475, 2008.
3. Vale M.G.R., Silva M.M., Welz B., Lima E.C. - *Determination of cadmium, copper and lead in mineral coal using solid sampling graphite furnace atomic absorption spectrometry,* Spectrochimica Acta, Part B, 56: 1873-1859, 2001.
4. Vale M.G.R., Oleszczuk N., dos Santos WNL, *Current status of direct solid sampling for electrothermal atomic absorption spectrometry – A critical review of the development between 1995 and 2005,* Appl. Spectr. Rev., 41: 377-400, 2006.
5. Welz B., Becker-Ross H., Florek S., Heitmann U., Vale, M.G.R. - *High-Resolution Continuum-source Atomic Absorption Spectrometry – What Can We Expect?,* J. Braz. Chem. Soc., Vol. 14, No. 2, 220-229, 2003.
6. Welz B., Vale M.G.R., Borges D.L.G., Heitmann U. - *Progress in direct solid sampling analysis using line source and high-resolution continuum source electrothermal atomic absorption spectrometry*, Anal Bioanal Chem, 389:2085–2095, 2007.

TOXIC EFFECT OF SEMINAL PLASMA LEVELS OF COPPER ON HUMAN SEMEN PARAMETERS

Maryam EIDI, Akram EIDI, Pouneh SHAHMOHAMMADI, Omid POUYAN, Reza FAZAELI, Massih BAHAR

Department of Biology, Varamin Branch, Islamic Azad University, Varamin, Iran

** E-mail : maryameidi@gmail.com*

ABSTRACT

The trace element Cu has been identified as a highly toxic element for sperm. To investigate the correlation between plasma seminal copper concentrations with semen parameters, a standardized semen specimen from 320 males. Samples were categorized into normospermic, oligozospermic, asthenozospermic, azospermic and varicocele groups according to their spermiograms. Total seminal plasma copper (Cu) concentration was determined by furnace atomic absorption spectrophotometer. The results showed that seminal plasma Cu concentrations in oligozospermic, asthenozospermic, azospermic and varicocele groups are significantly higher than normozospermic group. Also, negative correlations were found between seminal plasma Cu concentration and sperm count, sperm motility, sperm vitality, normal morphology and pH in all groups. Therefore, the determination of the Cu element in seminal plasma could be a critical parameter in evaluation of semen parameters.

Key words: copper, semen parameters, male infertility

INTRODUCTION

Exposure to environmental contaminants has been suggested to play a role in the pathophysiology of adverse reproductive health effects including decreased semen quality, sub-fertility, change in birth sex ratio, and an increase in the prevalence of developmental abnormalities of the male reproductive tract (Carlsen et al., 1992; Colborn et al., 1992; Swan 1997). Copper products act as components of large systems, such as building, magnet, motor vehicle and telecom wire, copper tube, sheet and strip and many alloy products (Spatari et al., 2002). Cu in tailings and smelter slag is a potential environmental hazard (Gordon 2002) and high Cu in drinking water transported through corroded Cu tube has been frequently observed (Barceloux 1999). The trace element Cu has been identified as a highly toxic element for sperm (Skandhan 1992). It is also known to affect sperm motility in humans (Mann 1981), and experimental implantation of copper in the epididymis, vas deferens, and scrotum of mammals has been demonstrated to affect fertility detrimentally (Mann 1981).

Sperm concentration, motility, vitality, morphology and seminal plasma fructose concentration are parameters used to evaluate potential male fertility. Since, Cu is believed to be important for spermatogenesis; we conducted a study to investigate the correlation between plasma seminal Cu concentration and human semen parameters.

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 53 - 57, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

MATERIALS AND METHODS

Ejaculates were provided from a total of 330 males (mean age 31.22 ± 5.3) attending the Omid Fertility Clinic, Tehran, Iran. Subjects could not have baby after 2 years conception. Participants provided semen samples in polypropylene containers, via masturbation after an abstinence period of 2 to 3 days at the laboratory. Aliquots were taken after liquefaction at 37 ºC. Semen analysis was performed according to the World Health Organization guidelines to obtain volume, pH, vitality, sperm concentration, motility, morphology and seminal plasma fructose concentration (World Health Organization, 2000). Sperm concentration was determined with a Neubauer® counting chamber. Motility was expressed as the percentage of motile spermatozoa into 4 categories (immotile, flagella, slow and rapid motile). Morphology was determined according to the WHO criteria using the papanicolaou's staining procedure. At least 300 cells were examined at a final magnification of 1000×. Following semen analysis, samples were centrifuged at 1000 g for 5 min and the seminal plasma supernatants were removed for measurement of fructose concentration. Concentrations of seminal fructose were determined using acid resorcinol colorimetric method. Copper concentration was measured by furnace atomic absorption spectrometry. Samples were diluted in high purity water (1:2). Wavelength was 324.8 nm. Calibration curve was delineated using suitable standard concentrations (10, 50 and 100 μg/L) by diluting standard $CuCl_2$, H_2O solution (Merck, Darmstadt). Statistical analyses were performed with the SPSS program. Pearson correlation coefficients were implemented to determine correlations between seminal plasma copper concentration and semen parameters. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

In the present study, seminal plasma copper concentration was compared among 5 groups. Figure 1 showed that seminal plasma copper concentration is higher in azospermic ($p<0.001$), oligozospermic ($p<0.001$), varicocele ($p<0.01$) and asthenozospermic ($p<0.01$) groups than normospermic males.

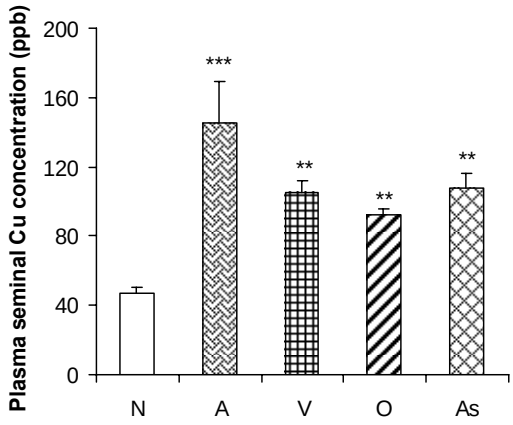


Fig. 1. Plasma seminal copper concentrations in normospermic (N), azospermic (A), oligozospermic (O), varicocele (V) and asthenozospermic (As) groups. ** $p<0.01$, *** $p<0.001$ different from normospermic group.

When studying the correlations between the seminal plasma copper concentration and semen parameters, significant negative correlations were found between seminal plasma copper concentration and pH ($r_s = -0.173$, $p<0.01$), vitality

(r_s = -0.391, p<0.01) (Fig. 2), sperm concentration (r_s= -0.114, p<0.05), total motility (r_s = - 0.399, p<0.01) (Fig. 3), normal morphology (r_s = -0.317, p<0.01), and fructose concentration (r_s = 0.116, p<0.05) (Fig. 4).

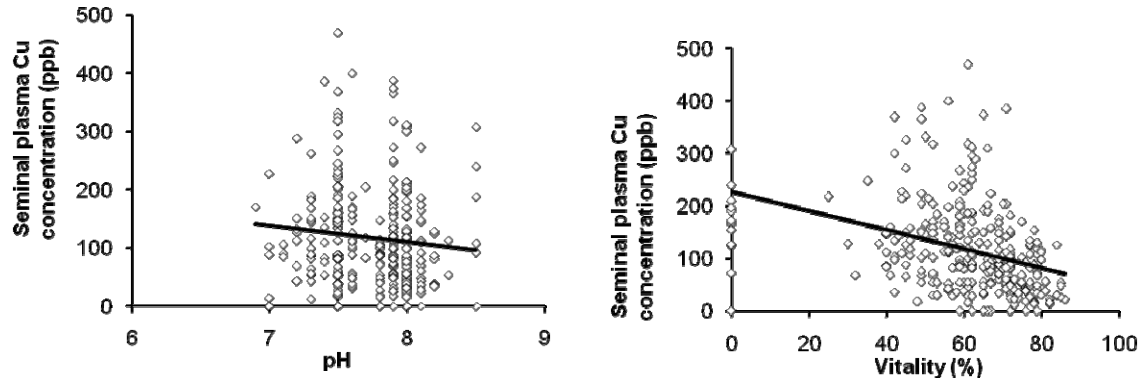


Fig. 2. Correlation between plasma seminal copper concentration and pH (left) and vitality (right).

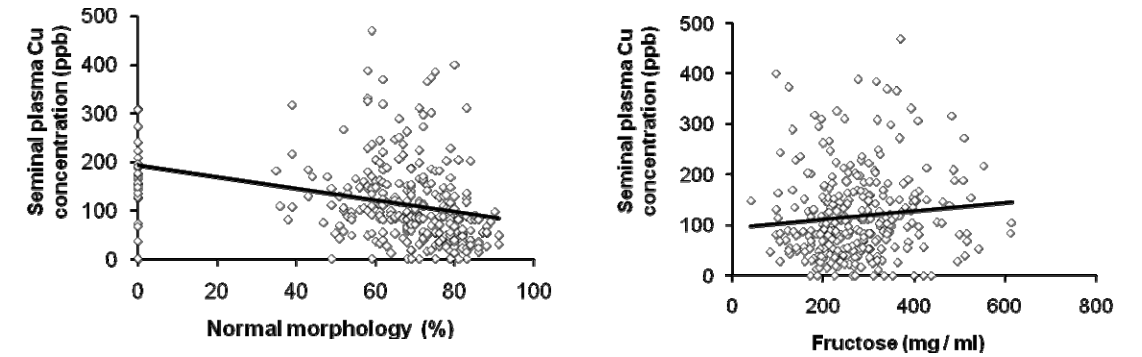


Fig. 4. Correlation between plasma seminal copper concentration and normal morphology (left) and fruvtose (right).

In recent years, it has been suggested that environmental factors may adversely affect the reproductive organs (Carlsen et al., 1992). Many metals are discharged as environmental pollutants from the combustion of fossil fuels, such as diesel fuels. While certain trace amounts of metals are essential for physiological homeostasis, it is well known that excessive or insufficient concentrations of these elements will induce toxicity and deficiency symptoms, respectively. The present study showed that significance differences of seminal plasma copper concentration between normospermic, varicocele, oligozospermic and asthenozospermic males. Also, our study demonstrated that significant correlations between seminal plasma Cu concentration and fructose concentration, pH, vitality, total motile, rapid motile, slow motile, sperm concentration, normal morphology and tail defects. Also, the present study showed that plasma seminal copper concentration attenuated sperm metabolism, so it could have increased plasma seminal fructose concentration.The role of Cu in male reproductive capacity appears to be largely unknown, although high concentration of Cu in seminal plasma is correlated with reduced sperm motility both in our study and other previous studies (Rebrelo et al., 1996). Excess levels of monovalent and divalent Cu ions in solution should result in lipid peroxidation in

sperm plasma membrane, an effect that may render sperm immotile. In a similar study, Jockenhövel et al (Jockenhövel et al., 1990) showed that a weak, though significant correlation was reported between seminal plasma Cu concentrations and sperm count, motility, and normal morphology. Also, Wong et al (Wong 2001) reported a weak but significant correlation between blood Cu concentrations and sperm motility. Katayose et al (Katayose et al., 2004) demonstrated that higher concentrations of copper had significant adverse effects on sperm motility. Ackerman et al (Ackerman et al., 1997) demonstrated an adverse effect of high concentrations of Cu on sperm morphology in impala living in the Kruger National Park, South Africa. This report and previous studies found that a large variety of sperm abnormalities are to be found in impala, both in control and in animals exposed to Cu. The frequency of occurrence of abnormalities in elevated Cu levels in the animals compared the normal, as presented by the liver Cu concentrations, revealed a statistically significant correlation between the occurrence of sperm neck vacuoles and Cu levels. It is reported that Cu acts as a catalyst in the formation of reactive oxygen species (ROS) that can lead to oxidative stress and destructive lipid peroxidative damage (19). It has been shown that Cu “in vitro” increased lipoprotein oxidation (Raveh et al., 2001; Abuja et al., 2001). Accordingly, it is plausible to consider plasma seminal copper concentration as a good marker for evaluating reactive oxygen radicals, sperm metabolism, vitality, motility and relevant semen parameters.

REFERENCES

1. Carlsen E., Giwercman A., Keiding N., Skakkebaek N. E. - Evidence for decreasing quality of semen during past 50 years. *Br Med J* 1992; 305: 609–613.
2. Colborn T., vom Saal F. S., Soto A. M. - Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* 1993; 101: 378–384.
3. Swan S., Elkin E. P., Fenster L. - Have sperm densities declined? A reanalysis of global trend data. *Environ Health Persp* 1997; 105: 1228–1232.
4. Spatari S., Bertram M., Fuse K., Graedel T. E., Rechberger H. - The contemporary European copper cycle: I year stocks and flows. *Ecological Economics* 2002; 42, 27-42.
5. Gordon R. B. - Production resides in copper technological cycles. *Resources Conservation Recycling* 2002; 36: 87-106.
6. Barceloux D. G. - Copper. *Journal Toxicological Clinical Toxicology* 1999; 37: 217-230.
7. Mann T., Lutwak-Mann C. - Male reproductive function and semen. Springer, Berlin; 1981.
8. Rebrelo L., Guadarrama A., Lopez T., Zegers H. F. - Effect of Cu ion on the motility, viability, acrosome reaction and fertilizing capacity of human spermatozoa in vitro. *Reprod Fertil Dev* 1996; 8: 871-874.
9. Jockenhövel F., Bals-Pratsch M., Bertram H. P., Nieschlag E. - Seminal lead and copper in fertile and infertile men. Andrologia 1990; 22: 503–11.
10. Wong W. Y., Flik G., Groenen P. M. W., Swinkels D.W., Thomas C. M. G., Copius-Peereboom J. H. J., Merkus H. M. W. M., Steegers-Theunissen R. P. M. - The impact of calcium, magnesium, zinc, and copper in blood and seminal plasma on semen parameters in men. *Reproductive Toxicology* 2001; 15: 131–136.

11. Katayose H., Shinohara A., Chiba M., Yamada H., Tominaga K., Sato A., Yanagida K. - Effects of various elements in seminal plasma on semen profiles. *J Mam Ova Res* 2004; 21: 141-148.
12. Ackerman D. J., Reinecke A. J., Els H. J. - Transmission electron microscopic observations of flagellum abnormalities in impala (Aepyceros melampus) sperm from the Kruger National Park. Koedoe 1997a; 40: 1-13.
13. Ackerman D. J., Reinecke A. J., Els H. J. -Transmission electron microscopic observations of acrosome and head abnormalities in impala (Aepyceros melampus) sperm from the Kruger National Park. Koedoe 1997b; 40: 15-30.
14. Raveh O., Pinchuk I., Fainaru M., Lichtenberg D. - Kinetics of lipid peroxidation in mixture of HDL and LDL, mutual effects. *Free Radical Biology and Medicine* 2001; 31: 1486-1497.
15. Abuja P. M., Albertini R. - Methods for monitoring oxidative stress, lipid peroxidation and oxidant resistance of lipoproteins. *Clinical Chimica Acta* 2001; 306: 1-17.

HEAVY METALS CONTENT OF SOIL AND PLANTS FROM CENTRAL PARKS (BUCHAREST, ROMANIA)

Marilena ONETE[1]*, Mihaela PAUCĂ-COMĂNESCU[1], Elisabeta BIANU[2], Stelian ION[3]

[1]*Ecology, Taxonomy and Nature Conservation Centre, Institute of Biology, Romanian Academy, Spl. Independentei 296, Sector 6, Bucharest, Romania.*
[2]*Toxicology-Chemistry Laboratory, Animal Diagnostic and Health Institute, Str. Dr. Staicovici, nr. 3, Sector 5, Bucharest, Romania.*
[3]*Institute of* Mathematical *Statistics and Applied* Mathematics*, Romanian Academy, Calea 13 Septembrie nr. 13, sector 5, Bucharest, Romania.*

** E-mail: m_onete@yahoo.com*

ABSTRACT

Bucharest is the biggest and the most polluted city from Romania. The city centre present an increased air and soil pollution due to traffic agglomeration and other diffuse sources. The central parks have been chosen in accord with their different structural diversity. Following the inventory of plants from all three parks, chemical analyses have been performed. The chemical analysis of soils and plants from the central parks from Bucharest revealed that the heavy metal content of these components of the urban environment is different according with: the site complexity, the proximity with the main traffic roads, parks management and plant species.

In the central parks from Bucharest (Cişmigiu, Izvor and Unirii), the heavy metal concentration in the soil is highly heterogeneous being different at the entire park level, micro-site level and even at the point-like level. The heavy metal input can be realized both from atmospheric dry and wet depositions and from the soil itself (usually being brought from other sites more or less polluted). The proximity of intense traffic roads as major and intensive source of air pollution, leads to depositions on the soil and vegetation, influencing the heavy metal concentration in the soil.

The content of heavy metals in plants distinguish that the metal uptake from soil is different with different plants species. In the same park, the uptake of the metals is different both with different sites and distance to the main traffic road.

Key words: heavy metals, soil, plants, parks, Bucharest

INTRODUCTION

The specific character of air pollution in Bucharest city is due to emission conditions, the existence of multiple sources with different height, and scattered distribution of the sources in the entire area of the city. The main air pollution sources in Bucharest city is traffic (AIR-AWARE Report, 2008).

Bucharest city, covering 260 km^2 of the central area of Romanian Plain, has a complex soil cover formed by eight types: fluviosols, regosols, entiantrosols, chernozems, preluvosols, luvosols, gleiosols and stagnosols. Entiantrosols has been

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 58 - 64, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

entirely anthropic occupying 48,5% from city area predominately in city centre where most of them are covered with asphalt and buildings. The other predominant soil types (clay-loamy and eu-bazic), mainly occurring outskirt and half-central areas of the city, are developed in semi-natural conditions (Lăcătuşu et al., 2008).

The origin of street dust in Bucharest is mainly accessory (intra-city) and less elementary (from external sources like: rivers margins, natural river terraces with loess levels, degraded soils, etc.). The highest values of soil Cu content are presented in the central area of the city and in the industrial platform areas. For Zn, high values have been found in Eastern and Southern Bucharest, in the centre (700 mg/kg) and belt line of the city, near by arterial roads. In the Eastern and centre of the city, they were recorded the highest values of Pb content in the soil (Lăcătuşu et al., 2008).

In Romania, the heavy metals content in different soil types is shown by Răuţă et al. (1985) and Băjescu & Chiriac (1962, 1968).

Heavy metals (Zn, Cu, Pb, Cd) occurs naturally in plants, playing or not a role in their metabolism. Plants are intermediate reservoirs through which heavy metals from soil, and partially from water and air, move to man and animals. They accumulate heavy metals, in or on their tissues, due to their great ability to adapt to variable chemical properties of the environment. The metal forms in plants seem to have a decisive role in metal transfer to other organisms. The fate of anthropogenic heavy metal in soils is hazardous for man and animals from two sources: food chain and inhalation of the soil dust. This represent the basic environmental problem relates to the quantities of accumulated metals in plant parts used as food. (Agrawal et al., 1988; Breckle et al., 1992; Pascoe et al., 1996; Szaro et al., 2002; Omasa at al., 2007). Certain soil and plants factors (low pH, low P content of soil, organic ligands) are known to promote both heavy metals uptakes by roots and their translocation into plants tops (Jimura et al., 1977).

Assessed against background values obtained for unpolluted vegetation, the chemical composition of plants may be a good indicator for contaminated areas (Kabata-Pendias & Dudka, 1991).

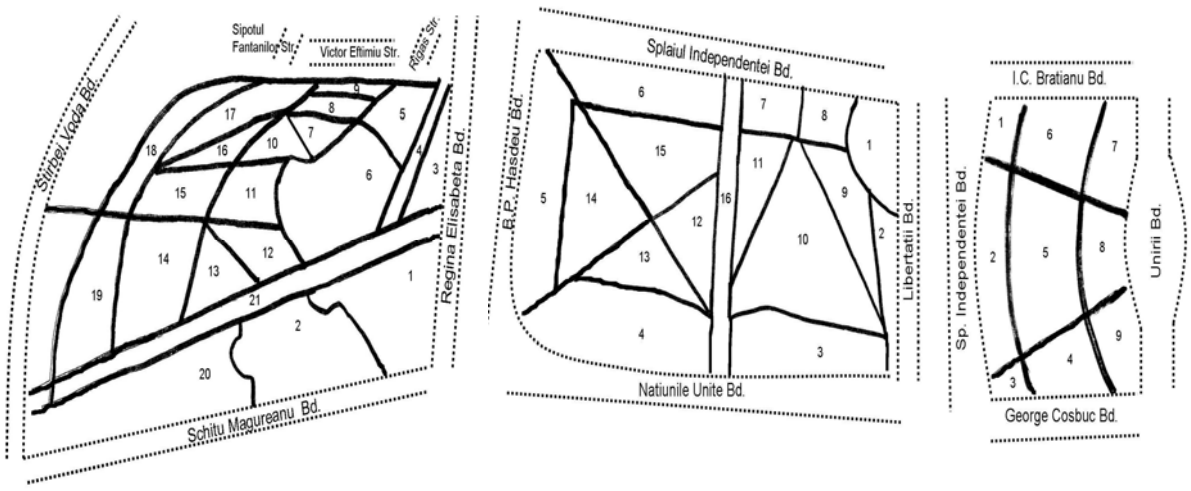
Soil chemical and physical properties and chemical elements' speciation are the determinant of the fate of heavy metals generated by various sources once elements reach the surface of the soil. Contaminants can persist in soil much longer than in other components of the biosphere. Metal accumulated in soils are depleted slowly by different processes (leaching, plant uptake, erosion, etc.). According to Jimura et al. (1977), the first half-life of heavy metals (calculated for soil in lysimetric conditions), varies greatly: 70-510 years (Zn), 13-1100 years (Cd), 310-1500 years (Cu), 740-5900 years (Pb). The complete removal of metallic contaminants from soil is almost impossible. In surface soils, the permissible level of trace elements, particularly heavy metals, depend on local conditions (Kabata-Pendias and Pendias, 1992).

Deficiencies and excesses of heavy metals are both the chemical stresses on which the plants react. The plants have developed during their evolution (phylogeny) and course of life (ontogeny) several biochemical mechanisms that have resulted in adaptation and tolerance of new or chemically imbalanced environments. The chemical composition of plants reflects, in general, the element composition of the growth media. Many different factors governed the highly variable relation between plant and soil.

MATERIALS AND METHODS

The plant species diversity has been recorded in areas shaped by man-made paths (asphalt alleys, walk paths) (Figure 1).

For chemical analysis have been used only leaves from trees and shrubs and the annual development of perennial herbaceous, recording the heavy metal intake by plant during 2007 vegetation season.



Cismigiu Park Izvor Park Unirii Park

Fig. 1. Diagrammatic division of the central parks from Bucharest city and the proximity of the main traffic roads.

Soil and plants samples for performing chemical analysis were collected on transects close to all edges of the park, by the major roads and on transects throughout the middle of the park. Due to the richness of the vegetation at the edge of the park, the air pollution should be diminished through the middle of the park (especially for Cişmigiu Park). The collection of plant samples was based not only on proximity of the main traffic roads but also on their presence in more sites. Where one species was present in only one site, the samples collection was based on the knowledge about the bioindicator status of the species.

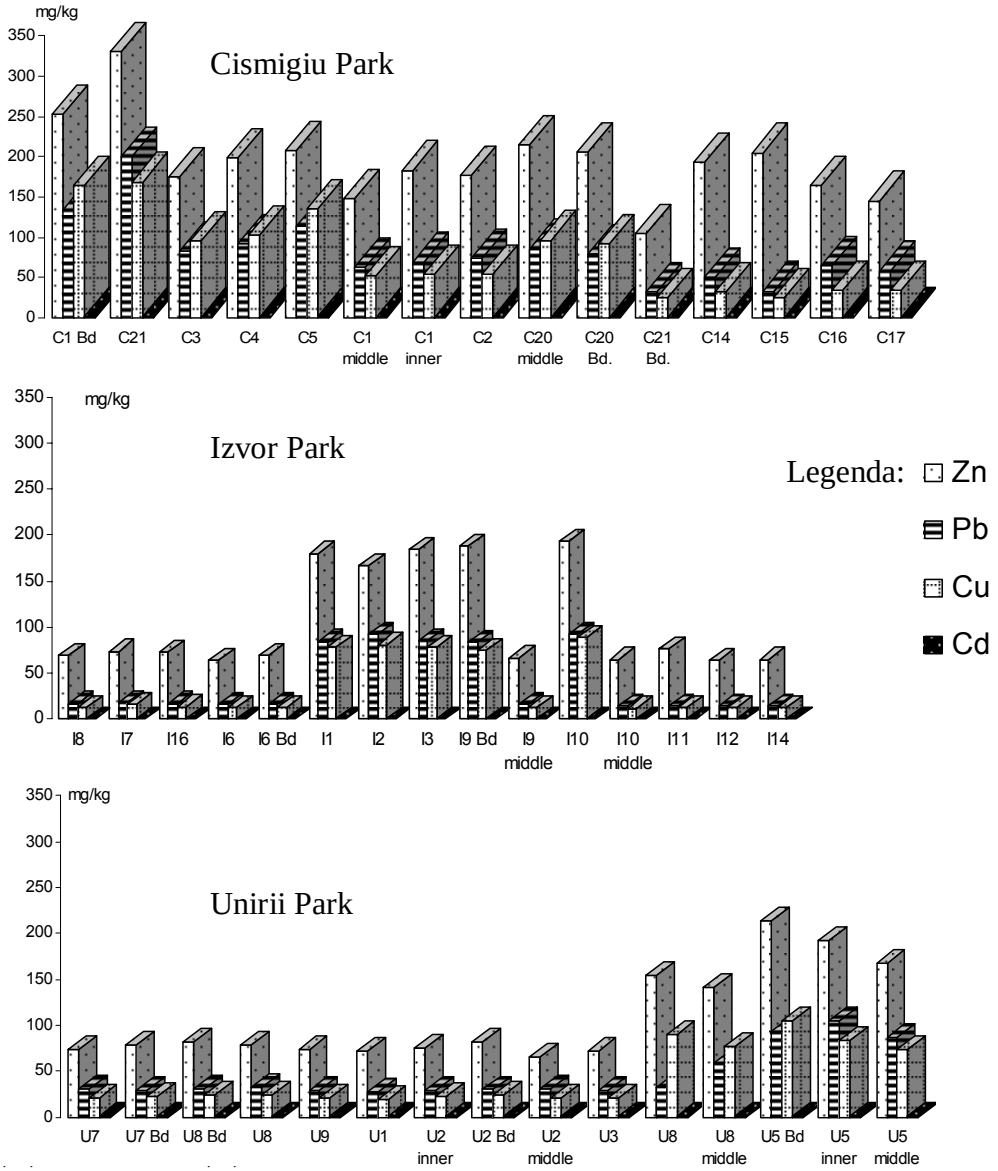
Soil samples have been collected using Corer devise with 10 cm diameter and 10 cm length.

Vegetation samples have been washed with double distilled water; the analysis of washed leave samples provides elemental concentration in leaf tissue. The samples were dried at 105^0 during 24 h, grinded with Planetary Ball Mill PM 100, sieved and kept in plastic bags for farther analysis, digested with HNO_3 and hydrogen-peroxide 30%. Heavy metals (Pb, Cd, Cu, Zn) from soil and plant samples have been analysed with Perkin Elmer AAnalyst 800 Atomic Absorption Spectrophotometer incorporating all spectrometer and atomizer components using graphite furnace or flame techniques.

RESULTS AND DISCUSSION

The chemical analysis of the soil revealed that the content in heavy metal is very different in different sites from all three parks (Figure 2).

Cd has very low values in all three parks but Pb, Cu, and Zn have the highest values close to the main traffic roads.



C1…C21 – Cişmigiu sites position according with Figure 1
I1…I16 – Izvor sites position according with Figure 1
U1…U9 - Unirii sites position according with Figure 1

Fig. 2. Heavy metal content (mg/kg d.w.) in soils from the central

We argue that in Cişmigiu Park, where the values of heavy metal content is increased comparing with other two studied parks, heavy metals have been accumulated in soil due to high plant diversity and wet or dry deposition. Due to Cişmigiu Park structural complexity, aerial depositions (wet and dry) are kept at the soil level, vegetation high diversity not allowing to the dust particles together with pollutant to re-suspend in the air.

In Unirii and Izvor Parks, where the highest values of heavy metals have been found in areas situated in the middle of the park, there is a possibility that heavy metals re-enter in the particles suspended in the air due to scarce vegetation and to the paths created by humans, trampled vegetation from both parks. The soil in these parks is sandy and dry clay and every wind blow brings in the air a lot of dust.

The range values show differences between max and min values from different site, their diversity on heavy metal contain. Zn has higher values in Cişmigiu comparing with Izvor and Unirii and also comparing with the values of content in different types of soils. Cu and Pb have also higher values in Cişmigiu comparing with Unirii and Izvor and also with the values of content in different types of soils.

Based on plant's tissue content of heavy metals (Table 1) we can argue that the metal uptake from soil is different with different plants species.

Tab. 1. Range content (mg/kg d.w.) of heavy metals in soil and plants' tissues

Name	Pb	Cd	Cu	Zn
Cismigiu soil	32,3 - 199,8	0,47 - 1,21	24,6 - 168,5	104,6 - 330,3
Unirii soil	27,68 - 104,6	0,36 - 0,8	19,16 - 105	64,81 - 214,5
Izvor soil	15,02 - 92,87	0,44 - 0,78	12,32 - 89,74	63,94 - 194
Normal range of metals in plants' tissues*	0,1 - 10	0,2 - 0,8	2 - 5	10 - 20
Trees				
Acer tataricum L.	0,47 - 0,9	0,22 - 0,3	1,02 - 2,47	8,77 - 18,49
Amygdalus communis L.	2 - 3,49	0,1 - 0,38	2,4 - 3,83	9,23 - 11,8
Catalpa bignonioides Walter	1,25 - 3,73	0,14 - 0,79	2,73 - 7,12	8,93 - 13,61
Fraxinus angustifolia Vahl.	1,57 - 7,77	0,22 - 0,41	1,73 - 2,6	5,22 - 7,6
Fraxinus excelsior L.	0,59 - 2,63	0,25 - 0,45	3,76 - 5,08	7,81 - 11,7
Malus medzweltzkyana Dieck	1,79 - 3,03	0,2 - 0,34	3,09 - 5,35	6,93 - 9,45
Pinus sylvestris L.	1,1 - 1,44	0,22 - 0,27	1,05 - 1,99	9,18 - 14,27
Platanus hispanica Miller ex. Muench.	1,16 - 2,28	0,18 - 0,60	1,74	7,90
Populus nigra L. cv. *italica* Moench.	1,16 - 4,96	0,33 - 0,47	2,81 - 3,67	11,4 - 44,43
Quercus cerris L.	0,43 - 0,78	0,31 - 0,33	3,43 - 3,67	13,34 - 16,19
Quercus robur L.	2,19 - 3,59	0,22 - 0,83	4,44 - 6,89	10,41 - 16,99
Quercus rubra L.	1,24 - 3,34	0,26 - 0,53	1,18 - 9,97	6,13 - 21,05
Thuja orientalis L.	1,38 - 8,74	0,13 - 0,56	2,75 - 6,69	11,50 - 17,34
Tilia argentea	3,05 - 3,55	0,23 - 0,29	3,56 - 4,6	9,08 - 9,56
Tilia cordata Miller	1,07 - 8,93	0,14 - 0,68	2 - 10,51	6,38 - 20,43
Tilia tomentosa Moench.	1,28 - 3,05	0,21 - 0,23	2,81 - 4,6	6,38 - 9,56
Ulmus minor Miller	2,32 - 5,33	0,22 - 0,47	3,63 - 6,44	12,84 - 15,36
Tree - Shrubs				
Buxus sempervirens L.	2,20 - 7,68	0,11 - 0,37	2,49 - 33,30	9,30 - 15,61
Crataegus monogyna Jacq.	0,38 - 4,51	0,58 - 0,92	2,53 - 3,28	8,18 - 13,28
Shrubs				
Cornus sanguinea L.	1,4 - 3,81	0,22 - 0,55	0,95 - 13,90	5,58 - 13,84
Corylus avellana L.	2,4 - 5,93	0,14 - 0,38	1,42 - 13,64	6,7 - 28,52
Ligustrum vulgare L.	0,1 - 0,36	0,12 - 0,48	1,42 - 3,44	7,59 - 12,77
Rosa canina L.	1,83 - 2,95	0,22 - 0,4	3,89 - 5,65	6,64 - 11,7
Rubus caesius L.	1,42 - 2,27	0,1 - 0,33	1,18 - 15,2	7,31 - 7,4
Lians				
Hedera helix L.	0,68 - 3,32	0,1 - 0,58	1,45 - 12,9	8,3 - 22,11
Herbaceous				
Cynodon dactylon (L.) Pers.	0,21 - 0,99	0,19 - 0,52	2,8 - 3,3	5,39 - 17,16
Geum urbanum L.	1,23 - 8,32	0,17 - 0,93	2 - 23,5	14,51 - 66,94
Glechoma hederacea L.	0,69 - 0,94	0,21 - 0,38	2,23 - 3,22	10,98 - 17,68
Plantago lanceolata L.	1,2 - 2,97	0,15 - 0,69	2,84 - 3,76	7,3 - 10,43
Polygonum aviculare L.	0,25 - 1,18	0,19 - 0,32	2,42 - 3,6	15,53 - 16,40
Portulaca oleracea L.	0,56 - 0,87	0,2 - 1,02	1,28 - 1,75	6,38 - 13,35
Setaria viridis (L.) Beauv.	1,21 - 2,05	0,27 - 1,02	2,63 - 2,73	22,66 - 32,70
Taraxacum officinale Weber ex. Wiggers	0,94 - 3,26	0,1 - 0,22	0,26 - 6,82	3,90 - 13,6

* according to Pais and Jones Jr., 1996, for Pb and Cd, and Kalra, 1998, for Cu and Zn

In the same park, the uptake of the metals is different both with different sites and distance to the main traffic road. For instance, *Buxus sempervirens* individual analysed from Cişmigiu Park, accumulate more Pb and Zn from the site near by traffic road than, the individuals from inside parks. The metal concentration in plants is affected by prevailing weather conditions, the water solubility of deposited metal-containing particulates, the nature of the plant surface, plants root uptake of the metals, etc. (Bache et al., 1991). Metal uptake in higher vascular plants takes place through their root system, additionally through the leaves and, therefore, it is difficult to distinguish whether the accumulated elements originate from the soil or from the air (Harrison and Chirgawi, 1989).

Exceeding the normal range of metals in plants means that the metals become sufficient or toxic and excessive (Pais and Jones Jr., 1996; Kalra, 1998). *Fraxinus angustifolia*, *Thuja orientalis, Tilia cordata* accumulated Pb in their tissues in a high range. Cd was accumulated in high range by *Catalpa bignonioides*, *Crataegus monogyna, Tilia cordata, Plantago lanceolata* and *Geum urbanum.* Cu was accumulated even more times more than the high vallue of normal range in: *Quercus rubra, Tilia cordata, Buxus sempervirens, Cornus sanguinea, Corylus avellana, Rubus caesius, Cynodon dactylon, Geum urbanum, Taraxacum officinale. Zn has high vallues in trees, shrubs and lian Populus nigra* cv. *Italica*, *Quercus rubra, Corylus avellana, Hedera helix* but much higher in herbaceous *Geum urbanum* and *Setaria viridis.*

Heavy metals (and other chemical) are taken up by plants from soils and transferred through the terrestrial invertebrates and vertebrates (including humans) via respiration and ingestion. Plants influence and are influenced by their environment. The chemical composition of plants reflects, in general, the element composition of the growth media.

In the central parks from Bucharest (Cişmigiu, Izvor and Unirii), the heavy metal concentration in the soil is highly heterogeneous being different at the entire park level, micro-site level and even at the point-like level. The proximity of intense traffic roads as major and intensive source of air pollution, leads to depositions on the soil and vegetation, influencing the heavy metal concentration in the soil.

Acknowledgements
Data obtained in the framework of project LIFE05 ENV/RO/000106 Air Pollution impact surveillance and warning system for urban environment. We express our gratitude to Rodica Iosif and Daniela Nica for field and lab work.

REFERENCES

1. ***AIR-AWARE Report, 2008, http://life-airaware.inmh.ro
2. Agrawal K.M., Sharma H.C., Aggarwal A.L., 1988, Heavy metal pollution of air, soil and plants around a zinc smelter: A case study, Indian Journal of Environmental Health, 30(3): 234-241.
3. Bache C.A., Gutenmann W.A., Chu G., Elfving D.C., Lisk D.J., 1991, Concentrations of metals in grasses in the vicinity of a municipal refuse incinerator, Archives of Environmental Contamination and Toxicology, 20(4): 538-542
4. Băjescu J., Chiriac A, 1962, Some aspects of the distribution of copper, cobalt, zinc and nickel in soils of the Dobruja, Anal. Inst. Cent. Cerc. Agr., 30A: 79-83
5. Băjescu J., Chiriac A, 1968, Trece element distribution in brun lessive and lessive soils, Stiinta Solului, 6: 45-57

6. Breckle S.W., Kahle H., 1992, Effects of toxic heavy metals cadmium and lead on growth and mineral nutrition of beech, *Fagus sylvatica* L., Vegetatio, 101(1): 43-53.
7. Harrison, R.M. and Chirgawi, M.B. 1989 The assessment of air and soil as contributors of some trace metals to vegetable plants. I. Use of a filtered air growth cabinet. Science of the Total Environment, 83 (1-2): 13-34.
8. Jimura K., Ito H., Chino M., Morishita T., Hirata H., 1977, Behavior of contaminants heavy metals in soil-plant system, Proc. Inst. Sem. SEFMIA, Tokyo, 357-373
9. Kabata-Pendias A., Dudka S., 1991, Trace metal contents of *Taraxacum officinale* dandelion as a convenient environmental indicator, Environmental and Geochemical Health, 13 (2): 108-113.
10. Kabata-Pendias, A., Pendias, H., 1992, *Trace Elements in Soils and Plants*, 2nd Edition, CRC Press, Boca Raton, Ann Arbor, London
11. Kalra Y.P., 1998, Handbook of reference methods for plant analysis, CRC press LLC
12. Lăcătuşu R., Anastasiu N., Popescu M., Enciu P., 2008, *Geo-atlas of Bucharest city*, Editura Est Falia, Bucharest
13. Omasa K., Nouchi I., De Kok L.J., 2007, Plant responses to air pollution and global changes, Springer
14. Pais I., Jones Jr. J.B., 1996, The handbook of trace elements, St. Lucie Press, Boca Raton
15. Pascoe G.A., Blanchet R.J., Linder G., 1996, Food chain analysis of exposures and risks to wildlife at a metals contaminated wetland, *Arch. Environ. Contam. Toxicol.*, 30: 306-318
16. Răuţă C., Cârstea S., Mihăilescu A., Lăcătuşu R., 1985, Some aspects of pedochemical and biogeochemical research in Romania, Proc. 1st Inst. Symp. On Geochemistry and health, Thornton, I, Ed. Imperial College, London, 236
17. Szaro R.C., Bytnerowicz A., Oszlányi, 2002, Effects of air pollution on forest heath and biodiversity in forests of the Carpathian Mountains, Series 1: Life and Behavioral Science, vol. 345, NATO Science Series

EFFECT OF ALCOHOLIC EXTRACT OF EUCALYPTUS GLOBULUS LABILL. LEAVES ON SODIUM AND POTASSIUM LEVELS IN HEALTHY AND DIABETIC RATS

Akram Eidi [1,*], Maryam Eidi [2], Mohammadhadi Givian Rad [3], Nasim Abaspour [1]

[1] *Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran*

[2] *Department of Biology, Varamin Branch, Islamic Azad University, Tehran, Iran*

[3] *Department of Chemistry, Science and Research Branch, Islamic Azad University, Tehran, Iran*

** E-mail : akram_eidi@yahoo.com*

ABSTRACT

Many herbal medicines have been recomended for the treatment of diabetes. In traditional medicine leaves of eucalyptus (Eucalyptus globules L.) posses interesting biological activities, such as antioxidation, antibacterial and antiviral. The activity of erythrocyte membrane Na+/K+-ATPase was significantly decreased in diabetes. In the present study, oral administration of 0.1, 0.2 and 0.4 g/kg body wt. of encalyptus leaves alchoholic extract for 14 days on the level of Na^+ and K^+ serum in healthy and streptozotocin–induced diabetic rats were evaluated. A significant decrease in serum sodium and a significant increase in serum potassium levels were observed in the STZ-diabetic groups. The results showed that treatment with eucalyptus extract increased the serum sodium and decreased potassium levels in STZ-diabetic rats insignificantly. It can serve as a good adjuvant in the present armamentarium of antidiabetic drugs. Further biochemical and pharmacological investigations should be carried out to elucidate in detail the mechanism of action of this plant.

Key words: Eucalyptus (*Eucalyptus globulus* Labill.), antidiabetic, Na^+, K^+-ATPase, rat

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease caused by inherited or acquired deficiency in insulin secretion and by decreased responsiveness of the organs to secreted insulin. Such a deficiency results in increased blood glucose level, which in turn can damage many of the body's systems, including blood vessels and nerves (Matsui et al., 2007). DM is currently one of the most costly and burdensome chronic diseases and is a condition that is increasing in epidemic proportions throughout the world (King et al., 1998). Diabetes affects about 5% of the global population (WHO, 2002) and the management of diabetes without any side effects is still a challenge to the medical system (Chakraborty et al., 2002; Kameswararao et al., 2003). Moreover, insulin has been shown to modulate vascular reactivity inducing vasodilatation (Zemel et al., 1992; Ozturk et al., 1996). This effect is partially linked to the activation

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 65 - 69, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

of the Na+,K+-ATPase which seems to contribute to the vasodilator effect of insulin (Ewart and Klip, 1995; Gupta et al., 1996; Tack et al., 1996)..

As for the developed countries, the use of herbal medicine by the suffers of chronic disease is encouraged because there is concern about the adverse effects of chemical drugs and treatment using medicines of natural origin appears to offer more gentle means of managing such disease (Hamdan et al., 2004; Klepser et al., 1999; WHO, 2002). Herbal drugs are prescribed widely because of their effectiveness, fewer side effects and relatively low cost.

Many species of the genus *Eucalyptus* from the Myrtaceae family are used in Brazilian folk medicine for a variety of medical conditions. *Eucalyptus globulus* (eucalyptus; blue gum tree) is traditionally used to treat diabetes (Duke 1985, Lewis 1949). The medicinal part of the plant is the leaves from which tea is made (Chiej 1988). The use of *Eucalyptus globulus* leaves in the treatment of diabetes mellitus was first advocated by Faulds (1902). Recent studies in streptozotocin-diabetic mice confirmed the antihyperglycemic efficacy of *Eucalyptus globulus* (Swanston-Flatt et al. 1990). The aim of the present work is to evaluate the effect of eucalyptus ethanolic extract on the level of Na^+ and K^+ serum in healthy and streptozotocin–induced diabetic rats.

MATERIALS AND METHODS

Adult male Wistar rats with body weights of 200-230 g were used in the study. The animals were maintained in an air-conditioned animal house at a temperature of 22ºC ±2ºC, relative humidity of 57±2% and photo-cycle of 12:12 h light and dark periods. Water and food pellet were provided ad libitum.

Eucalyptus leaves were collected from Ilam area in summer and scientifically approved in the department of botany of Islamic Azad University. The plant was cleaned, shed dried at 25°C, and the dried leaves of the plant were ground with a blender. Dried and ground leaves (about 60 g) were submitted to extraction with 300 ml ethanol (80%) in a Soxhlet apparatus for 72 h. After extraction, the solvents were filtered and then evaporated by Rotavapor.

Male adult Wistar rats were injected with streptozotocin (70 mg/kg, i.p.). Five days after injection, the rats with fasting blood glucose higher than 180 mg/dl were used for the experiments. Eight rats were used in each experiment. Each animal was used once only in all of experiments. The food and water were removed from cages 12 h before testing.

Eucalyptus leaves extract was suspended in distilled water and administrated orally through orogastric tubes at the following doses of 0.1, 0.2 and 0.4 g/kg body wt. The volume of the above three doses was kept constant at 1 ml.

In the experiment, a total of 48 rats (24 diabetic rats, 24 normal rats) were used. Diabetes was induced in rats 5 days before starting the treatment. The rats were divided into nine groups. In the experiment six rats were used in each group.

- Group 1: Normal control rats were administrated 1 ml of distilled water;
- Groups 2,3,4: Normal rats were administrated eucalyptus leaves alcoholic extract (0.1, 0.2 and 0.4 g/kg body wt.) daily using an intragastric tube for 14 days;
- Group 5: Diabetic control rats were administrated 1 ml of distilled water;
    - Groups 6,7,8: Diabetic rats were administrated eucalyptus leaves alcoholic extract (0.1, 0.2 and 0.4 g/kg body wt.) daily using an intragastric tube for 14 days.

After 14 days of treatments, blood samples were drawn from heart. Serum sodium and potassium levels were determined. Sodium and potassium levels were assayed in serum by flame photometry.

All the data were expressed as mean ± S.E.M. Statistical analysis was carried out using one-way ANOVA followed by Tukey post hoc test. The criterion for statistical significance was $p<0.05$.

RESULTS AND DISCUSSION

There was a significant elevation in serum potassium while the serum sodium level significantly decreased in the diabetic rats.

Figure 1 showed that the effect of the eucalyptus leaves extract on serum sodium levels in normal and diabetic rats. The results showed that administration of the eucalyptus leaves extract at dose of 0.4 g/kg body wt., tended to bring serum sodium significantly toward normal values, while normal rats did not exhibit any significant alterations in this parameter levels duration of the experiment.

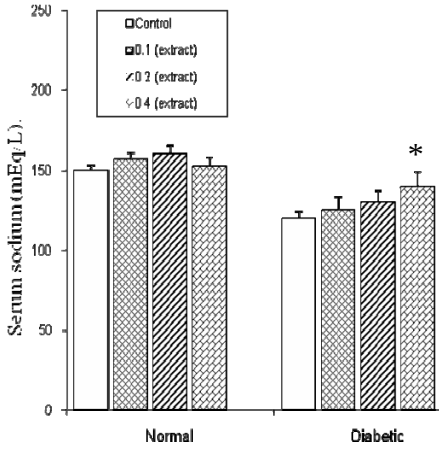


Fig 1. Effect of oral administration of eucalyptus leaves extract at doses of 0.1, 0.2 and 0.4 g/kg body weight on serum sodium levels in normal and diabetic rats. Each column represents mean ± S.E.M. for 8 rats. Control group administrated with distilled water as a vehicle. * $p<0.05$ different from control diabetic group.

Figure 2 showed that the effect of the eucalyptus leaves extract on serum potassium levels in normal and diabetic rats. The results showed that administration of the eucalyptus leaves extract at dose of 0.4 g/kg body wt., tended to bring serum potassium significantly toward normal values, while normal rats did not exhibit any significant alterations in this parameter levels duration of the experiment.

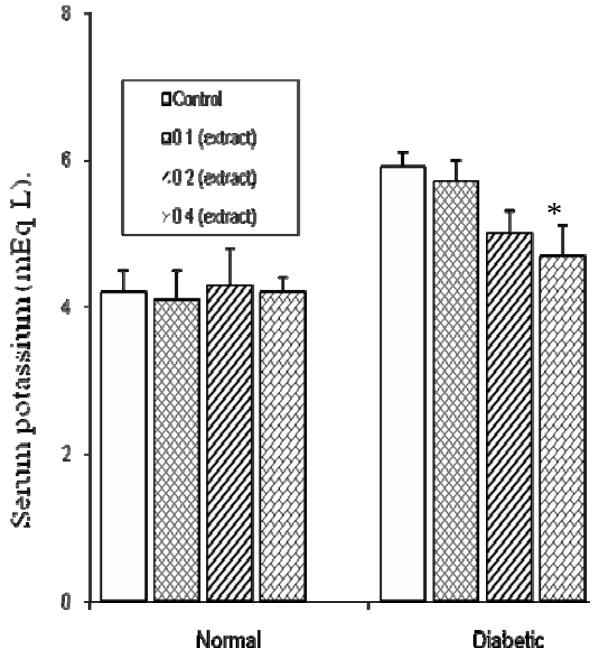


Fig. 2. Effect of oral administration of eucalyptus leaves extract at doses of 0.1, 0.2 and 0.4 g/kg body weight on serum potassium levels in normal and diabetic rats. Each column represents mean ± S.E.M. for 8 rats. Control group administrated with distilled water as a vehicle. * $p<0.05$ different from control diabetic group.

Several studies have shown changed levels of sodium and potassium in serum of diabetic rats (Karam et al., 2004; Nagahama et al., 2004). In our study, serum sodium level increased, but serum potassium levels decreased in the groups given eucalyptus extract. As a result, it may be concluded that the eucalyptus extract may be of use as a antidiabetic agent.

REFERENCES

1. Chakraborty R., Rajagopalan R. Diabetes and insulin resistance associated disorders: Disease and therapy. Current Science, 2002, 83, 1533-1538.
2. Chiej R. The MacDonald Encyclopedia of Medicinal Plants. MacDonald & Co. Ltd., London, UK, 1988.
3. Duke J.A. CRC Handbook of Medicinal Herbs. CRC Press, Florida, US, 1985.
4. Ewart H.S., Klip A. Hormonal regulation of the Naq,Kq-ATPase: mechanism underlying rapid and sustained changes in pump activity. Am. J. Physiol., 1995, 269, C295-C311.
5. Faulds G. Eucalyptus in the treatment of diabetes. Glasgow Med. J. 1902, 57, 342–348.
6. Gupta S., Phipps K., Ruderman N.B. Differential stimulation of Naq pump activity by insulin and nitric oxide in rabbit aorta. Am. J. Physiol., 1996, 270, H1287-H1293.
7. Hamdan I.I., Afifi F.U. Studies on the in vitro and in vivo hypoglycemic activities of some medicinal plants used in treatment of diabetes in Jordanian traditional medicine. Journal of Ethnopharmacology, 2004, 93, 117-121.

8. Kameswararao B., Kesavulu M.M., Apparao, C. Evaluation of antidiabetic effect of Momordica cymbalariafruit inalloxan-diabetic rats. Fitoterapia, 2003, 74, 7-13.
9. Karam G.A., Reisi M., Kaseb A.A., Khaksari M., Mohammadi A., Mahmoodi M. Effects of opium addiction on some serum factors in addicts with non-insulin-dependent diabetes mellitus. Addiction Biology, 2004, 9, 53-58.
10. King H., Aubert R.E., Herman W.H. Global burden of diabetes, 1995–2025; prevalence, numerical estimates, and projections. Diabetes Care, 1998, 21, 1414-1431.
11. Klepser T.B., Klepser, M.E. Unsafe and potentially safe herbal therapies. American Journal of Health System Pharmacy, 1999, 56, 125-138.
12. Matsui T., Tanaka T., Tamura S., Toshima A., Miyata Y., Tanaka K. Alpha-glucosidase inhibitory profile of catechins and theaflavins. Journal of Agricultural and Food Chemistry, 2007, 55, 99-105.
13. Nagahama K., Iseki K., Inoue T., Touma T., Ikemiya Y., Takishita S. Hyperuricemia and cardiovascular risk factor clustering in a screened cohort in Okinawa. Japan Hypertension Research, 2004, 27, 227-233.
14. Ozturk Y., Melih Altan V., Yildizoglu-Ari N. Effects of experimental diabetes and insulin on smooth muscle functions. Pharmacol. Ver., 1996, 48, 69-112.
15. Swanston-Flatt S.K., Day C., Bailey C.J., Flatt P.R. Traditional plant treatments for diabetes. Studies in normal and streptozotocin diabetic mice. Diabetologia, 1990, 33, 462-464.
16. Tack C.J.J., Lutterman J.A., Vervoot G., Thien T., Smits P. Activation of the sodium–potassium pump contributes to insulin-induced vasodilation in humans. Hypertension, 1996, 28, 426-432.
17. WHO. Traditional medicine strategy 2002–2005. WHO Publications, 2002, pp. 1-6.
18. Zemel M.B., Johnson B.A., Ambrozy S.A. Insulin-stimulated vascular relaxation: role of Ca2q-ATPase. Am. J. Hypertens., 1992, 5, 637-641.

DETERMINATION AND ASSESSMENT OF HEAVY METAL CONTENT IN FISH IN MUREŞ RIVER.

Claudiu-Nicolae ŞIMONAŢI

,,Aurel Vlaicu"University of Arad,F.I.A.T.P.M Faculty,Elena Dragoi Street,No.2,Zip code 310330,Arad,ROMANIA

ABSTRACT

Levels of lead (0.064µg/l), iron (0.81µg/l), zinc (4.82µg/l) ,copper (0.19µg/l), arsenic (0.05µg/l), manganese(0.03µg/l), chromium(0.005µg/l) and mercury(0.01µg/l) were determined in the water and in some fish from Mureş river. They were found to be below the maximum allowable levels set by the Environmental Protection Agency (EPA), except for lead, iron and mercury. This implies that waste assimilation capacity of the river is high, a phenomenon that could be ascribed to dilution, sedimentation and continuous water exchange. Empirical evidence shows that the metals were at low levels in the organisms.

***KEYWORDS*:** Heavy metals, pollution, waste assimilation, lead.

INTRODUCTION

The enrichment of heavy metals in Mureş river has been reported [7]. However, allochthonous and autochthonous influences could make concentrations of heavy metals in the water high enough to be of ecological significance. Moreover, bioconcentration and magnification could lead to toxic levels of these metals in organisms, even when the exposure level is low [9]. The proven toxicity of high concentrations of heavy metals in water to fisheries and other aquatic life poses the problem of an ultimate dis-equilibrium in the natural ecological balance [4], [2]. Under such conditions, the toxicity of a moderately toxic metal could be enhanced by synergism [12] and fish populations may decline [7]. Apart from destabilizing the ecosystem, the accumulation of these toxic metals in the aquatic food is a potent threat to public health. The Minamata Bay epidemics in Japan remains a classic example[8].

Dumping of wastes in rivers contributes to the larger problem of river pollution, which can seriously damage the marine environment and cause health hazard to people in some areas.One study reported that the concentrations of heavy metals including zinc, lead, and copper in the upper 3mm (or microlayer) are from 10 to 1000 times higher than in the deeper waters. There is fear that disproportionate pollution of the microlayer will have especially serious effect on marine organisms [9].

Marine pollution can also have major impacts on people and society. Contaminated marine organisms may transmit toxic elements or diseases to people who eat them. When solid waste, oil, and other materials pollute beaches and harbours, there is a loss of visual appeal and other amenities from the river.

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 70 - 75, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

Economic loss is considerable.

MATERIALS AND METHODS

Sampling

Five different water samples were each collected with 1 litre sterile polyvinyl chloride (PVC) plastic water bottles from the five designated sampling points in Mureş river. At each point, three water samples were drawn at random from three points and pooled. Analysis of fish and shellfish harvested from Mureş river and Bega river for comparative studies were done. The fish includes *Mugil cephalus* and *Tilapia guineensis*. Bega river is a urban river that is located in Timişoara county.The fish species used in the analysis were selected based on their abundance in the river and their popularity in our local diets.

Analysis

Recovery experiments done with 1% oxine solution in chloroform (3g in 200ml) showed optimum recoveries ranging from 61% for copper (cu) to 76% for iron (Fe) at pH7 in a one step recovery of all the metals. The pH of each unfiltered water sample was adjusted to 7 using drops of dilute HCL and the metals were recovered from 500ml aliquots by shaking with 25ml of 1% oxine in chloroform for 2min. The bottom layer was decanted into a 100ml beaker and the remaining chloroform in solution evaporated at low temperature in water bath. The residue was dissolved with 5ml 1:1HCL, filtered into 25ml standard flask using Whatman no.1 fitter paper and the solution made up to the mark with distilled water [10].

Metal free water for preparing blank and standard solutions were obtained by shaken each aqueous layer with 25ml of 1% oxine solution. The chloroform layer was discarded, leaving the condensate. A 500ml aliquot of the “metal free’ water was shaken with 25ml of the oxine solution and the extract subjected through the same procedures as the sample extracts. The resulting solution was set aside as a blank. Three 500ml serially diluted standard solutions containing all the metals in parts per million (ppm) ranges were prepared using the “metal free” water as diluent. These were also extracted with 25ml of the oxine solution and the extracts subjected through the same procedures as sample and blank extracts.

The fish samples were de-scaled, washed with distilled water and left to thaw enough for skinning and cutting of muscle fillets to be completed before the onset of drainage of body fluid. Muscle samples of *M.cephalus* and *T.guineensis* were obtained from 8 and 5 fish respectively. The samples were homogenised separately in a mortar and weighed accurately in a porcelain crucible. Before ashing, 1ml conc.HNO_3 was added to the samples and allowed to predigest overnight in order to reduce losses of volatile metals. The samples were charred on electric hot plate before ashing in a muffle furnace at 550^{o}c for 4hr. The white ash was dissolved in 5ml 1:1HCl, and solution made in 25ml standard flask [10].

The metal concentrations of the samples were read against appropriate blank and standard solutions using a Perkin-Elmer model 306 Atomic Absorption spectrophotometer (AAS) with an oxy-acetylene flame. Blank solution for the biotic samples was made by diluting 1ml conc. HNO_3 + 5ml 1:1 HCL to 25ml with distilled water. Empirical data generated were analyzed statistically.

RESULTS AND DISCUSSION

The average levels of zinc, copper, manganese, chromium, mercury and arsenic were below the Romanian states Environmental Protection Agency (EPA) maxima, except for lead and iron. They were either not comparable to those obtained in the Danube Delta waters (Table 1).[7]. Though, levels of lead and iron were high, they were not significant. Analysis of variance (ANOVA) did not reveal significant spatial variations in the levels of any of the metals, neither did the least significant difference (LSD) reveal any significant seasonal variations ($P<0.05$). The low levels of metals determined could be as a result of dilution, sedimentation and continuous water exchange. Though the water flow in the Mureş, river is mainly slow with little or no upwelling during the rainy season, immense volumes of fresh water passes through the river and out to the sea.

The obtained data on the analysis of the fish are presented in Table 2, and were used for comparative studies on heavy metals accumulation in biotic components having different ecological characteristics.

The metal level is comparable to those determined in fish which feed by picking food near the water surface. The levels in *T. guineensis* and *M.cephalus*, which are deposit feeders are high ($P<0.05$).

Fish head, a favourite delicacy in this part of the world was analysed to ascertain the content of heavy metal in consumed fish.

The empirical figures in Table 3 are at variance with that of [7], as a result of rapid urbanization and industrialization that is associated with Romanian State. These has brought with it an alarming unmanageable, inevitable and persistent problems associated with environmental degradation.

The impact of waste disposal on the rivers were assessed by comparing the metal levels in the Mureş river fish with those determined in fish from Bega river, a urban water, and with some literature values in Table 3. Comparative studies of "metal enrichment" in the rivers shows relative presence of the heavy metals in the Mureş river fish while that of Bega river were below detection. This confirms the earlier report concerning a significant impact which urban and industrial waste disposal has had on heavy metal baseline levels in Mureş river.

Table 1: Seasonal mean values of heavy metal levels in the surface sediments of Mureş river, Romania.[7].

VARIABLES	UPSTREAM1	UPSTR-EAM2	MUREŞ	DOWN STREAM1	DOWN STREAM 2	MEAN	EPA MAXIMA[a]	DANUBE DELTA
Pb								
Dry season	0.04	0.12	0.08	0.08	0.06	0.08±0.03		
Rainy season	0.04	0.08	0.04	0.04	0.06	0.05±0.02		
MEAN	0.04	0.1	0.06	0.06	0.06	0.064	0.05	Nd-22.45
Fe								
Dry season	0.82	0.96	0.88	0.84	0.84	0.89±0.06		
Rainy season	0.84	0.68	0.68	0.88	0.64	0.74±0.12		
MEAN	0.83	0.82	0.78	0.86	0.74	0.81	0.1	94.35-901.00
Zn								
Dry season	3.80	6.80	6.40	4.20	4.20	5.08±1.04		
Rainy season	3.64	4.62	6.48	3.80	4.20	4.55±1.44		
MEAN	3.72	5.71	6.44	4.0	4.20	4.82	5.0	4.50-42.86
Cu								
Dry season	0.18	0.24	0.24	0.20	0.18	0.24±0.03		
Rainy season	0.08	0.20	0.12	0.18	0.12	0.14±0.05		
MEAN	1.13	0.22	0.18	0.19	0.15	0.19	1.0	3.1-34.03
As								
Dry season	0	0.12	0.06	0.06	0.04	0.06±0.04		
Rainy season	0	0.04	0.02	0.04	0.04	0.03±0.02		
MEAN	0	0.08	0.04	0.05	0.04	0.05	0.05	-
Mn								
Dry season	0	0	0.04	0.04	0	0.02±0.02		
Rainy season	0	0.02	0.04	0.06	0.02	0.03±0.02		
MEAN	0	0.01	0.04	0.05	0.01	0.03	0.05	-
Cr								
Dry season	Nd	0.04	Nd	nd	Nd	0.01±0.02		
Rainy season	Nd	0.02	Nd	nd	Nd	0.00±0		
MEAN	Nd	0.03	Nd	nd	Nd	0.005	0.05	-
Hg								
Dry season	0.01	Nd	Nd	0.02	0.02	0.01±0.02		
Rainy season	0	Nd	Nd	0.02	0.02	0.01±0.02		
MEAN	0.01	Nd	Nd	0.02	0.02	0.01	0.002	-

All values are in µg/l,A :source; EPA(1996),nd :not detectable

Table 2: Heavy metals levels in fish in Mureş river, Romanian

FISH	Pb	Fe	Zn	Cu	As	Mn	Cr	Hg
M.cephalus	0.01±0.02	0.3±0.02	1.5±0.8	0.14±0.61	0.2±0.12	0.01±0.04	0.0±0	0.01±0.04
T.guineensis	0.02±0.02	0.35±0.02	2.5±0.02	0.03±0.03	0.01±0.04	0.2±0.02	0.0±0	0.01±0.04

Table 3: Some literature standards[a] and metal levels in Bega river fish compared with the levels in Mureş river fish.

	Pb	Fe	Zn	Cu	As	Mn	Cr	Hg
Lit. std.	2.0 [b,c]	-	**40 [b],50 [c]**	**20[b]30[c]**	-	-	-	-
Bega River fish ***M.cephalus***	Nd	0.02	0.05	Nd	nd	nd	nd	nd
Mureş River Fish ***M.cephalus***	0.01	0.30	1.5	0.14	0.2	0.01	nd	0.01

All values are in µg/g, wet wt.
A:source:Stănescu (2004)
b : New Zealand
c : United Kingdom
Lit. std.: Literature standard.

CONCLUSIONS

High lead levels in Mureş river fish could be traced to urban and industrial waste. Waste management in urban and industrial centers in the country, especially around Mureş has remained very unsatisfactory.

REFERENCES

[1] Arah, R.O. (1985). "Lead-free gasoline in Russian by the year 2000", *Proceedings of Seminar on Petroleum Industry and the Russian environment.* 339-346.

[2] Babich, H. and Stoczky, G. (1985). Heavy metals toxicity to microbemediated ecologic processes: a review and potential application to regulatory policies". *Journal of Envrronmental Resources 36,*111-115.

[3] Botkin,D.B. and Keller, E.A. (1998). *Environmental Science; Earth as a living Planet.* ,2nd Edition. Canada, John Wiley and sons,Inc.

[4] Cain, J.R, Paschal, D.C.and Hayden, C.M. (1980). "Toxicity and bioaccumulation of cadmium in the colonial green algae (*Scenedesmus obliquus)" Archieves of Environmental contamination. Toxicolog9,* 9-16.

[5] Council on Environmental Quality. (1970). *Ocean dumping: a national policy; report to the President.* Washington D.C., Government printing office.

[6] Environmental Protection Agency (EPA). (1976). *"Quality criteria for water", USEPA, 440(*9), 76-123. Washington, D.C.

[7] Stănescu, O. and Grigorie, A.. (2004). Studies on the effect of Abattoir and Industrial effluents on the heavy metals and microbial quality of Danubius Delta

in Romanian.*Romanian Journal of Biotechnology. 4(*3), 266-272.
[8] Laws, E.A.(1981). *Aquatic pollution*. New York, John Wiley and Sons.
[9] Lenssen, N. (1989). The ocean blues. *World watch*, July/Aug., pp. 26-35.
[10] Nauen, C.E. (1983). "Compilation of legal limits for hazardous substances in fish and fishery products" *, FAO Fish Circulation, 764*, 5-91.

DETERMINATION OF MANGANESE IN SOIL AND SUNFLOWER *(Helianthus annuus L.)* PLANT PARTS AFTER MICROWAVE-ASSISTED DIGESTIONS BY ICP-OES

Gulay SEREN and Ozan YORUK

Department of Chemistry, Trakya University, 22030 Edirne, Turkey

E-mail: gulayseren@trakya.edu.tr

ABSTRACT

This study concerns the problem of trace elements in the environment, especially in soil and plant samples. Trace elements are harmful to humans, animals and tend to bioaccumulation into the food chain. Sunflower (Helianthus annuus L.) samples were taken from five locations. In total, 72 samples from 24 sampling points, including soil, sunflower roots and sunflower head parts in Edirne. Turkey. The concentrations of manganese in soil and sunflower plant samples were determined by Inductively Coupled Plasma Optic Emission Spectroscopy (ICP-OES) after microwave-assisted digestions. In this study, the concentrations of manganese in soil samples were observed under the limits of Soil Contamination Control Administration. The manganese concentrations in heads were higher than in roots of sunflower but these concentrations were observed under the limits of phytotoxic.

Keywords: *manganese, soil, sunflower, inductively coupled plasma spectrometry*

INTRODUCTION

Manganese is the second most abundant heavy metal; it is present to about 0.1% in the Earth's crust and is in the twelfth in the abundance list of the elements. It is to be found as a trace element in virtually all soils, and frequently together with iron in ground waters (Welz et al., 1999).

Manganese is an essential trace element that is present in all living cells. It is resorbed by plants in the form of Mn(II) salts and plays a major role in photosynthesis. It is also an essential trace element in animals and is present in numerous oxidoreductases and other enzymes (Welz et al., 1999).

Trace elements because of atmospheric and industrial pollution accumulate in the soil, and influence the ecosystem nearby (Al-Radady et al., 1994). Hence, the investigation of trace elements in soil and plant samples is very important in the point of environmental pollution, especially for plants with nutritional requirements. The bioaccumulation of trace elements over large territories and long time periods, which may result in the gradual damage of living organisms, necessitates careful monitoring of the input, mobility and effects of these pollutants. Metal-accumulating plants, such as *Brassica* species and *Helianthus annuus L*., can accumulate unusually high concentrations of trace elements in both shoots and roots from polluted soil and waters (Dushenkov et al., 1995). Hence, it is necessary to obtain more information about the plants which grow on soils with high concentration of metals, in order to

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 76 - 78, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

determine their potential for the management of polluted soils and, especially, for metal extraction.

The purpose of the present work was to determine the levels of manganese by ICP-OES, in soil and roots, heads of sunflower plants collected from Edirne, Turkey.

MATERIAL AND METHODS

Soil and sunflower plant samples were taken from five locations as shown in Figure 1. In total, 72 samples from 24 sampling points including soil, sunflower roots and sunflower head parts.

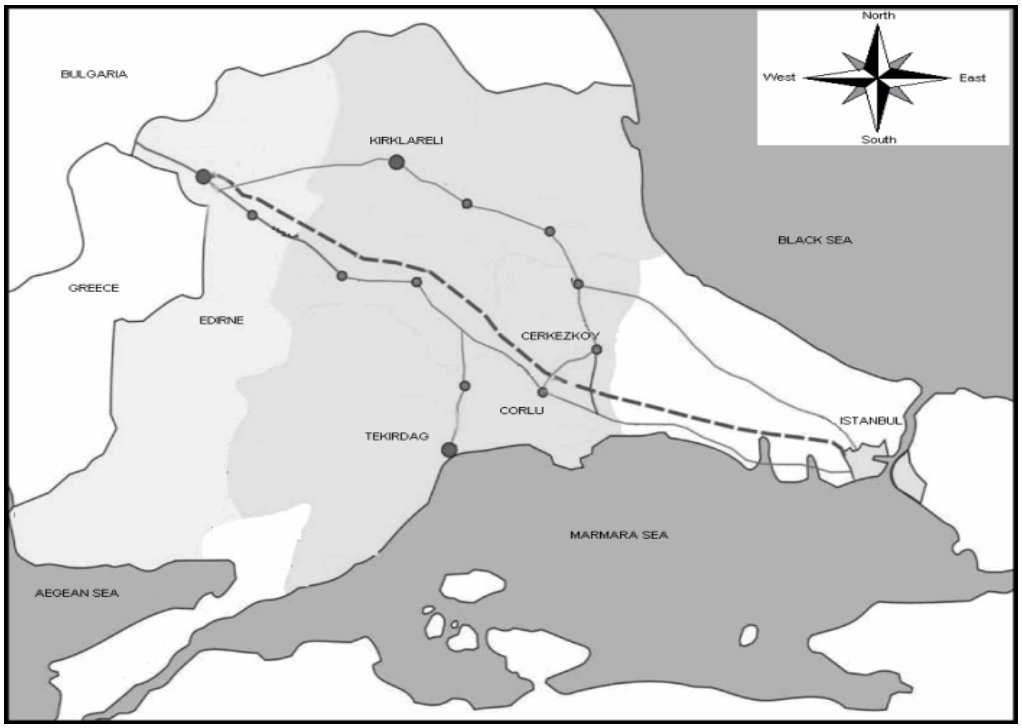


Figure 1. The map is the sampling area of plant and soil samples.

Samples were dried at 60ºC for 48 hours. The dried samples were ground and homogenized (Welz et al., 1999). For digestion of the samples; 0.25 g samples were digested with conc.HCl-conc.HNO_3 acid mixture for plant parts and conc.HNO_3-conc.H_2O_2 acid mixture for soil in microwave digestions system (CEM Mars5 Model).

In the final solutions, the Mn concentrations in soil and plant samples were determined by Perkin Elmer 5300DV Model ICP-OES.

RESULTS AND DISCUSSION

The Mn concentration in sunflower heads was higher than in roots, and also with in the normal levels as shown in Table 1 (Blamey et al., 1997).

The Mn concentration in soil samples varied from 98.68 mg kg^{-1} to 797.75 mg kg^{-1} and under the limits of Soil Contamination Control Administration (Bowen et al., 1979).

Table 1. Normal ranges in plants and phytotoxic concentration of some trace elements.

Element	Normal levels, $mgkg^{-1}$ dry foliage	Phytotoxic levels, $mgkg^{-1}$ dry foliage
Cu	3-20	25-40
Mn	15-150	400-2000
Zn	15-150	500-1500

In the other study (Sabudak et al., 2007) we observed the phytotoxic Mn concentrations in sunflower leaves collected from industrial area (This area is located where the most power of industrialization is in Trakya region). This could have been the result of industrial pollution, as trace elements could have been easily absorbed from air in this case, and translocated to plant leaves or seeds.

In the present work, Mn concentrations were found to be low in both of soil and plant samples (Fig.2) because of selected area only agricultural in Trakya region .

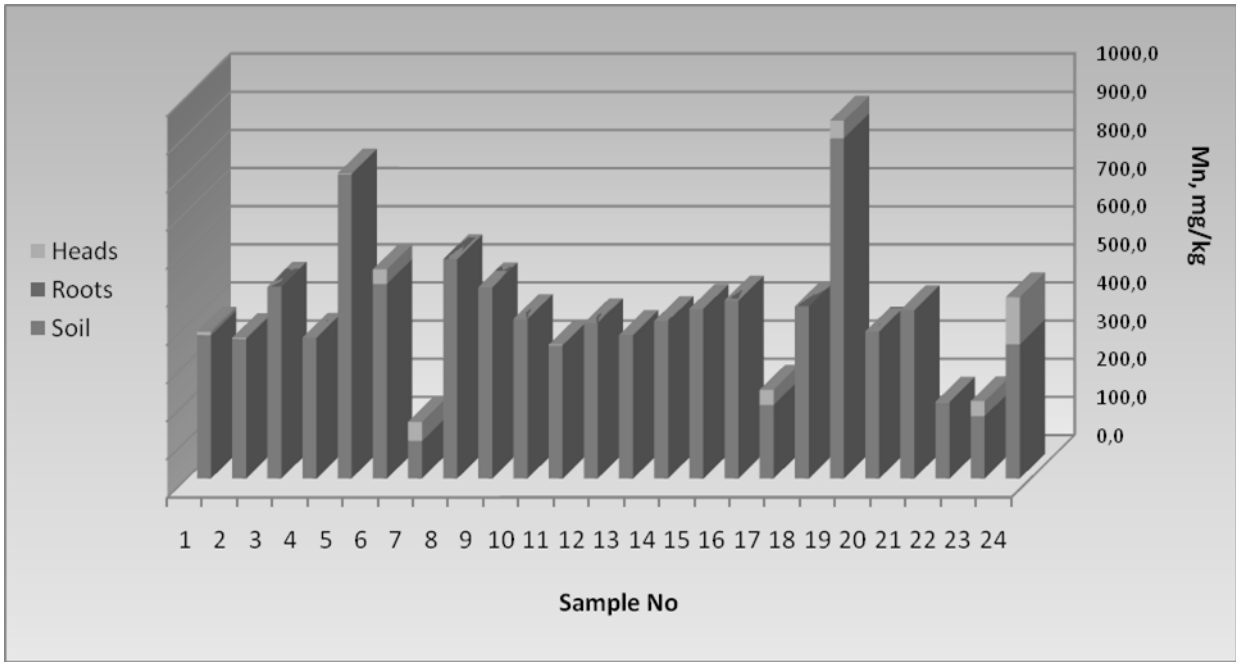


Figure 2. Manganese concentrations in plant parts and soil samples

REFERENCES

1. Welz B., and Sperling M. - Atomic Absorption Spectrometry. Wiley-VCH Verlag GmbH, Germany, 1999.
2. Al-Radady A.S., Davies B.E. and French M.J. - Distribution of lead inside home: case studies in the North of England. Science of the Total Environment, 1994, 145, 143-156.
3. Dushenkov V., Kumar P.B.A., Motto H. and Raskin I. -Rhizofiltration the use of plants to remove heavy metals from aqueous streams. Environmental Science&Technology, 1995, 29, 1239-1245.
4. Bowen H.J.M. - Environmental Chemistry of the Elements. Academic Press, London, 1979, 333.
5. Blamey F.P.C., Zollinger R.K., and Schniter A.A. - Sunflower Production and Culture. Madison, USA:ASA, 1997, 595-670.
6. Sabudak T., Seren G., Kaykıoglu G., Dinçer A.R. - Determination of trace elements in soil and sunflower (Helianthus annuus L.) plant parts, 2007, 16, 1274-1278.

THE INFLUENCE OF SOME NATURAL POLYPHENOLS ON *RHODOTORULA* SPP. GROWTH IN THE PRESENCE OF COPPER IONS

Anca HAINAL, Ioana IGNAT, Alina STINGU, Irina VOLF, Valentin I. POPA*

"Gheorghe Asachi" Technical University of Iasi, Faculty of Chemical Engineering, Departments of Pulp and Paper and Environmental Engineering and Management, 71D. Mangeron Bld., 700050 Iasi, Romania

**Email: vipopa@tuiasi.ro;vipopa15dece@yahoo.com;www.vipopa.ro*

ABSTRACT

The natural polyphenols are known to have microbiocide activities against a huge number of pathogenic microorganisms. On the other hand it is well known that Rhodotorula spp. can metabolize metal ions in different concentration while the phenolic compounds presents chelating properties. The aim of this study was to evaluate the growth response of a Rhodotorula strain, under the influence of polyphenolic extracts from spruce bark, and Asclepias syriaca in the presence of copper ions. To establish the behavior of the yeasts in these conditions the total phenolic content, the quantity of wet biomass and copper ions concentrations were periodically evaluated during the process.

Keywords: polyphenols, biosorption, red yeasts, copper ions.

INTRODUCTION

There is currently a great interest in phenolics research due to the possibility of improved public health through diet, where preventative health care can be promoted through the consumption of fruit and vegetables. (Caridi et al., 2007) Polyphenols possess well-known pharmacological properties and strong biological functions such as anti-inflammatory and antioxidant activities (Kong et al., 2003).In addition, they have a metal chelating potential.

The acknowledged potential of yeasts for the removal of heavy metal cations from aqueous solutions is well known and also it was recently reported that *Rhodotorula* spp. are resistant to heavy metals and could play an important part in the processes of mineral cycling (Li and Yuan, 2006). The cell wall structure is responsible for biosorption of heavy metals by *Rhodotorula* yeast as by the destruction of these structures with alkali showed a noticeable decrease in the amount of metals biosorption. Polyphenolic extracts present antimicrobial activity which depends on the type of microorganisms used and the bioactive compounds concentration. Therefore, a higher inhibitory rate in a lower germ concentration is justified because it is already known in literature that there is a correlation between the inhibitory effect of an active product's concentration and the tested germ's concentration (Popa et al., 2007).That is why, in this paper simultaneous influence of polyphenols and copper ions on *Rhodotorula* spp. is investigated.

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 79 – 83, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009

Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

MATERIALS AND METHODS

Plant material (spruce bark and *Asclepias syriaca*) was grinded in a mill and reduced to a fine powder 0.5 mm.10g of dried ground material were extracted (three times) with 125 mL water in water bath at 80-90°C for 45 min. The aqueous filtrates were collected and made to a volume of 500 mL with distilled water.

Dry matter content in the extracts was determined by evaporation of 25 mL extract on water bath and drying at 105°C till constant weight in accordance with the Polish Standard PN–90/A75101/03. After cooling at room temperature in a dessicator, the 105°C dried sample was placed into a muffle furnace at 600°C for 6 hours. The heating was repeated for one hour periods until the weight after cooling in desiccator is constant to within 0.3 mg.

The total phenolics were assayed colorimetrically by the Folin–Ciocalteu method described by Lapornik et. al., 2005.

Copper ions concentrations were evaluated by AAS (Atomic Absorption Spectrometry-GBC Avanta 2003) directly in the culture medium.

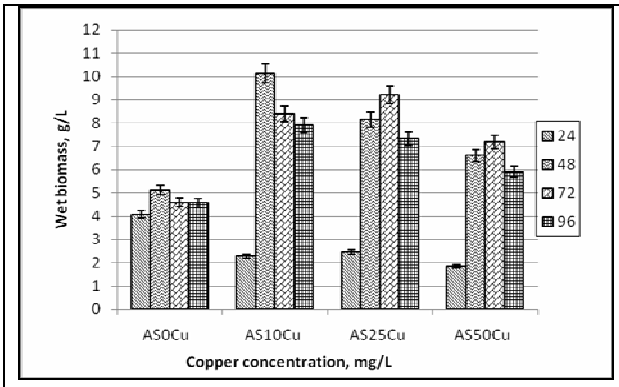
One strain of *Rhodotorula* spp. yeast was selected and purchased by Biorechnology Applied in Food Industry – Integrated Center for Research and Education – Bioaliment, "Dunarea de Jos" University, Galati. The cells were pre-cultured in basal medium composed of ($g \cdot L^{-1}$), glucose 10, peptone 5, yeast extract 3, malt extract 3. After 24h, 4mL of this culture (A_{620}=0.5) were used as inoculum for 100 mL of synthetic medium containing ($g\ L^{-1}$): glucose 1, $CaCl_2$ 0.05, $(NH_4)_2SO_4$ 1, KH_2SO_4 1.The synthetic medium was prepared in vegetal extracts containing different concentrations of copper (10, 25, 50 mg/L Cu^{2+}). Then, the Erlenmeyer flasks were incubated on an orbital shaker at 120 rpm and 27ºC for 96 h, and the pH was not adjusted during the fermentation process. Every 24 h, one sample flask was collected and the cells were harvested by centrifugation (4000 rpm from 15 min), and washed with distilled water until this was colourless. The wet mass of cells was gravimetrically determined.

RESULTS AND DISCUSSION

The dry matter content showed to be higher in the case of *Asclepias syriaca* extracts (1.85 g/L) comparing with spruce bark extract (1.00 g/L). At the same time, the organic matter content represents approximately 96% for spruce bark extracts and 90% for *Asclepias syriaca*.

The presence of copper ions introduced in the vegetal extracts seems to have a stimulating effect on the wet biomass production. The higher content in wet biomass was reported in the case of *Asclepias syriaca* extract (Fig.1, left) containing 10mg/L Cu^{2+}. Not the same thing could be said about the spruce bark extract (Fig.1 right) which presents a wet biomass stimulation in the case of 50 mg/L Cu^{2+} concentration.

The analysis of the results provides a significant decreasing (almost 50%) of copper amounts in the culture medium before the inoculation process (0 h).This can be explained by a potential chelating effect of polyphenolic compounds with copper ions. During the fermentation process, copper level remains almost constant (Fig.2).



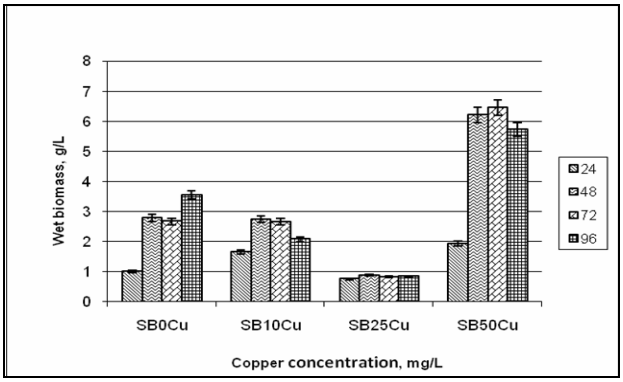
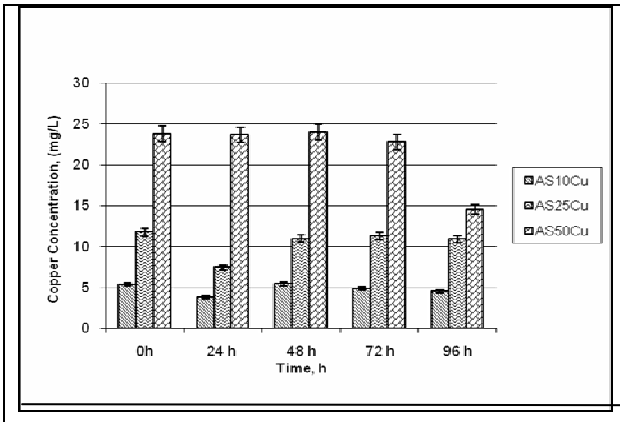


Fig.1. Variation of wet biomass (g/L) as a function of copper concentration (mg/L).



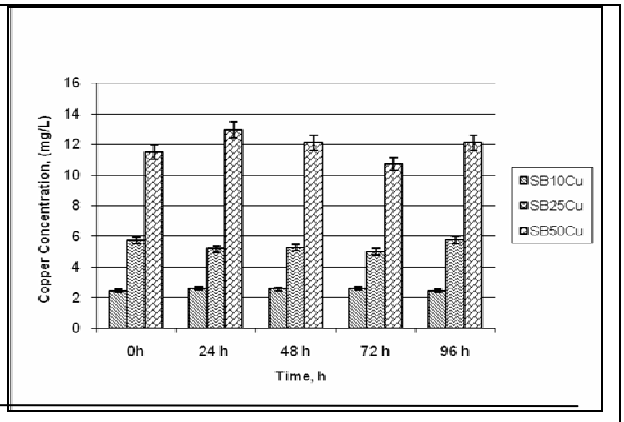


Fig.2. Variation of copper concentration (mg/L) in growth medium as a function of time (h).

The total phenolic content of *A. syriaca* extracts seems to be almost constant during the yeast cultivation (Fig. 3, left). On the other side, the total phenolic content as a function of copper concentration shows a decreasing trend (Fig.3 right).

The decreasing values obtained may be due to the complex forming of phenolic compounds with copper ions or due to the consumption of polyphenols as a carbon source by the yeasts.

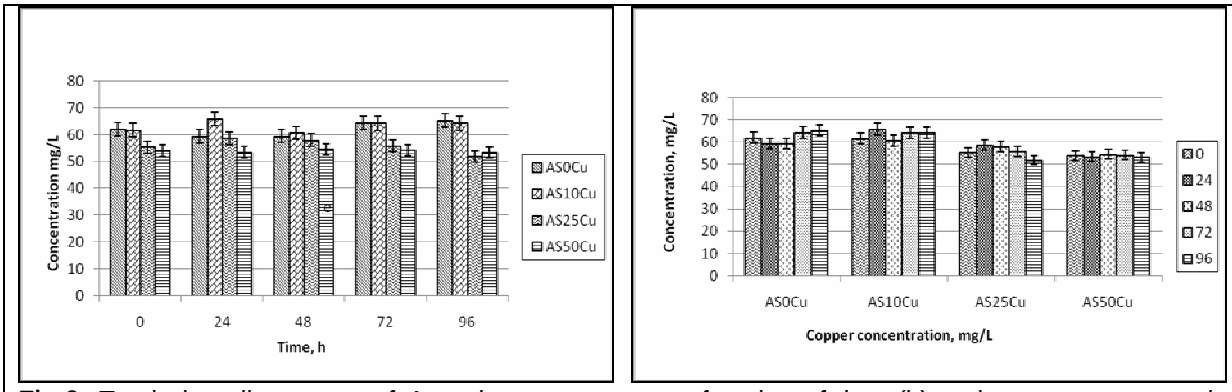


Fig.3. Total phenolic content of *A. syriaca* extracts as a function of time (h) and copper concentration mg/L

Regarding the total phenolic content in the culture medium of spruce bark extract, it cannot be observed a significant variation as function of time in terms of concentrations. The content of total phenolic compounds reported to different copper concentrations applied shows decreasing values (Fig. 4) with increasing copper concentrations. The lowest concentrations was reported for 50 mg/L Cu^{2+} at 48, 72 and 96 respectively.

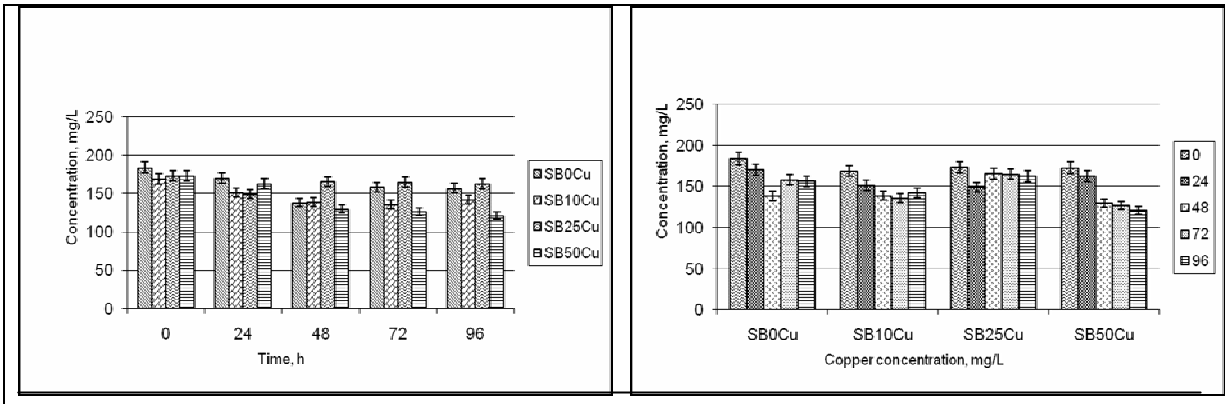


Fig.4. Total phenolic content of spruce bark extracts as a function of time (h) and copper concentration (mg/L)

AS10Cu, AS25Cu, AS50, SB10Cu, SB25Cu, SB50Cu- the synthetic culture medium prepared in A.syriaca (AS) and spruce bark (SB) vegetal extracts containing different concentrations of copper 10, 25, 50 mg/L Cu^{2+}).

Therefore, the behavior of *Rhodotorula* spp. investigated in this paper depends on the copper ion concentration and composition of polypohenols determined by the source of their isolation.

ACKNOWLEDGEMENTS

This paper was supported by BRAIN "Doctoral scholarships as an investment in intelligence" project, financed by the European Social Found and Romanian Government.

REFERENCES

1. Caridi D., Trenerry V.C., Rochfort S., Duong S., Laugher D., Jones R.- Profiling and quantifying quercetin glucosides in onion (*Allium cepa* L.) varieties using capillary zone electrophoresis and high performance liquid chromatography, Food Chem., 2007,105 691–699.

2. Kong, J. M., Chia, L. S., Goh, N. K., Chia, T. F., Brouillard, R.- Analysis and biological activities of anthocyanins, Phytochem., 2003, 64, 923–933.
3. Lapornik B., Prosek M., Golc Wondra A.- Comparison of extracts prepared from plant by-products using different solvents and extraction time, J.Food Eng., 2005, 71, 214–222.
4. Li Z., Yuan H. – Characterization of cadmium removal by *Rhodotorula* sp. Y11, Appl. Microbiol. Biotechnol., 2006, 73, 458-463.
5. Popa V.I., Anghel N., Dănăilă M., - Natural polyphenolic compounds. Structure, bioavailability and microbial interactions, Proceedings of the 8 th ILI Forum, Rome, May-2007, 10-12, 97-100.

EFFECTS OF METALS ON BENTHIC FORAMINIFERAL TESTS IN ASALOOYE (PERSIAN GULF)

Masoomeh SOHRABI-MOLLAYOUSEFI[1]*, Maryam SAHBA[2]

1-Department of Geology, Faculty of Science, Islamic Azad University, Islamshahr Branch, Tehran, Iran
** E-mail : m_sohrabi14@yahoo.com*

2- Department of Geology, Faculty of Science, Islamic Azad University, Science and research branch, Tehran, Iran
E-mail : msahba224@yahoo.com

ABSTRACT

Asalooye is located in Booshehr province in south of Iran. The studied region includes the gas refineries and Petrochemicals Companies that are the most important source of toxic and heavy metals. It is known that benthic foraminifera show great sensitivity to environmental changes. Their sensitivity to pollutants is expressed by the development of morphological abnormalities, which are induced by deleterious effects of toxins on the cell.

The faunal studies were accomplished by geochemical analyses of surface sediments and measurement of physicochemical properties of water. Physico-chemical properties included the salinity, temperature, dissolved oxygen and acidity of water was measured on board. Samples for geochemical analysis were powdered and concentration of Ba, Sr, Cu, Zn, Pb, Ni and Cr was determined. The samples for foraminifer's analysis were stored in a fridge for 8 hours to effect a sufficient staining with Rose Bengal. After drying foraminifera tests were picked out, then normal and abnormal tests were counted separately. The abundances were expressed as a number of specimens per 1cm^3 of sediment. The main species were photographed by using a Scanning Electronic Microscope (SEM). The living benthic foraminifera communities in sediments were dominated Elphidium ceraticulatum.

Key words: Booshehr, sediment, foraminifera test, acidity, salinity, Heavy metals

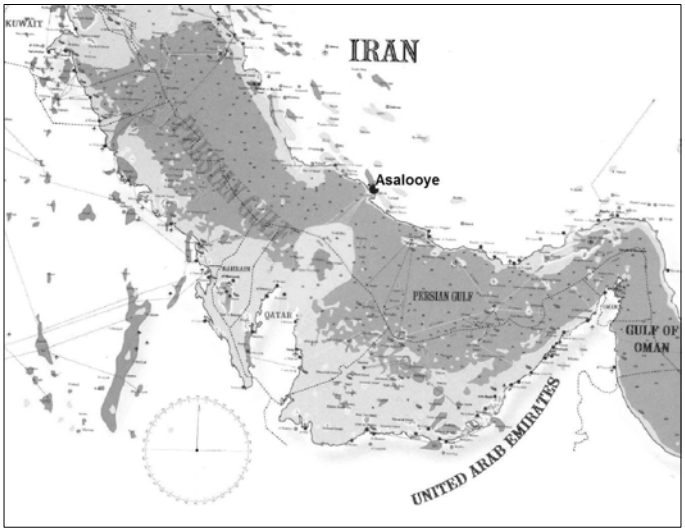
INTRODUCTION

Booshehr province is one of the south west parts of Iran, and its coastline has much pollution of hydro-carbon and oil. Gas refineries and Petrochemicals industries are located in Asalooye city of Booshehr province. The aim of this study is to determine the possible causes of abnormality on benthic foraminifer's tests.

Benthic foraminifera are single-celled organisms similar to amoeboid organisms in cell structure. Foraminifera are covered with an organic test that varies from a single chamber with an aperture to calcite wall or agglomeration of mineral grains. Foraminifera occupy a wide range of marine environments, from brackish estuaries to the deep ocean basins (Ernest et al, 2006). They are good biomarkers for environmental pollution of natural or artificial causes.

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 84 - 89, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

The heavy metals adversely affect the biota and cause morphological abnormalities in individuals. Benthic foraminifera of the Persian Gulf were investigated in 2007-08. By systematical and ecological studies were determined foraminiferal assemblages in coastline sediments of Asalooye.



Map 1- map of studied region

MATERIALS AND METHODS

The current study is based on 14 surface sediment samples collected in May 2008 on 2 cruises. The surface samples were placed into a glass vial thoroughly mixed and subsamples for organic and inorganic geochemical analyses were taken from this mixture at first. The remaining was transferred to a PVC vial, and preserved and stained with a solution of 2 grams Rose Bengal per litre ethanol in order to mark living foraminifers (Murray et al, 2000).

According to Physicochemical properties measurements the salinity, temperature, acidity and dissolved oxygen content of the super standing water in core tube were measured on board.

For geochemical analysis subsamples were freeze-dried and powdered and amount of Ba, Sr, Cu, Zn, Pb, Ni and Cr was determined by X.R.F examinations.

The subsamples for foraminifera analysis were stored in a fridge for 8 hours to effect a sufficient staining with Rose Bangal. Living individuals are recognized by staining (Biocenosis).

The samples were first passed and washed through 4 sieves (50 μ, 100μ, 0.5mm and 1 mm). After drying foraminifera tests were picked out, both normal and abnormal tests were counted separately.

RESULTS AND DISCUSSION

The temperature and salinity showed a pronounced seasonality. Average of temperature was determined 23.5 ^{o} C. Acidity of water didn't show important changes. Average of dissolved oxygen exceeded 6.1 mg/lit (tables 1,2).

The concentrations of Cu, Zn, Pb, Ni and Cr in subsamples of Asalooye were showed in table 3. Accumulations of elements have relation to grain size of sediments, mean' while amount of them in muddy sediments is more than sandy.

The surface sediment pollution by Cu, Zn, Pb, Ni and Cr principally could be considered as moderate because the levels of metals are comparable to elsewhere in Persian Gulf.

According to previous studies mean concentration of Cu is increased but the others didn't show important changes.

The living benthic foraminiferal communities were dominated by: *Elphidium ceraticulatum*. The other common species of benthic foraminifera in Asalooye coast line sediments are *Ammonia beccarii, Amphistegina lobifora, Cymaloporetta sp., Dendritina ambiguea, Penereplis planatus, Pseudohauerinella sp. Pseudohauerinella dissidens, Quniqueloqulina agglotinanas, Quniqulina seminulum Rotalia trochidiformis, Rupertinella rupertiana, Spiroculina depressa, Spiroloculina excavata, Triloculina inflata, Triloculina tricarinata* (table 4, plate 1).

Our results infer that genra or species with hylaine tests have more abundancy than the porcelanose or aglutina tests. Abundance of living individuals (or biocenosis) more than dead forms of foraminifera (or taphocenosis). Investigation on test size of foraminidera showed living individuals in studied region is smaller than normal size.

The results of a comparison between dimensions of test and grain size of sedimentary bed showed silty or muddy beds have small or tiny tests. In this type of beds the amount of total organic matter is high and concentration of metals, especially Cu, is higher than in sandy beds.

According to Alve (1995) abundant and geographically widespread species are to be considered as most tolerant to environmental pollution. *Ammonia beccarii* is commonly frequent in coastal environments (Stouff et al. 1999). Species of Ammonia beccarii is aboundance because its opportunistic behavior and high potential to survive under high input of nutrients and metals concentration. In our studied region *Elphidium ceraticulatum* has high abundance but with abnormal test. The majority of abnormal tests were observed in *Ammonia beccarii* and *Elphidium ceraticulatum*. However during spring time we observed an increase in abundance of abnormal tests, which was correlated to high metal levels. We suggest that this mirrors the production of benthic foraminifera during spring, and the juveniles were especially sensitive to environmental stress.

Table 1- Physicochemical properties of water in sampling stations, May2008 (ST1-ST7)

STATION NO.	ST1	ST2	ST3	ST4	ST5	ST6	ST7
TEMPRETURE (^{o}C)	22.8	24.9	22.3	22.6	22.9	24.5	23.3
pH	8.1	8	8.2	8.1	7.9	7.8	8
SALAINITY (gr/lit)	38.87	38.5	39.1	38.5	38.78	38.9	39
DISSOLVED OXIJEN (mg/lit)	6.45	6.27	6.38	6.1	6.08	5.98	5.85

Table 2- Physicochemical properties of water in sampling stations, May2008 (ST8_ST14)

STATION NO.	ST8	ST9	ST10	ST11	ST12	ST13	ST14
TEMPRETURE (^{o} C)	24.1	24.9	23	24.1	23.7	22.4	23.6
PH	7.9	8.2	8.1	7.9	8.1	8	7.8
SALAINITY (gr/lit)	38.4	38.58	38.94	38.5	38.5	39.15	38.9
DISSOLVED OXIJEN (mg/lit)	6.25	6.1	5.8	6.22	6.3	5.46	6.24

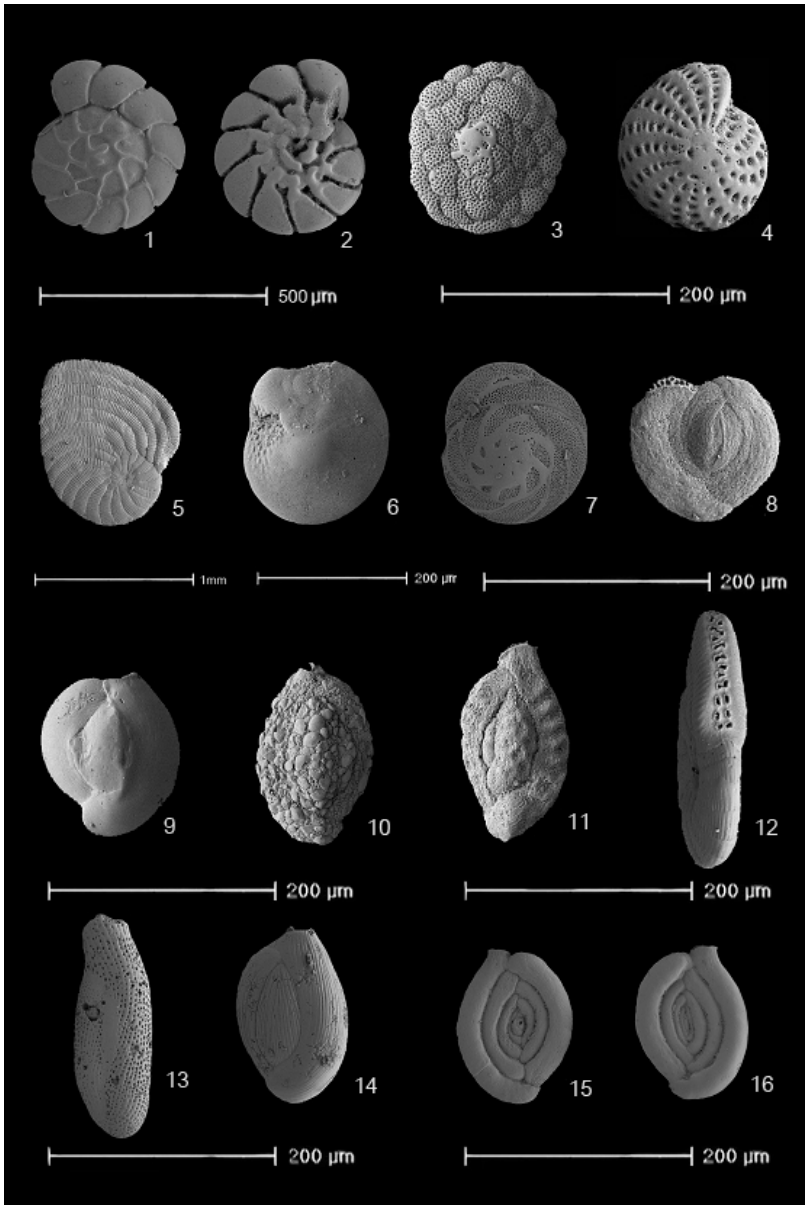
Table 3 - Mean concentration of Cu, Zn, Pb, Ni and Cr (ppm)

Cu	Zn	Pb	Ni	Cr
33	34	4	17	4

Table 4- Summary of foraminifer's population in studied region

NO.	Genera & Species	Biocensis	Taphocenosis
1	*Elphidium ceraticulatum*	70	12
2	*Ammonia beccarii*	20	-
3	*Amphistegina lobifora*	54	23
4	*Cymaloporetta sp.*	22	5
5	*Dendritina ambiguea*	43	12
6	*Penereplis planatus*	28	8
7	*Pseudohauerinella sp.*	11	1
8	*Pseudohauerinella dissidens*	25	3
9	*Quniqueloqulina agglotinanas,*	30	11
10	*Quniqueloqulina seminulum*	40	20
11	*Rotalia trochidiformis*	21	10
12	*Rupertinella rupertiana,*	5	-
13	*Spiroculina depressa*	7	-
14	*Spiroloculina excavata*	6	-
15	*Triloculina inflata*	21	18
16	*Triloculina tricarinata*	33	21

Plate 1



1, 2: *Ammonia beccarii,* 3: *Cymbaloporetta* sp., 4: *Elphidium craticulatum*, 5: *Peneroplis planatus,* 6: *Amphistegina lobifera,* 7: *Rotalia trochidiformis,* 8: *Pseudohauerina* sp*.,* 9: *Quinqueloculina seminulum,* 10: *Quinqueloculina agglutinans,* 11: *Pseudohauerinella dissidens,* 12: *Dendritina ambigua*, 13: *Rupertianella rupertiana,* 14: *Triloculina inflate,* 15, 16 *Spiroloculina depressa*

REFERENCES

1. Alev, E. – Benthic foraminiferal responses to estuarine pollution, J.Foramin Res., 1995, 25, 3, 190-203.
2. Alev, E. Murray, J.W. - Marginal marine environment of Skagerrak and Kattegat. Paleogeogr.Paleoklimatol.Paleoecolo,1999, 146, 171-193
3. Boyle, E. A., Cadmium, - zinc, copper, and barium in foraminifera tests, Earth Planet. Sci. Lett.1981,53, 11 –35.
4. Ernest, S. R., Morvan, J., Geslin, E., Le Bihan, A., and Jorissen, F. J. - Benthic foraminiferal response to experimentally induced Erika oil pollution, 2006, 61, 76-93.
5. Dmitrenko, O.B., - Environmental reconstruction based on heavy metals, Diatoms and Benthic foraminifers in the Isahaya Reclamation Are, Nagasaki, Japan, 2004, J.JEM, 1, 83-105.
6. Le Cadre, V. and Debenay, J. P. - Morphological and cytological responses of Ammonia (foraminifera) to copper contamination: Implication for the use of foraminifera as bioindicators of pollution, Environ, Pollut. 2006, 143, 304–317.
7. Murray, J.W. –Ecology and pale ecology of Benthic Foraminifera, 1991, Bath press, Avon, 397.
8. Sch¨onfeld, J. and Numberger, L. - The benthic foraminiferal response to the 2004 spring bloom in the western Baltic Sea, 2007, Mar. Micropaleontol., 65, 78–95.
9. Stouff, V., Geslin, E., Debenay, J.-P., and Lesourd, M.,- Origin of morphological abnormalities in *Ammonia* (foraminifera): studies in laboratory and natural environments, J. Foramin. Res. 1999, 29(2), 152–170.
10. Yanko, V., Ahmad, M., and Kaminski, M. - Morphological deformities of benthic foraminiferal tests in response to pollution by heavy metals: implications for

BEHAVIOR OF TOMATO PLANT DURING CULTIVATION IN THE PRESENCE OF COPPER IN FOREST AND SANDY SOILS

Alina STINGU, Ioana IGNAT, Anca HAINAL, Irina VOLF, Valentin I. POPA*

"Gheorghe Asachi "Technical University, Faculty of Chemical Engineering and Environmental Protection, 71 A Mangeron Blvd., 700050 Iasi, Romania

** E-mail: vipopa@ch.tuiasi.com;vipopa15dece@yahoo.com;www.vipopa.ro*

ABSTRACT

Application of copper compounds is a routine disease control practice in organic tomato production and it is somewhat controversial because excessive use can result in the buildup to phytotoxic (crop damaging) levels in the soil. Copper toxicity in tomatoes plants was studied in pots experiment induced pollution in two different types of soils (sandy and forest soils). Changes in tomatoes growth were quantified through biometric and quantitative analysis, chlorophyll assimilation assay, copper accumulation in plant organs, % recovery degree.

Key words: physiological changes, heavy metal content, bioaccumulation coefficient

INTRODUCTION

Copper toxicity in crops has become a serious problem, especially in the developed countries. Copper accumulation in soils may come from different sources, including air pollutants and soil applications of commercial fertilizers, sewage sludge, manure and lime (McLaughlin et al., 1996; Kidd et al., 2007). Also, industrial effluents may contain a wide variety of pollutants depending on the industries involved, and in many cases high concentrations of heavy metals have been reported (Iribar et al., 2000). Most of the information available about Cu physiology in plants comes from studies with the Cu-hyperaccumulator and Cu-tolerant plants but less information is available in commercial crops such as tomato.

Metals such as Zn, Cd, Pb, Fe, Cu, Mn and Mo may be phytotoxic and/or if accumulated in the fruit will impose a health risk to humans. Human health risks, such as parasitic infections and transfer of heavy metals in the food chain, represent the main limitations which led many countries to issue more rigid regulations with respect to pathogenic and heavy metal contents in wastewater and sludge reused in agriculture, as well as to establish maximum permissible limits for heavy metals in soil (Lahham et al., 2007).

The effects of Cu have been investigated in tomato (*Lycopersicon esculentum*) plants grown in a controlled environment using solution of concentrations of 10, 25, 50 mg/L $CuSO_4$ and the results are presented in this paper.

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 90 - 93, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009

Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

MATERIALS AND METHODS

Tomato plants seedlings were transferred in vegetative pots with forest or sandy soil and watered 2 times per week with tap water.Twenty days after seedling tomato plants were moved in separately vegetative pots.

The influence of copper ions on tomatoes growth and development were analyzed in different environments (sandy/forest soils). Five replicates of each vegetative pot (2 tomato plant/ pot) were watered every two days with copper ions solution of different concentration (10, 25, 50mg/L $CuSO_4$).Treatments applied to tomato plants in different conditions were suggestively marked as follows: Cu-10-S, Cu-25-S, Cu-50-S (copper solution applied in 10, 25, 50 mg/L to sandy soil) and Cu-10-F, Cu-25-F, Cu-50-F (copper solution applied in 10, 25, 50 mg/L to forest soil). At the end of the experiment the value of total copper concentration, in the growth medium, was 2.3mg/L, 5.75mg/L, 11.5mg/L $CuSO_4$.

Plants were harvested and divided into three fractions, leaves, stems and roots. Biometric (length measurements) and quantitative determinations (fresh - FW and dry - DW weights) were performed for each fraction.

All plant tissue were washed with distilled water, excepting root fraction which were washed twice with tap water and twice with distilled water to remove any trace of sand/ soil. Samples were oven dried at 70 °C for 72 h until constant weight.

Assimilator pigments were determined in 80% acetone extract, by colorimetry at 663 nm, 646 nm and 470 nm wavelengths. Results were calculated based on formulas developed by Yamazaki (1982) and values expressed in mg/100g plant material.

For Cu analysis, roots, stems and leaves dried samples were digested with nitric acid and hydrogen peroxide (0.25 g in 5mL HNO_3 and 1.5mL H_2O_2) on a hot plate for at least 5 hours (Smith et al., 2008).The digested samples were analyzed by atomic absorption spectrometry (GBC Avanta 2003 - www.ch.tuiasi.ro/cercetare/PNCDI/MEDRES-LAB/index.php) for copper content analysis in the plant tissue.

Different parameters were calculated by the following the formula:

Bioaccumulation coefficient = Metal content/g dry Plant tissue/ Metal content mL /nutrient solution (Singh et al. 2009).

% Recovery = Metal content in shoot or root/ Metal content in medium (Hajiboland, 2005).

RESULTS AND DISCUSSION

Tomatoes roots were strongly developed in sandy soil than in the forest soil, mean while stems, in terms of length and dry weight, showed a better development comparing with control and sandy soil samples in the presence of copper ions (10mg/L, 25 mg/L $CuSO_4$).Tomatoes roots dry weight and stems length were inhibited in 50 mg/L $CuSO_4$ contaminated forest soil. Biomass production of the growing stems and leaves was depressed at high metal levels in sandy soil. The presence of copper ions in forest soil induced no visible injurious effect on *Lycopersicon esculentum* growth and development comparing with the others grown in sandy soils which were visible smaller and weak developed (results not shown).

Copper sulphate in forest soil growth medium stimulated the total chlorophyll (Chl) assimilation in tomato leaves (Table 1). The highest value for chlorophyll total content was obtained in the presence of 50 mg/L $CuSO_4$. Chlorophyll a, b content in tomato was much higher for plants grown in forest soil than for those grown in sandy

soil. This could be correlated with leaves color, dark green for sandy soil plant and fresh green for the others tomato plants.

Table 1.Content in chlorophyll a, b and total, the ratio of chlorophyll a and b of tomato leaves studied

Plant and Growth Medium	Tratament Solution	Chl a	Chl b	Total Chl content	Chl a/b
Tomato Sandy soil	Control	40.8	13.2	54	3.09
	Cu -10mg/L	25.4	6	31.4	4.23
	Cu -25mg/L	26.2	8.34	34.54	3.14
	Cu- 50mg/L	31.5	6.94	38.44	4.53
Tomato Forest soil	Control	87.6	28.2	115.8	3.10
	Cu -10mg/L	217.2	64.6	281.8	3.36
	Cu-25mg/L	205.2	69.2	274.4	2.96
	Cu-50mg/L	161.6	47.6	209.2	3.39

Regarding Cu (II) concentration, it could be said that copper ions were predominantly accumulated into the roots for plants cultivated both sandy and forest soil tomatoes and increased with increasing concentrations of copper solution applied (Fig.1, left). Copper concentration as well as copper content was higher in tomatoes roots plants grown in sandy soil comparing with those harvested from forest soil (Fig.1, right). Bioaccumulation coefficient presented the highest value for sandy soil contaminated with 10 mg/L $CuSO_4$ and the smallest one for forest soil treated with 50 mg/L solution of $CuSO_4$ (Fig. 2, left).

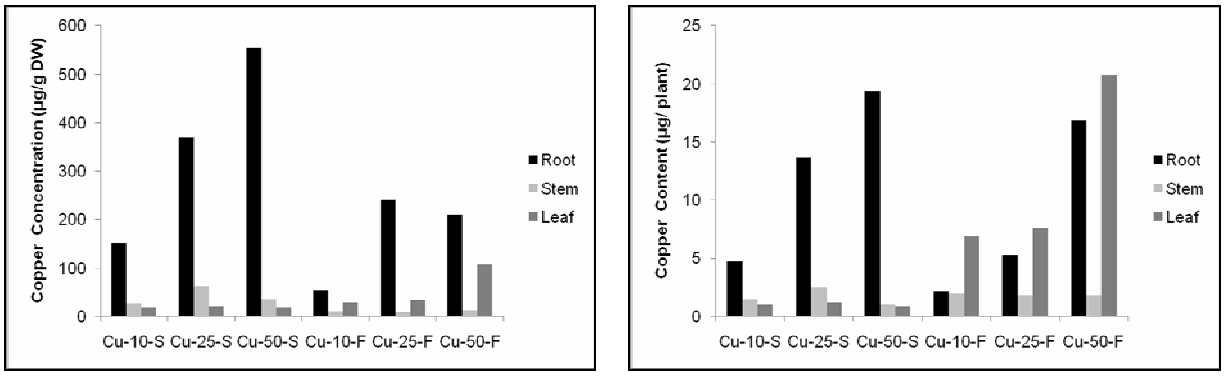


Fig. 1. Copper concentration, Copper content for tomato plant grown in sandy/forest soil induced polluted.

Recovery percentages showed total opposite values comparing with bioaccumulation coefficient and the highest recovery was obtained for tomatoes plants cultivated in the presence of 10mg/L copper solution contaminated forest soil. The smallest degree of recovery was observed for 50mg/L $CuSO_4$ in the sandy soil medium.

That could be explained by the low biomass yield of tomato plants in sandy soil (Fig.2, right). Therefore, tomato plants responses were different depending on various factors, including plant morphological element, site specific soil factors (pH,

organic matter, dissolved organic carbon, etc.), Cu concentration in the growth medium

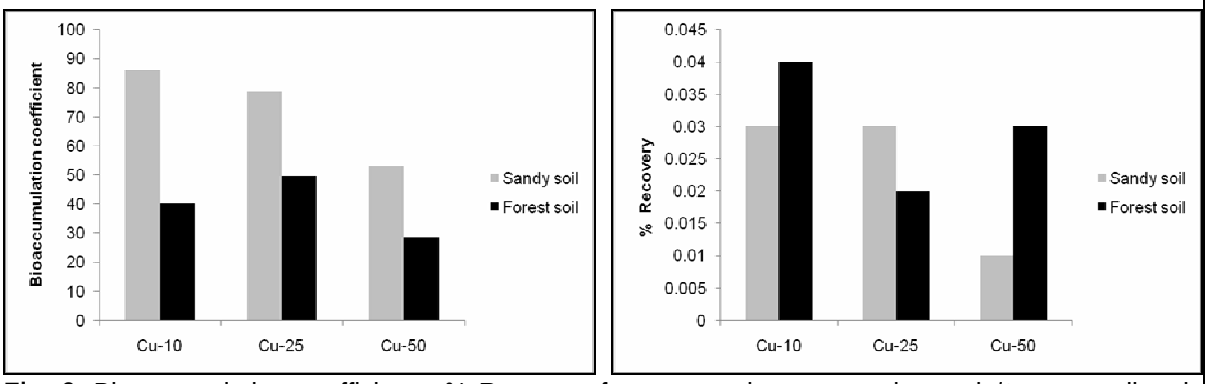


Fig. 2. Bioaccumulation coefficient, % Recovery for tomato plant grown in sandy/forest polluted soil.

AKNOWLEDGEMENTS

This paper was supported by BRAIN "Doctoral Scholarships as an investment in intelligence" project, financed by The European Social Found and Romanian Government.

REFERENCES

1. Hajiboland R.- An evaluation of the efficiency of cultural plants to remove heavy metals from growing medium, Plant Soil Environ., 2005, 51, 156–164.
2. Iribar V., Izco F., Tames P., Antiguedad I., da Silva A. - Water contamination and remedial measures at the Troya abandoned Pb–Zn mine (The Basque Country, Northern Spain), Environ. Geol., 2000, 39, 800–806.
3. Kidd P.S., Domínguez-Rodríguez M.J., Díez J., Monterroso C.- Bioavailability and plant accumulation of heavy metals and phosphorus in agricultural soils amended by long-term application of sewage sludge, Chemosphere, 2007, 66, 1458–1467.
4. Lahham O. A., El Assi N.M., Fayyad M. - Translocation of heavy metals to tomato (*Solanum lycopersicom* L.) fruit irrigated with treatedwastewater,Sci.Hort.,2007, 113, 250–254.
5. McLaughlin M.J.,Tiller K.G., Naidu R., Stevens D.P. - Review: the behavior and environmental impact of contaminants in fertilizers, Aust. J. Soil Res, 1996, 34, 1–54.
6. Singh A., Eapen S., Fulekar M.H. - Potential of *Medicago sativa* for uptake of cadmium from contaminated environment, Rom. Biotech. Lett., 2009, 14, 4164-4169.
7. Smith P.G., Koch I., Reimer K.J. - Uptake, transport and transformation of arsenate in radishes (*Raphanus sativus*), Sci. Total Environ., 2008, 390, 188-197.
8. Yamazaki K. - Nutrient Solution Culture (Japanese). Pak-kyo co., Tokyo, 1982, 251.

STUDY OF C-H···O INTERACTIONS BEETWEN COORDINATED WATER MOLECULE AND C_6-AROMATIC GROUP

Marija CIZLER[a], Dušan VELJKOVIĆ[b],Goran V. JANJIĆ[b] and Snežana D. ZARIĆ[a]

[a]Department of Chemistry, University of Belgrade,Studentski trg 16, 11000 Belgrade, Serbia , e-mail: szaric@chem.bg.ac.yu
[b] IHTM, Njegoševa 12, 11000 Belgrade, Serbia

** E-mail : szaric@chem.bg.ac.rs*

ABSTRACT

The coordinated water molecule can interact in a few ways with aromatic groups. It can interact with π–system of aromatic group, forming metal ligand aromatic cation-π (MLACπ) interactions, or C-H groups of aromatic fragment can interact with oxygen forming C-H···O interactions. Here we present results of intermolecular C-H···O interactions between coordinated water molecules and C_6-aromatic groups. Study of interactions was based on analysis of crystal structures and on ab initio calculation. Crystal structures archived in the Cambridge Structural Database involving coordinated water molecules and C_6-aryl groups were screened for intermolecular contacts. It was observed that in C-H...O interactions between C_6-aromatic group and coordinated water molecule linear arrangement is not favored. Visual analysis of structures and ab initio calculations showed that deviation from linear arrangement is consequence of tendency of water molecule to make energetically more *stable bifurcated interaction with two adjoining hydrogen atoms from aromatic group.*

Key words: C-H···O interactions, ab initio calculation, coordinated water

INTRODUCTION

The conformations and functions of molecules depend on the interactions with the surrounding solvent, in particular with water molecules. The interactions of water molecule with aromatic groups play important role in interactions of polar solvent with nonpolar molecules or fragments. The investigation of OH/π interactions between water molecule and the aromatic groups of amino acids in crystal structures of proteins confirmed relatively frequent occurence of aromatic interactions [1].

The coordinated water molecule can interact in a few ways with aromatic groups. It can interact with π–system of aromatic group, forming metal ligand aromatic cation-π (MLACπ) interactions[2], or C-H groups of aromatic fragment can interact with oxygen forming C-H···O interactions. The interactions of coordinated water molecules and π –system of C_6-aromatic group, called metal ligand aromatic cation-π (MLACπ) interactions were recognized and studied in crystal structures of metalloproteins and metal complexes [2]. Here we present results for intermolecular C-H···O interactions between coordinated water molecules and C_6-aromatic groups.

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 94 - 98, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

Study of the interactions was based on statistical analysis of crystal structures and on ab initio calculation.

MATERIALS AND METHODS

The statistical study is based on the crystal structures archived in the Cambridge Structural Database. The crystal structures involving coordinated water molecules and C_6-aryl groups were screened for intermolecular contacts. We searched for structures in which the distance between the O atom of the water molecule and the H atom of the C_6- aromatic ring is less than 3.2 Å, and the angle α larger than 110°. Among the CSD crystal structures we found 2355 short intermolecular contacts between a water and a C_6- aromatic ring which satisfy these criteria.

For the purpose of this analysis, we used parameters (Figure 1) which were directly retrieved from CSD (H···O distance d, angle α), as well as parameters which were derived (distances R, r and angle φ).

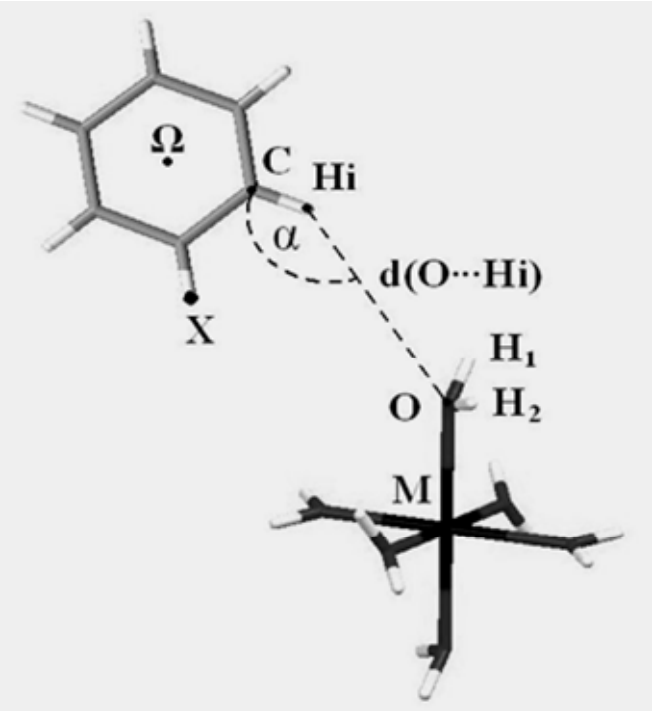


Fig. 1. The geometric parameters and atom labeling used for the description CH···O of interaction.

Ab initio calculations were done on the water-benzene model systems based on the geometries observed in the crystal structures.

RESULTS AND DISCUSSION

The results of searching crystal structures from CSD showed that H···O distances occur in the interval from 2.2 Å to 3.2 Å with maximum in the interval from 2.7 Å to 3.0Å (Figure 2). Histrogam for distribution of angle α, with maximum between 120° and 140° (Figure 2), indicate that linear arrangement is not favored.

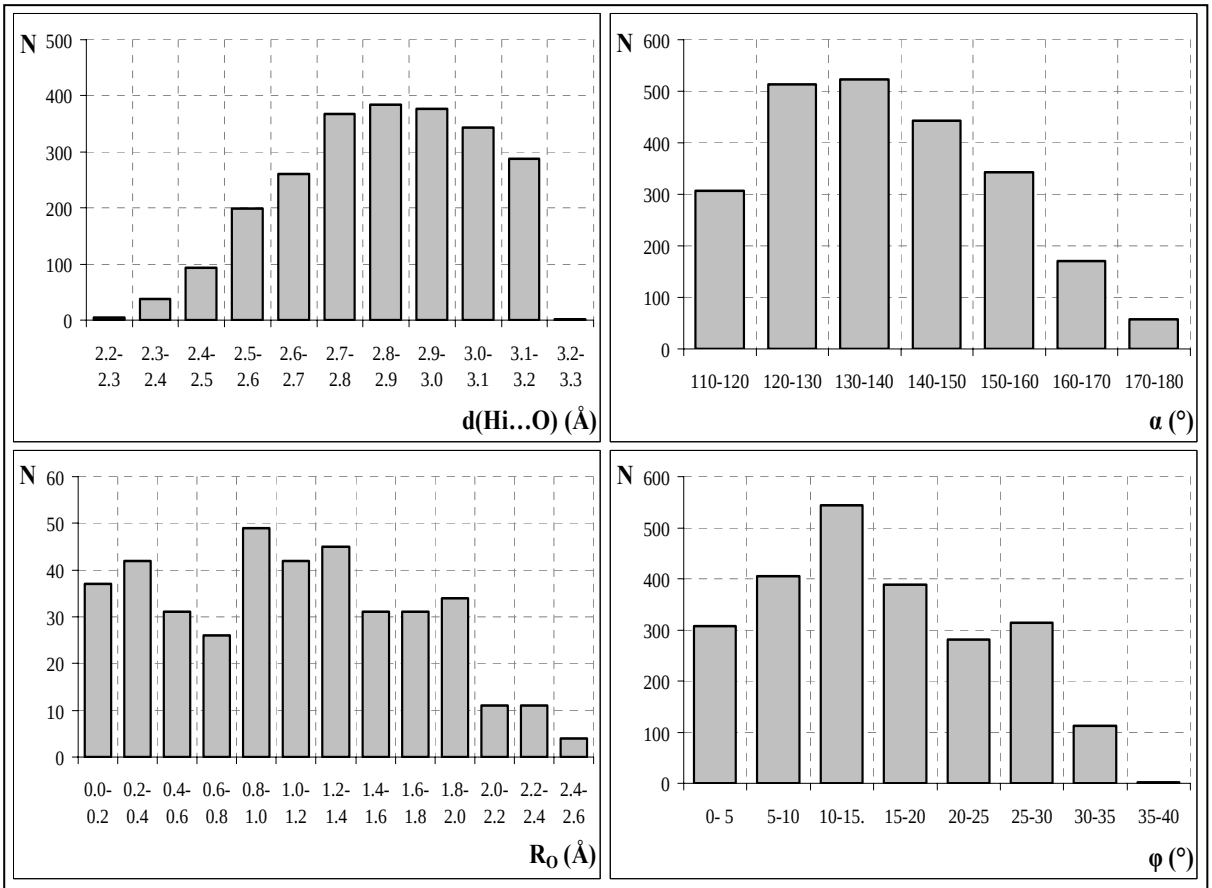


Fig. 2. The frequency distributions of d, α, Ro and φ parameters.

Histogram of the distribution of normal distances, Ro, shows that in large number of structures oxygen atom is not in the ring plane (Figure 2). The values of φ angle are between 0° and 40°, with pronounced peak for the region 10-15° (Figure 2). The frequency distribution of Ro and φ parameters also indicate that linear arrangement is not favored.

Visual analysis of structures showed that one of the reason for non linear arrangement is steric effect, because interacting species are usually voluminous.

The neighbouring X atoms in C_6- aromatic ring give possibility for bifurcuareted C-H···O hydrogen bond. However, these interactions are possible only in cases where X is hydrogen atom (Figure 1), while in studied structures some other atoms can be at X position. Since the substituent, on the neighboring carbon atom, can interact with the water molecule the analysis of the type of atom at X position is done. In the largest number of the structures on the X position is hydrogen atom, then carbon atom, oxygen atom and nitrogen atom (Table 1).

Table 1. Number of interactions for contacts with various atoms on the X position

X	Number of interactions
H	855
C	629
O	179
N	107

The frequency distributions of angle φ, for the structures in which hydrogen atom is on the X position, implies that in these structures there is a similar number of simultaneous and bifurcated C-H···O interactions (Figure 3).

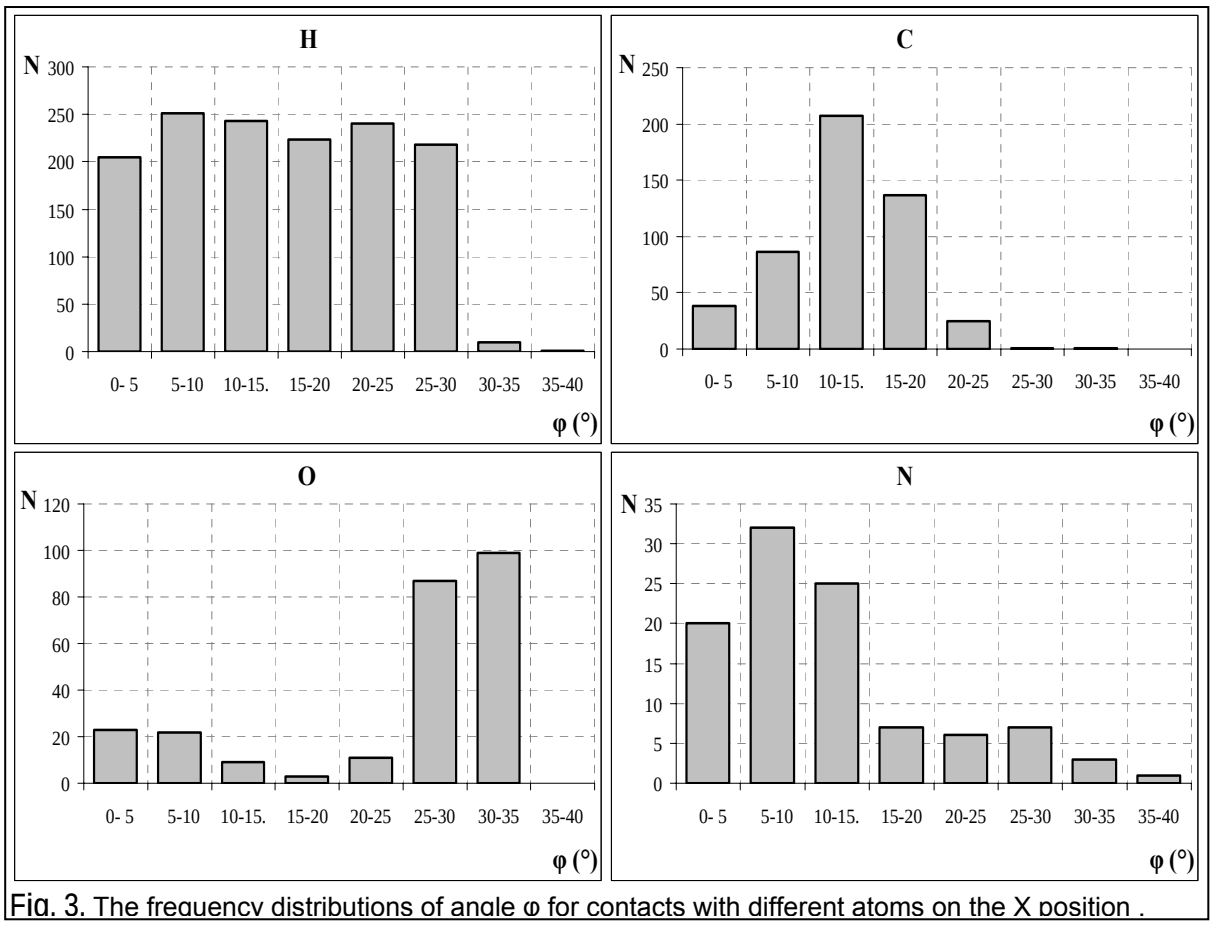


Fig. 3. The frequency distributions of angle φ for contacts with different atoms on the X position .

The analogous graph, for the structures in which oxygen atom is on the X position (Figure 3), is not similar. In these structures, the most of the contacts have angle φ values about 30°. The graph for the structures, in which carbon atom is on the X position, shows that in most of the structures angle φ values are smaller than 25° (Figure 4). Accumulation of spots in the range from 15° to 25° implies that oxygen atom from water interacts with substituent on the neighboring carbon atom. In the structures, in which nitrogen atom is on the X position, the most of the contacts have angle φ values smaller than 15° (Figure 3), indicating that in these structures there is no a tendency for simultaneous interactions.

Ab initio calculations showed that bifurcated C-H···O interaction (Figure 4) is more stable. This is consequence of tendency of water molecule to make energetically more stable interaction with two adjoining hydrogen atoms from aromatic group.

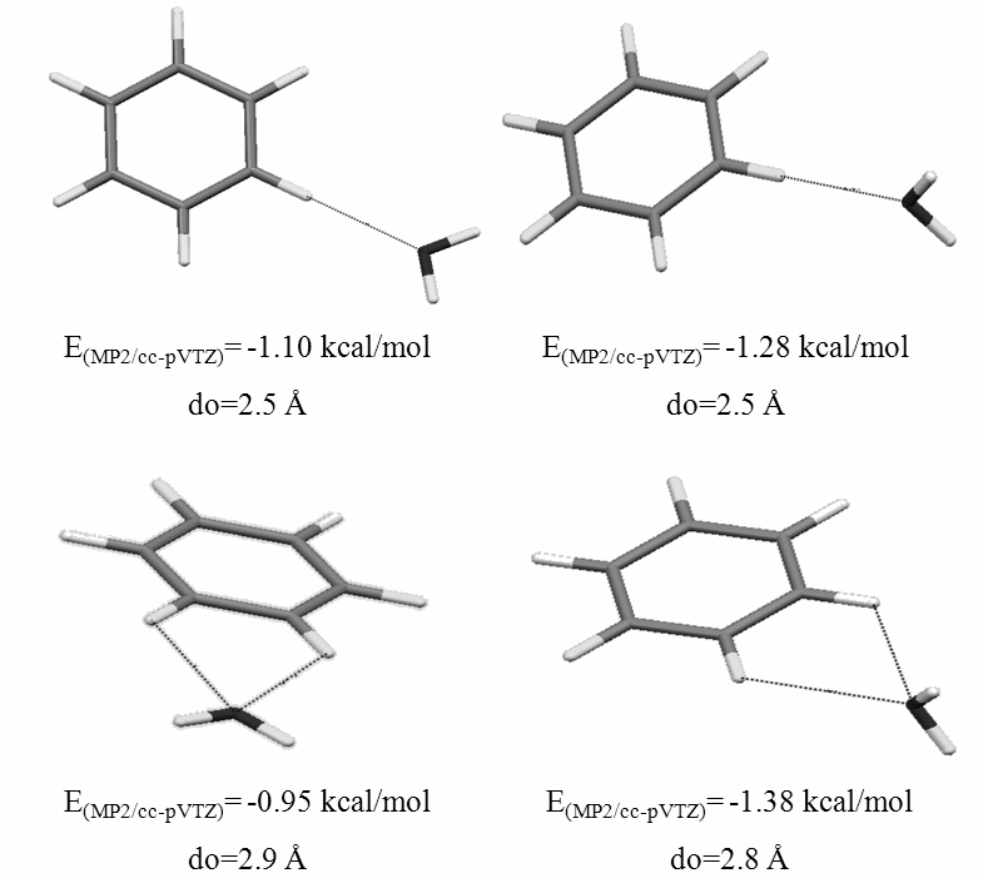


Figure 4. The model systems of the water-benzene dimers used for the ab initio calculations of the d distances and the interactions energies.

REFERENCES

1. S. D. Zarić, D. Popović, E. W. Knapp, *Chem. Eur. J.* **(2000),** 6, 213.
2. M. K. Milčić, S. D. Zarić, *Eur. J. Inorg. Chem.*, **(2001)**, 2143.

Hemoglobin peroxidase activity: interaction with hydroquinone and anthracene

Mariann-Kinga Árkosi, Florina Deac, Radu Silaghi-Dumitrescu

Department of Chemistry and Chemical Engineering, "Babes-Bolyai" University, 11 Arany Janos str, Cluj-Napoca RO-400028, Romania

e-mail : arkosi.mariann@gmail.com

ABSTRACT

The ascorbate peroxidase reactivity of hemoglobin and myoglobin has already been investigated and demonstrated in previous studies. Affinity toward urate, another natural antioxidant of plasma was analyzed, too. This study reviews the effect of two exogenous reducing agents, hydroquinone and anthracene on strong oxidant ferryl species of bovine hemoglobin. Anthracene appears to act as an inhibitor since the rate of ascorbate decay in ascorbate peroxidase catalytic cycle linearly decreases with anthracene concentration. Kinetic data suggest that inhibition can not be competitive in nature and anthracene can not act as a substrate in the peroxidase catalytic cycle of hemoglobin. Besides, it is shown here that hydroquinone efficiently protects hemoglobin from peroxide-induced free radical damage and the peroxidase activity of hemoglobin toward hydroquinone has well-measurable Michaelis-Menten parameters. The low K_m, close to physiological ranges is indicative of in vivo relevance. Moreover, hydroquinone appears to be a ferric iron reductant. Therefore a multifunctional role of hydroquinone in limiting the redox toxicity of Hb and maintaining its functional oxy state is proposed here.

Key words: hemoglobin, blood, oxidative stress, peroxidase, ferryl, Compound II, antioxidants, ascorbate, hydroquinone, anthracene

Introduction

The two 'traditional' members of globins family, hemoglobin (Hb) and myoglobin (Mb), have long been known as respiratory proteins for their reversible dioxygen handling function (transport and storage). However, Hb as well as Mb have recently been reported to be involved in redox side-reactions which show entirely enzymatic nature [1-8].

The general key to all catalase and heme peroxidase (such as myeloperoxidae, thyroid peroxidase, horse radish peroxidase and prostaglandin H synthase [6, 9]) mechanisms are two, highly oxidizing intermediates [6]. When hydrogen peroxide or other organic hydroperoxides accept two electrons from ferric Hb, one converts ferric into ferryl heme iron (classically termed Compound II, $Fe^{4+}=O^{2-}$), and the other results in the formation of an associated protein-bound free cationic radical (Compound I, Fe^{5+} formally) [7, 9].

Hb initiates free radical chemistry [7] and so has the potential to be an oxidative stressor [6]: the autoxidation of oxyHb (the ferrous heme-oxygen

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 99 - 110, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

complex) leads to metHb (ferric Hb) and superoxide ion ($O_2•^-$) formation which subsequently dismutates to generate peroxide [6, 8, 10].

High-valent intermediates are generated from metHb by peroxides (hydrogen peroxide, lipid hydroperoxides, peroxynitrite) [1, 11], similarly to what is seen with typical peroxidases. These reactive species (Compound I and Compound II) can then be reduced by a reducing agent. It appears that small molecule plasma reductants such as ascorbate, urate or glutathione [2, 6, 12, 13] are sacrificed to prevent any toxicity or damage to the heme [14-18] or other nearby biological targets, such as lipids, nucleic and amino acids [19-21].

It was suggested that Hb is especially designed to interact with ascorbate as it is the one protein with the highest affinity for ascorbate, of all those known so far, from any species [6]. The K_m value for ascorbate peroxidase activity of Hb was indicated at ~ 20 μM [22, 23], thus is similar to classical ascorbate peroxidases such as those found in plants [24, 25]. The maximal catalytic turnover (ascorbate oxidized per heme per minute) is much lower than that for classical ascorbate peroxidases, ~ 20 catalytic cycle/min [22]. This indicates that peroxidase activity of mammalian Hb has not been evolved to remove peroxide from biological systems and is “just” a defense mechanism against damaging oxidative consequences of superoxide release.

Although at a rather low rate, ascorbate is known to be the only plasma reductant that can effectively reduce metHb back to oxyHb. In other words, it is capable to avoid the loss of effectiveness of Hb converting the non-oxygen carrying met form to the ferrous, oxygen carrying form [6, 7, 26]. As opposed to ascorbate, urate and glutathione are less effective plasma antioxidants: they cannot cope with the ferric/ferrous couple. This ability of ascorbate to reduce met to oxy seems to be essential in case of cell-free Hb, where methemoglobin reductase (also known as cytochrome b5 reductase) cannot action. Interestingly, a cytochrome b561 was found on the surface of erythrocyte of non-ascorbate producers (human and guinea pig) that catalyses the dehydroascorbate (oxidized ascorbate) recycle to ascorbate [6, 7, 27-30] and thus can be involved in extracellular metHb reduction.

Our studies focused on the redox activity of Hb and a range of several oxidizable substrates that may control this reactivity, assuming that the electrons required for reduction of Compound II can be supplied by other small organic molecule reducing agents. Two exogenous ligands, hydroquinone and anthracene were considered to be worthwhile to investigate in further experiments. Hence, the role of these two compounds in the reduction of oxidized species of Hb is interpreted here.

Anthracene is used in the artificial production of the red dye alizarin, and it is also used in wood preservatives, insecticides and coating materials. Unlike other polycyclic aromatic hydrocarbons, anthracene is not carcinogenic but has been recently included in the Substances of Very High Concern list because being considered persistent, bioaccumulative and toxic for freshwater and marine ecosystems. It is generated during combustion processes: exposure to human happens mainly through tobacco smoke and ingestion of food contaminated with combustion products [31].

Hydroquinone can undergo mild oxidation to convert to benzoquinone. Reduction of benzoquinone reverses this reaction back to hydroquinone. Some biochemical compounds in nature have hydroquinone / quinone

moieties in their structures, such as coenzyme Q (also known as ubiquinone) and can undergo similar redox interconversions. Generally speaking, quinones are among the toxic products of oxidative metabolism of aromatic hydrocarbons [32]. The dietary quinones are known to be detoxificated by quinone reductases type 1 (QR1 or NAD(P)H:quinone reductases). These are FAD-containing cytosolic enzymes that catalyze the reduction of a broad range of quinones to hydroquinones using reducing equivalents provided by NAD(P)H [29].

Materials and methods

Bovine hemoglobin was purified following a variation of the general protocol of Antonini and Brunori [33]. Bovine blood, freshly drawn on citrate, was centrifuged 15 minutes at 5000 rpm (g) to separate the red blood cells, which were then washed three times with 5 mM phosphate pH 7.4 + 150 mM NaCl.

The peroxidase function proposed for heme proteins with peroxides is known to involve ferric, not ferrous, heme [3, 34]. Hence, after purified oxyHb had been obtained, it was converted to the fully ferric (met) state. Therefore oxyHb (ferrous, oxygen carrying state) was oxidized to MetHb (ferric, non-oxygen carrying state) by the addition of a 1.5 excess (mole oxidant:mole heme) of potassium ferricyanide [7, 35, 36]. The ferrocyanide and the excess ferricyanide were removed from the protein solution by filtration via passage down through a PD-10 Desalting column containing Sephadex^{TM} G-25 Medium.

Hydroquinone stock solution was an aqueous solution whilst anthracene was dissolved in methanol since it is not soluble in water.

Previous studies reported enzymatic activity of Hb to be greatly enhanced at acid pH [4, 6, 22]. Thus, experiments relating to peroxidase activity were carried out in pH5 buffer solution (50 mM sodium acetate) where the reaction intermediate ferryl is highly reactive due to its protonation (forming Fe^{4+}−OH with pKa=4.7) [5]. Other experiments were carried out in pH7 buffer solution (50 mM monosodium phosphate).

UV-Vis spectra and enzyme kinetics measurements were recorded on Cary 50 (Varian, Inc) and Agilent 8453 (Agilent, Inc.) instruments. Michaelis–Menten curve was fit with the aid of Excel 2003 using the Solver tool.

Results and discussion

The first set of experiments focused on determining whether hydroquinone or anthracene has any influence on ascorbate peroxidase activity of Hb. Therefore ascorbate, hydrogen peroxide, hydroquinone or anthracene and metHb were mixed sequentially. Peroxidase activity was measured monitoring the time course of absorbance at 290 nm (specific for ascorbate). The diminution in time of absorbance is considered to be due to enzymatic consumption of ascorbate. Zero order reaction rate coefficients were measured. To compare reaction rate coefficients relative to each other an inhibition ratio was defined as the ratio between the coefficient of hydroquinone- or anthracene-free experiment and that of hydroquinone or anthracene containing one.

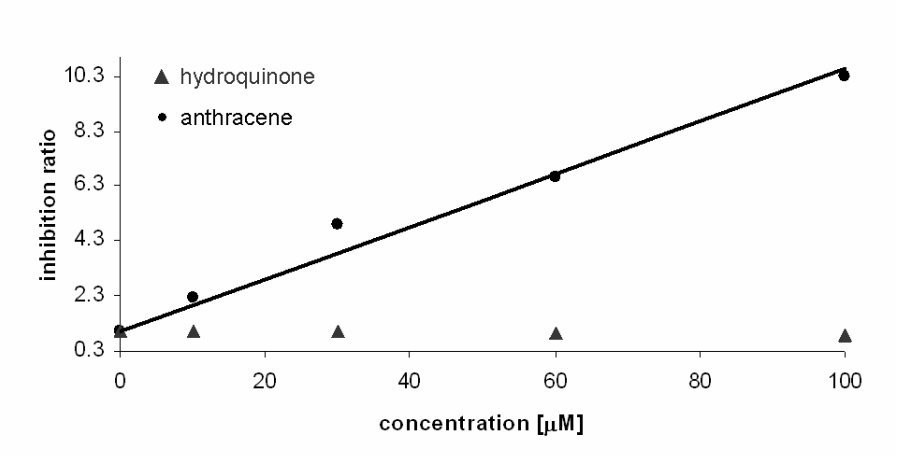


Figure 1. Comparison of reaction rate coefficients of ascorbate consumption – inhibition of ascorbate consumption in presence of anthracene. Conditions: buffer pH5, ascorbate 400 μM, hydrogen peroxide 1000 μM, hydroquinone or anthracene at concentrations varying from 0 to 100 μM, metHb 8 μM

As Figure 1 indicates, the presence of hydroquinone has slightly influenced the decay rate of ascorbate. Nevertheless, an increase in absorbance at 235-260 nm region is observed once the better part of ascorbate is consumed. Considering the rate of absorbance decrease at 290 and 245 nm (Figure 2.) it becomes clear that this happens due to involvement of hydroquinone in peroxidase catalytic cycle only after consumption of ascorbate (lower affinity of hydroquinone toward high valent heme iron) (Figure 3.) and not because a lower turnover number compared to ascorbate peroxidase catalytic cycle (in this case the increase of absorbance owing to benzoquinone formation would be hidden by the decrease of absorbance due to ascorbate consumption and should result in different rates of absorbance decrease at 290 and 245 nm).

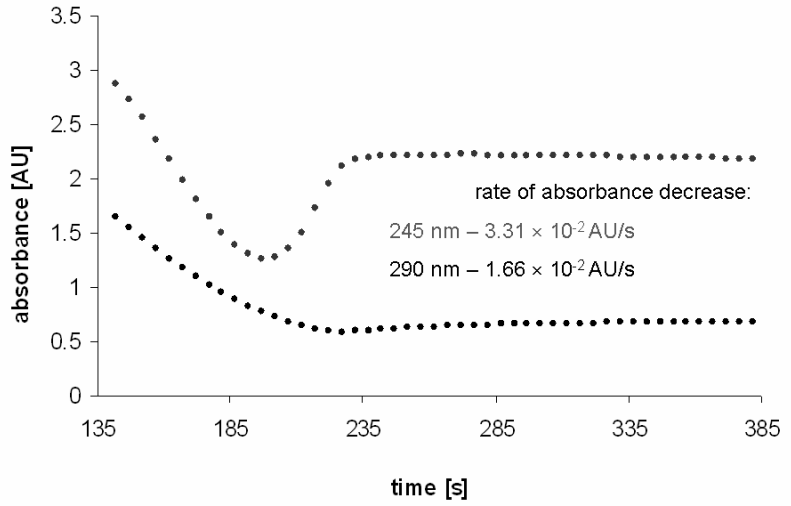


Figure 2. Absorbance decrease at 290 and 245 nm curse time. Conditions: buffer pH5, ascorbate 400 μM, hydrogen peroxide 1000 μM, hydroquinone 60 μM, metHb 8 μM

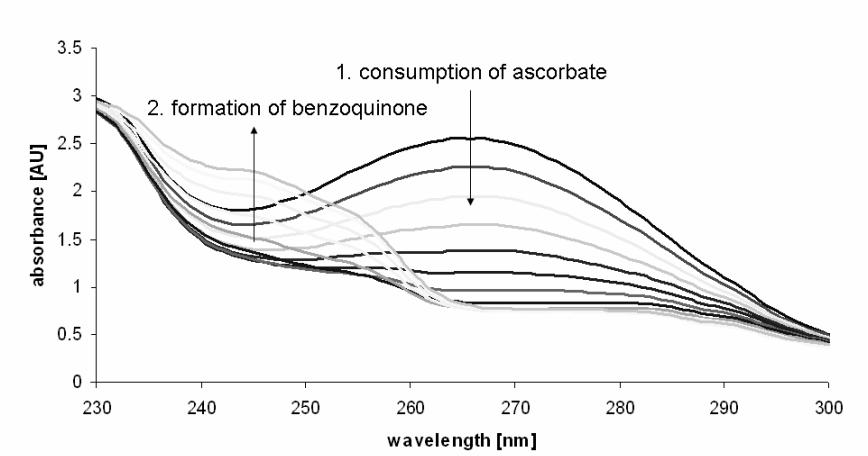


Figure 3. Involvement of hydroquinone in peroxidase catalytic cycle after consumption of ascorbate. Conditions: buffer pH5, ascorbate 400 μM, hydrogen peroxide 1000 μM, hydroquinone 60 μM, metHb 8 μM

On the other hand, in case of anthracene the rate of ascorbate consumption was significantly diminished: the higher the concentration of anthracene was the higher the value of inhibition ratio became. Moreover, the relation between inhibition ratio and concentration appears to be linear (Figure 1.). Spectra representing the ascorbate consumption in anthracene-free and anthracene-containing (10 μM) experiment are showed below (figure 4.a. and Figure 4.b.). Spectra are recorded in the first 60 seconds after mixing the components.

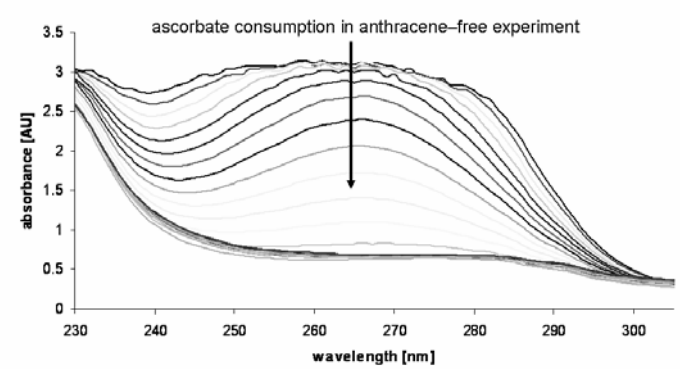


Figure 4.a. Decrease of absorbance in 290 nm region in athracene-free experiment. Conditions: buffer pH5, 400 μM, hydrogen peroxide 1000 μM, metHb 8 μM

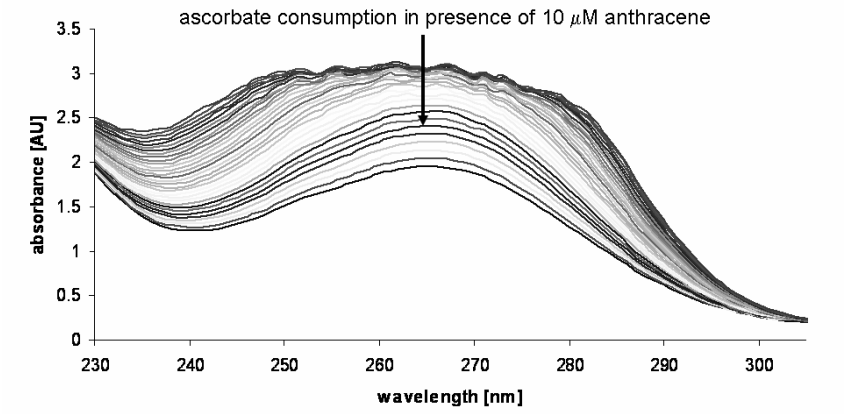


Figure 4.b. Decrease of absorbance in 290 nm region in anthracene-containing experiment. Conditions: buffer pH5, ascorbate 400 μM, hydrogen peroxide 1000 μM, anthracene 10 μM, metHb 8 μM

Following experiments where ferryl had been generated and anthracene added sequentially showed that the decay rate of ferryl was not remarkably accelerated in presence of anthracene (data not shown).

The maximum rate of ascorbate peroxidase catalytic cycle and affinity of the catalyst (metHb) for the two substrates (ascorbate and hydrogen peroxide) in anthracene-free and anthracene-containing experiments were measured and compared. The peroxidase activity appraisal supposed to modify the concentration of a single substrate (ascorbate or hydrogen peroxide), preserving the concentration of all other components (the other substrate, metHb and anthracene, if required). Absorbance was monitored at 290 nm. Zero order reaction rate coefficients of ascorbate consumption defined in the first thirty seconds after mixing the protein with the other compounds were transformed in μmole ascorbate consumed in a minute with an ε = 2107 $L \times cm^{-1} \times mol^{-1}$ extinction coefficient.

Figure 5.a. supports the idea of a non-competitive inhibition since the maximum speed of the reaction is decreased in presence of anthracene, whilst the affinity of ascorbate to the binding site, K_m, is substantially unchanged. The data of Figure 5.b. do not permit to draw an unambiguous conclusion because the Michaelis-Menten curves are too flattened to be thoroughly reliable. Parameters are indicating of a same, non-competitive inhibition (that is to say anthracene binds to an allosteric site and not to the enzyme's active site), but however, the possibility of a mixed inhibition can not be excluded.

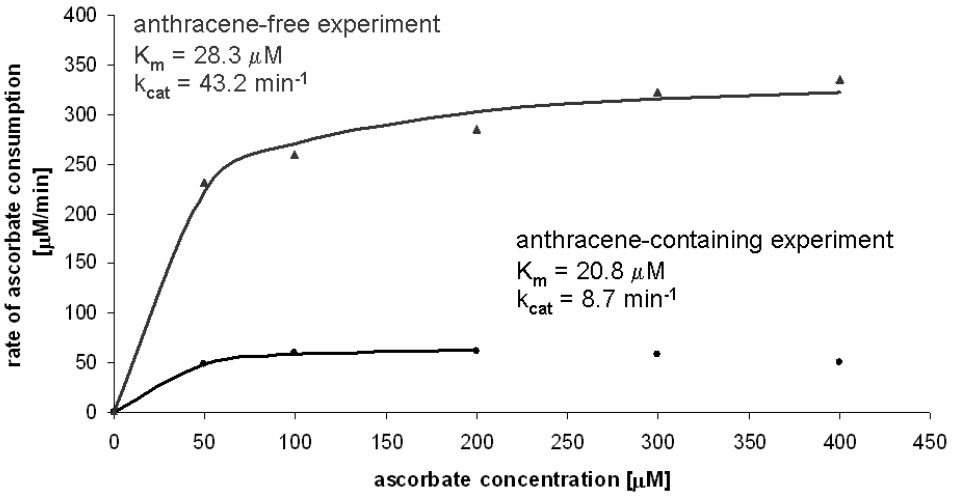


Figure 5.a. Study of influence of ascorbate concentration on ascorbate peroxidase activity. Conditions: buffer pH5, ascorbate at concentrations varying from 50 to 400 μM, hydrogen peroxide 1000 μM, anthracene 50 μM, metHb 8 μM

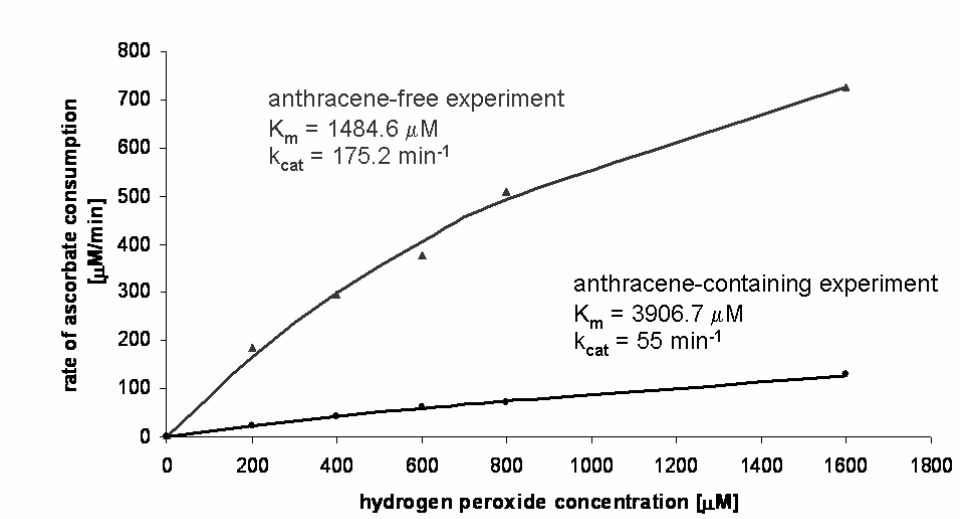


Figure 5.b. Study of influence of hydrogen peroxide concentration on ascorbate peroxidase activity. Conditions: buffer pH5, ascorbate 400 μM, hydrogen peroxide at concentrations varying from 200 to 1600 μM, anthracene 50 μM, metHb 8 μM

The second set of experiments were similar to those accomplished for purpose of investigate the peroxidase activity toward ascorbate and urate [6, 22]. This time hydroquinone was used as reducing substrate and not ascorbate or urate. Hydroquinone was added in concentrations ranging between 0-1000 μM. Catalytic cycle resulted in recovery of metHb at the end of reaction and consist in (i) hydrogen peroxide oxidation of ferric Hb to Compound II (ferryl) and (ii) reduction of compound II by hydroquinone. Hence, in presence of hydroquinone ferryl formation could be slowed down (Figure 6.a.)

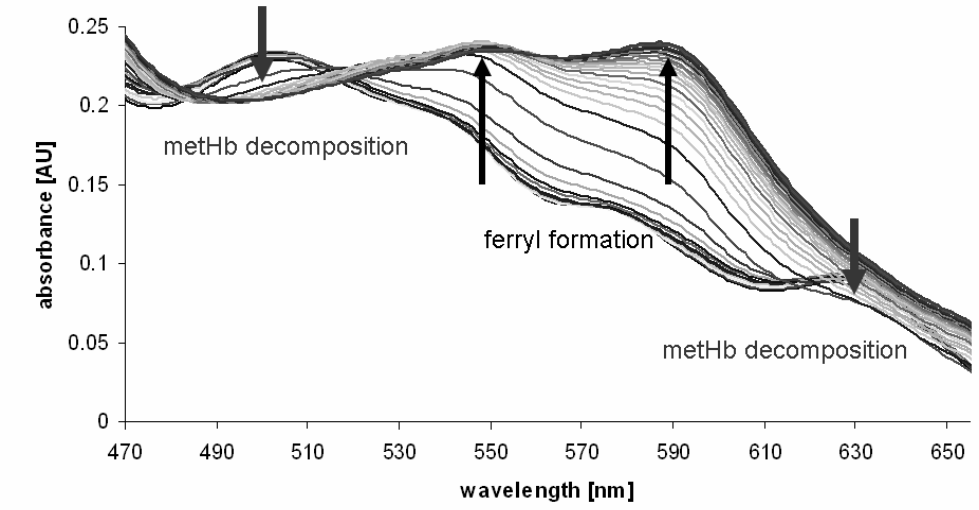


Figure 6.a. Formation of ferryl in presence of hydroquinone. Conditions: buffer pH5, hydroquinone 500 μM, hydrogen peroxide 1000 μM, metHb 8 μM. Spectra are recorded in the first 60 seconds after mixing the components.

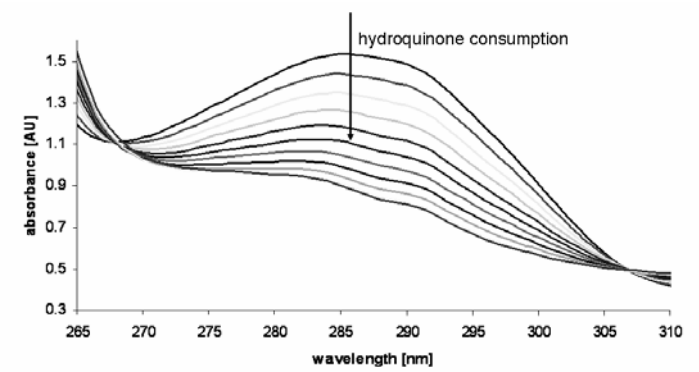


Figure 6.b. Hydroquinone consumption due to involvement of hydroquinone in peroxidase catalytic cycle. Conditions: buffer pH5, hydroquinone 500 μM, hydrogen peroxide 1000 μM, metHb 8 μM. Spectra are recorded in the first 50 seconds after mixing the components.

The decrease of absorbance at 240-300 nm region (including thus 290 nm) (Figure 6.b.) is owing to benzoquinone formation and confirms that the decrease of absorbance at 290 nm in the previous set of experiments is due to ascorbate consumption, not to ascorbate and hydroquinone simultaneous consumption (absorbance decrement at 290 nm was not accelerated by the presence of hydroquinone).

At low hydroquinone concentrations the rate of hydroquinone consumption could not be measured because hydroquinone was used up too rapidly. Therefore kinetic data related to the rate of benzoquinone absorbance (concentration) increment (first set of experiments) was used to determine the concentration of benzoquinone produced in a minute. In case of higher concentrations (250-1000 μM) the hydroquinone absorbance decrement was transformed in μM hydroquinone consumed in a minute, using 287.5 $L \times cm^{-1} \times mol^{-1}$ as extinction coefficient for hydroquinone at 290 nm. The representation of dependence of hydroquinone consumed and benzoquinone produced in a minute on hydroquinone concentration reveals a true enzymatic peroxidase activity of Hb toward hydroquinone with measurable K_m and k_{cat} parameters (Figure 7.). Consequently it is shown here that in presence of peroxide and hydroquinone, metHb is able to catalyze the oxidation of hydroquinone to benzoquinone and hence preserve the oxidative stability of Hb. With a relatively low K_m, 73 μM, affinity falls within the range of attainable physiological concentrations, although this constant is almost four time higher than that of ascorbate peroxidase cycle. The same as in case of ascorbate, turnover number (the maximum number of molecules of hydroquinone that Hb can convert per catalytic site per unit of time) is relatively low, k_{cat}=50 min^{-1}. Different peroxidase activities observed in the first set of experiments are reflected thus in different Michaelis-Menten parameters.

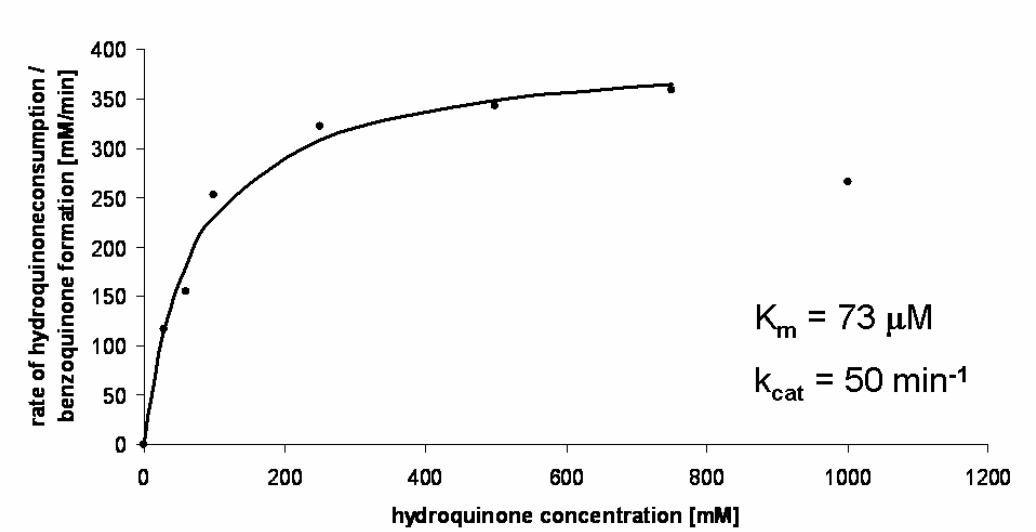


Figure 7. Peroxidase activity of Hb toward hydroquinone. Conditions: • buffer pH5, ascorbate 400 μM, hydrogen peroxide 1000 μM, hydroquinone (0, 30 or 60 μM), metHb 8 μM; • buffer pH5, hydroquinone (250, 500, 750, 1000 μM), hydrogen peroxide 1000 μM, metHb 8 μM

At elevated concentration of HQ, the enzyme activity is inhibited, which happens when the substrate is binding to a second, non-active site of the enzyme and thus block its activity (excess substrate inhibition). The same effect was observed at high concentrations of ascorbate [22].

Reducing ferryl to met may play a role in antioxidant defence of Hb against oxidative stress and toxicities, as ascorbate and urate do [6, 7]. As Figure 8. indicates it was revealed that hydroquinone can not only reduce ferryl to met, but it may contribute to maintain the functional oxy state of autooxidation more exposed acellular Hb. This probably makes hydroquinone capable to avoid the toxicity induced by the oxidative side reactions of Hb-based oxygen carriers (HBOCs, blood substitutes) [7, 37] and to maintain their efficacy in oxygen delivering through met/oxy transition.

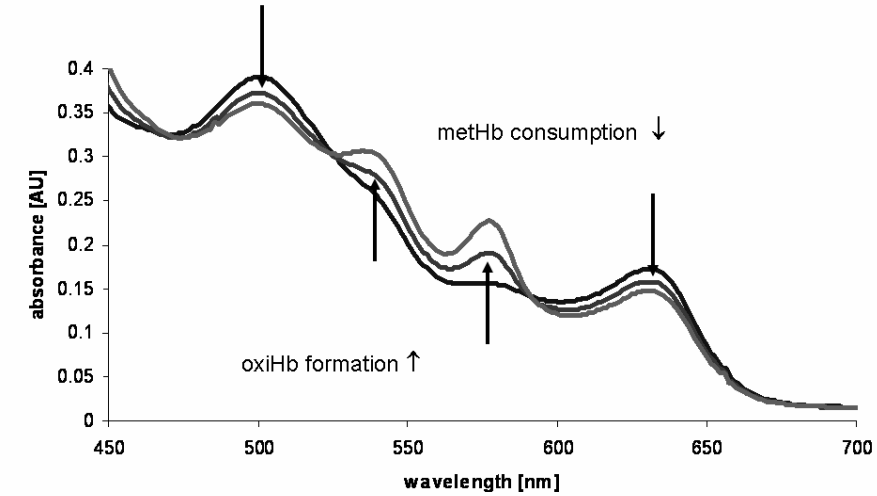


Figure 8. Reduction of ferric Hb to ferrous Hb in presence of hydroquinone. Conditions: buffer pH 7, metHb 8 μM, hydroquinone 500 μM. Spectra are recorded before hydroquinone was added, and in the 8th and 15th minutes after mixing the components.

Pseudo first order reaction rate coefficients of oxy formation were measured at concentrations of hydroquinone ranging from 100 to 5000 μM

(large excess as compared to 8 μM protein). As Figure 9. reveals, the reaction rate coefficient dependence on hydroquinone concentration appears to give a straight line intercepting (0;0). Thus, it can be concluded here that second order reduction of met to oxy in presence of hydroquinone has a 0.5466 $L \times M^{-1} \times s^{-1}$ rate constant and it is a simple A+B process (and not A+2B, for example). In other words, hydroquinone donates a single electron and the semiquinone does not interact with the protein. Most likely this semiquinone disproportionate in the solution.

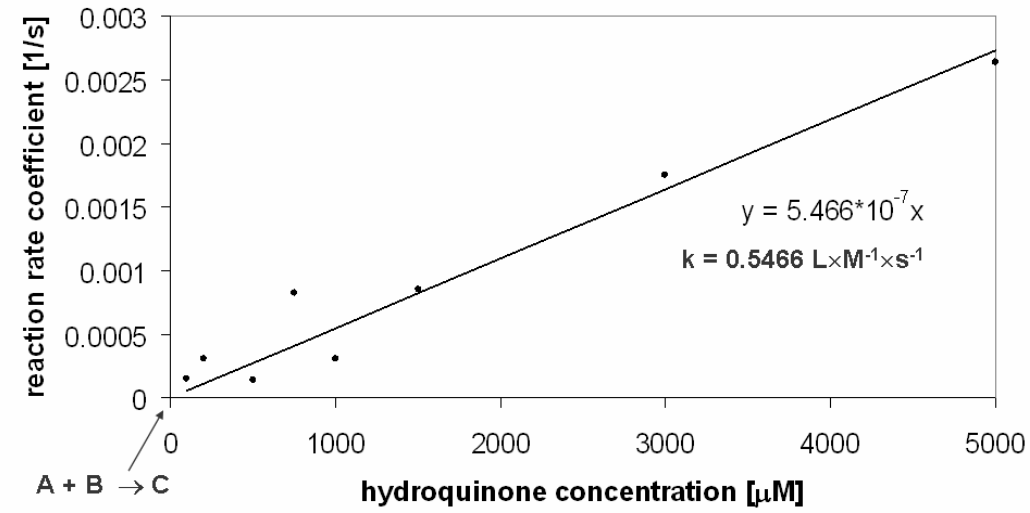


Figure 9. Pseudo first order reaction rate coefficient dependence of metHb reduction on hydroquinone concentration. Conditions: buffer pH 7, metHb 8 μM, hydroquinone at concentrations varying from 100 to 5000 μM

Acknowledgements: the work shown here has been supported by the Romanian Ministry for Education and Research (ID565/2007).

References

1. Giulivi, C. and E. Cadenas, *The reaction of ascorbic acid with different heme iron redox states of myoglobin. Antioxidant and prooxidant aspects.* FEBS Lett., 1993. **332**(3): p. 287-290.
2. Galaris, D. and P. Korantzopoulos, *On the Molecular Mechanism of Metmyoglobin-Catalyzed Reduction of Hydrogen Peroxide by Ascorbate.* Free Radic. Biol. Med., 1997. **22**(4): p. 657-667.
3. Silaghi-Dumitrescu, R., *Heme ferrous-hydroperoxo complexes.* Arch. Biochem. Biophys., 2004. **424**: p. 137-140.
4. Cooper, C.E., et al., *On the formation, nature, stability and biological relevance of the primary reaction intermediates of myoglobins with hydrogen peroxide.* Dalton Trans., 2005: p. 3483-3488.
5. Silaghi-Dumitrescu, R., et al., *Ferryl haem protonation gates peroxidatic reactivity in globins.* Biochem J, 2007. **403**(3): p. 391-5.
6. Cooper, C.E., et al., *Peroxidase activity of hemoglobin towards ascorbate and urate: a synergistic protective strategy against toxicity of Hemoglobin-Based Oxygen Carriers (HBOC).* Biochim Biophys Acta, 2008. **1784**(10): p. 1415-20.

7. Dunne, J., et al., *Ascorbate removes key precursors to oxidative damage by cell-free haemoglobin in vitro and in vivo.* Biochem J, 2006. **399**(3): p. 513-24.
8. A. I. Alayash, R.P.P., R. E. Cashon, *Redox reactions of hemoglobin and myoglobin: biological and toxicological implications. Antioxid Redox Signal.* 2001. **3**(2): p. 313-327.
9. J. Everse, K.E.E., M.B. Grisham (Eds.), *Peroxidases in Chemistry and Biology.* 1991. **2**: p. 1–137.
10. Reeder, B.J., et al., *The radical and redox chemistry of myoglobin and hemoglobin: from in vitro studies to human pathology.* Antioxid. Redox Sign., 2004. **6**(6): p. 954-966.
11. Cooper, C.E., *Radical reactions of haem proteins.* In Critical Care Focus., 2002. **8** - Blood and Blood Transfusion (Galley, H. F., ed.): p. 66-79.
12. Ames, B.N., et al., *Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis.* Proc Natl Acad Sci U S A, 1981. **78**(11): p. 6858-62.
13. Sies, H., *Total antioxidant capacity: appraisal of a concept.* J. Nutr., 2007. **137**: p. 1493-1495.
14. Vollaard, N.B., et al., *A new sensitive assay reveals that hemoglobin is oxidatively modified in vivo.* Free Radic Biol Med, 2005. **39**(9): p. 1216-28.
15. Catalano, C.E., Y.S. Choe, and P.R.O. Demontellano, *Reactions of the Protein Radical in Peroxide-Treated Myoglobin - Formation of a Heme-Protein Cross-Link.* J. Biol. Chem., 1989. **264**(18): p. 10534-10541.
16. Tew, D.a.O.d.M., P. R., *The myoglobin protein radical: coupling of Tyr-103 to Tyr-151 in the H2O2-mediated cross-linking of sperm whale myoglobin.* J. Biol. Chem., 1988. **263**(17880-17886).
17. Reeder, B.J., et al., *Characteristics and mechanism of formation of peroxide-induced heme to protein cross-linking in myoglobin.* Biochemistry, 2002. **41**(1): p. 367-375.
18. Rice-Evans, C., Green, E., Paganga, G., Cooper, C. and Wrigglesworth, J., *Oxidised low density lipoproteins induce iron release from activated myoglobin.* FEBS Lett., 1993. **326**: p. 177-182.
19. M. Ciuffi, L.T., S. Mugnai, S. Franchi-Micheli, L. Zilletti, *Hemoglobin affects lipid peroxidation and prostaglandin E2 formation in rat corticocerebral tissues in vitro.* Biochem. Pharmacol., 1996. **52**: p. 97-103.
20. Newman, E.S.R., C.A. Rice-Evans, and M.J. Davies, *Identification of initiating agents in myoglobin-induced lipid-peroxidation.* Biochem. Biophys. Res. Commun., 1991. **179**(3): p. 1414-1419.
21. Reeder, B.J. and M.T. Wilson, *The Effects of pH on the Mechanism of Hydrogen Peroxide and Lipid Hydroperoxide Consumption by Myoglobin: A Role for the Protonated Ferryl Species.* Free Rad. Biol. Med., 2001. **30**(11): p. 1311-1318.
22. Deac, F., *Activitatea peroxidazica a Mioblobinei si Hemoglobinei - lucrare de licenta.*
23. Florina-Violeta Deac, A.T., Ana Maria Bolfa, Paula Podea, Petronela Petrar, Radu Silaghi-Dumitrescu, *High-Affinity Binding Of Ascorbate To Globins.*

24. Raven, E.L., *Understanding functional diversity and substrate specificity in haem peroxidases: what can we learn from ascorbate peroxidase?* Nat. Prod. Rep, 2003. **20**: p. 367-381.
25. L. Lad, M.M., E.L. Raven, *Substrate binding and catalytic mechanism in ascorbate peroxidase: evidence for two ascorbate binding sites.* Biochemistry, 2002. **41**: p. 13774-13781.
26. Dorman, S.C., Kenny, C. F., Miller, L., Hirsch, R. E. and Harrington, J. P., *Role of redox potential of hemoglobin-based oxygen carriers on methemoglobin reduction by plasma components.* Artif. Cells Blood Substitutes Immobilization Biotechnol., 2002. **30**: p. 39-51.
27. McGown, E.L., et al., *Reduction of Extracellular Methemoglobin by Erythrocytes.* Biochim et Biophys Acta, 1990. **1036**(3): p. 202-206.
28. Mendiratta, S., Qu, Z. C. and May, J. M., *Erythrocyte ascorbate recycling: antioxidant effects in blood.* Free Radical Biol. Med., 1998. **24**: p. 789-797.
29. May, J.M., Z.C. Qu, and C.E. Cobb, *Recycling of the Ascorbate Free Radical by Human Erythrocyte Membranes.* Free Rad. Biol. Med., 2001. **31**(1): p. 117-124.
30. D. Su, J.M.M., M.J. Koury, H. Asard, *Human erythrocyte membranes contain a cytochrome b561 that may be involved in extracellular ascorbate recycling.* J. Biol. Chem., 2006. **281**: p. 39852-39859.
31. http://en.wikipedia.org/wiki/Anthracene.
32. Ann M. Benson, M.J.H., and Paul Talalay, *Increase of NAD(P)H:quinone reductase by dietary antioxidants: Possible role in protection against carcinogenesis and toxicity.* Proc. Nat!. Acad. Sci. USA, 1980. **77/9**: p. 5216-5220.
33. Antonini, E. and M. Brunori, *Hemoglobin and Myoglobin in their Reaction with Ligands*. Frontiers in Biology, ed. A. Neuberger and E.L. Tatum. 1971: North-Holland, Amsterdam.
34. Silaghi-Dumitrescu, R., *A density functional study of heme-peroxynitrite adducts.* J. Mol. Struct. THEOCHEM, 2005. **722**: p. 233-237.
35. Reeder, B.J., et al., *Characteristics and Mechanism of Formation of Peroxide-Induced Heme to Protein Cross-Linking in Myoglobin.* Biochemistry, 2002. **41**: p. 367-375.
36. Dunne, J., et al., *Reactions of cross-linked methaemoglobins with hydrogen peroxide.* Oxygen Transport to Tissue Xxi, 1999. **471**: p. 9-15.
37. Alayash, A.I., *Oxygen therapeutics: can we tame haemoglobin?* Nat Rev Drug Discov, 2004. **3**(2): p. 152-9.

CADMIUM REMOVAL FROM WASTEWATERS USING Ca-ALGINATE IMMOBILIZED BENTONITE AS ADSORBENT

Cornelia MAJDIK *, Andrada MĂICĂNEANU, Cerasella INDOLEAN, Silvia BURCA, Maria STANCA

Department of Chemical Technology, "Babeş-Bolyai" University, Faculty of Chemistry and Chemical Engineering, 11 Arany Janos st., RO-400028 Cluj-Napoca, Romania.

** e-mail: majdik@chem.ubbcluj.ro*

ABSTRACT

A commercial bentonite sample from Fort Benton (distributed by Interker-Wein kft., Hungary) was used to remove cadmium ions from synthetic wastewaters. Cadmium has no essential biological function and is extremely toxic to humans. In chronic exposure, it also accumulates in the body, particularly in the kidneys and the liver. These properties, along with its common usage make cadmium one of the commonest environmental metal poisonings. The bentonite sample was used as powder, (d < 0.2 mm), without any chemical treatment. We studied the influence of the bentonite quantity, temperature and pH over the process efficiency. An increase of the bentonite quantity immobilized in the Ca-alginate beads led to an increase of the removal process efficiency, while an increase of the temperature led to a slight decrease of the process efficiency. When the removal process was realised in acidic or basic environments the process efficiency also decreased. The bentonite sample we used proved to be efficient for the removal of cadmium from synthetic wastewaters; removal efficiencies up to 100% were reached.

Key words: cadmium, health effects, adsorption, bentonite, Ca-alginate beads

INTRODUCTION

Cadmium, a naturally occurring element found in the Earth's crust, was discovered in 1817, but was not used commercially until the end of the 19th century. This soft, silver-white metal was first used in paint pigments and as a substitute for tin in World War I (www.osha.gov). Cadmium is not usually present in the environment as a pure metal, but as a mineral combined with other elements such as oxygen (cadmium oxide), chlorine (cadmium chloride), or sulfur (cadmium sulfate, cadmium sulfide). Cadmium is most often present in nature as complex oxides, sulfides, and carbonates in zinc, lead, and copper ores. It is rarely present in large quantities as the chlorides and sulfates. These different forms of cadmium compounds are solids that dissolve in water to varying degrees. The chlorides and sulfates are the forms that most easily dissolve in water. One example of mineral containing cadmium is *cadmium smithsonite*, which is a yellow or yellow-green *smithsonite* colored by cadmium impurities.

Cadmium may change forms, but the cadmium metal itself does not disappear from the environment. Knowing the particular form of cadmium, however, is very

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 111 - 115, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

important when determining the risk of potential adverse health effects (www.epa.usa).

Today, about three-fourths of cadmium is used as an electrode component in alkaline batteries, in pigments, metal coatings for example protective coatings on steel and platings, and as a stabilizer for plastics. Cadmium is also a constituent of alloys. The cadmium may occur naturally or as a contaminant including sewage sludge, fertilizers, polluted groundwater and mining effluents.

Workers in many industries face potential exposure to cadmium. The potential for exposure is highest among workers in electroplating, metal machining, plastics, ceramics, paint, and welding operations. Occupational exposure may occur from the manufacture of these products and from welding, and smelting of lead, zinc and copper as these occur in mixed ores with cadmium. The main exposure routes are through inhalation of dust and fumes and the incidental ingestion of dust from contaminated hands, food or cigarettes. Workers may also be exposed to cadmium from the smelting and refining of metals or from air in industrial plants that manufacture batteries, coatings, or plastics (Hu, 1998; Wiliams et al., 1999).

Another source is from ingestion of grown foodstuffs, especially grain and leafy vegetables, which readily absorb cadmium from the soil (Hu, 1998; Wiliams et al., 1999).

Cadmium is also found in cigarette fumes (0.007 to 0.35 µg per cigarette) and fumes from vehicles. Residential sites may be contaminated by municipal waste or leaks from hazardous waste sites (Wiliams et al., 1999; Baldwin et al., 1999; Timbrell, 1995). Humans have a daily intake of cadmium from ingestion and inhalation which is around 20 to 40 µg per day, but only 5 to 10% of this is absorbed (Hu, 1998).

After absorption, cadmium is transported in the blood bound to albumin. It is taken up by the liver, and due to its similarity to zinc, causes this organ to induce the synthesis of the protein metallotheionin (MT) to which it binds. The cadmium-metallotheionin complex is transported to the kidneys, and it is filtered at the glomerulus, but is reabsorbed at the proximal tubule (Hu, 1998; Baldwin et al., 1999). Within the renal tubular cells, the cadmium-MT complex becomes degraded by digestive enzymes, which releases the cadmium. Renal tubular cells deal with the release of this toxic substance by synthesizing MT to neutralize it, but eventually the kidneys loose their synthetic capacity for MT. At this point, the cadmium has accumulated to a high level in the renal tubular cells, and irreversible cell damage occurs (Baldwin et al., 1999; Timbrell, 1995). Threfore it can be concluded that the renal cells do not have an effective elimination pathway for the cadmium complex, which means that the half life in the kidney is between 15 and 30 years (Hu, 1998; Baldwin et al., 1999). The toxic effects of cadmium are due to its inhibition of various enzyme systems. Like similar heavy metals, it is able to inactivate enzymes containing sulphydryl groups and it can also produce uncoupling of oxidative phosphorylation in mitochondria (Goldwater et al., 1972). Cadmium may also compete with other metals such as zinc and selenium for inclusion into metallo-enzymes and it may compete with calcium for binding sites on regulatory proteins such as calmodulin (Baldwin et al., 1999). Cadmium may also contaminate fish (Wiliams et al., 1999).

While metals cannot be broken down into non-toxic components like organic compounds, remediation can be used to stabilize, extract, or reduce the toxicity of soil, surface water and groundwater contaminated by acid mine drainages and other heavy metal containing wastewaters. Remediation strategies are often more

beneficial than traditional "pump and treat" strategies because it can be implemented *in situ*, providing a simpler, less intrusive, and cheaper method of remediation.

The purpose of this study was to evaluate the performances of a commercial bentonite immobilized in Ca-alginate beads in the cadmium removal process from a synthetic wastewater, as an assay to remediate the damages produced by different industries.

MATERIALS AND METHODS

We used a commercial bentonite sample from Fort Benton distributed by Interker-Wein kft., Hungary. The bentonite sample was used as powder, (d < 0.2 mm), without any chemical treatment. All chemicals used in this study were analytical reagent grade ($Cd(NO_3)_2 \times 4H_2O$, alginic acid sodium salt and $CaCl_2$).

In order to obtain the bentonite immobilized in alginate beads we used the cross-linking procedure with calcium alginate, which is an adapted version of the method for treatment of fungi biomass outlined by Schiewer et al. (1995) and Zhao and Duncan (1997).

For bentonite immobilization, various quantities of bentonite, 2 to 10 g were suspended in 50 ml distilled water. This suspension was next blended with a mixture formed from 1 g Na-alginate and 2 ml ethanol. The mixture was then dropped with a peristaltic pump into a 0.2 M $CaCl_2$ solution. During this process, alginate-bentonite mixture drops were gelled into beads with a diameter of 4.0±0.2 mm. The Ca-alginate immobilized bentonite beads were stored in 0.2 M $CaCl_2$ solution at 4°C for 1 hour to cure and to form the cross-linking bonds. The beads were rinsed with distilled water for remove excess of calcium ions and stored at 4°C prior to use.

For the heavy metal ion removal study we used synthetic monocomponent solutions containing cadmium ions, 48 mg Cd^{2+}/L. The concentration of cadmium ions in solution was determined using a flame atomic absorption spectrophotometer (SensAA Dual GBS Scientific Equipment, Australia).

The heavy metal ions removal process was realised in a batch reactor under magnetic stirring (825 rpm), using 100 ml of cadmium solution in which Ca-alginate bentonite beads obtained from the desired quantity of bentonite (2 to 10 grams) were suspended.

In order to determine the exact concentration of cadmium ions and establish the evolution of the removal process, samples of 100 μL (dilution in each case was 50) from the supernatant were collected at different time intervals, every 5 minutes for the first 20 minutes and next every 10 minutes until equilibrium was reached.

We studied the influence of the bentonite quantity (2, 4, 6, 8 and 10 g), temperature (20, 40 and 60°C) and pH (2.2, 5.4 – cadmium initial solution, 9.4) over the process efficiency.

Removal efficiencies (%) were calculated, eq. 1, in order to establish the effectiveness of the considered bentonite sample in the heavy metal ion removal process (the calculated values of removal efficiencies and adsorption capacities should be regarded according to the precision of the determination methods we used).

$$\mathbf{E} = \frac{\mathbf{C_i} - \mathbf{C_t}}{\mathbf{C_i}} \cdot \mathbf{100} \qquad (1)$$

where,

C_i is cadmium initial concentration, in mg Cd^{2+}/L

C_t is cadmium concentration at moment t, in mg Cd^{2+}/L.

RESULTS AND DISCUSSION

The results obtained in case of cadmium removal from monocomponent model solutions on Ca-alginate immobilized bentonite are presented and discussed in terms of removal efficiency.

In Fig. 1a, evolution of cadmium removal efficiency in time is presented for the experiments conducted with Ca-alginate beads containing different quantities of bentonite (2 to 10 g).

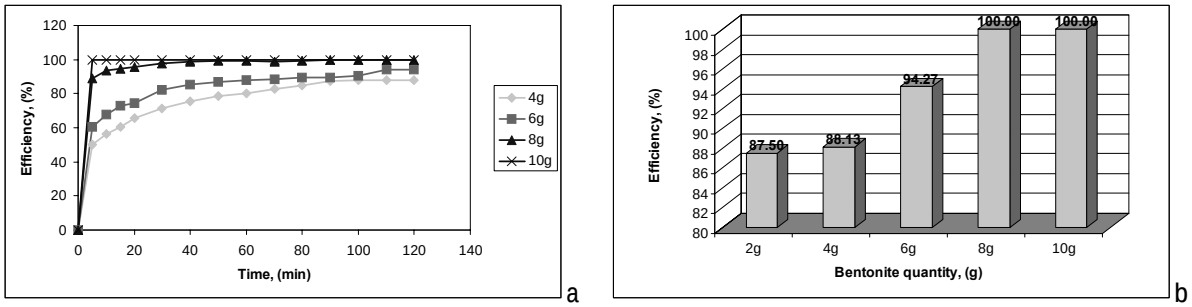


Fig. 1. Influence of the bentonite quantity over the cadmium removal efficiency evolution in time (a) and maximum removal efficiencies (b) in cadmium removal process on immobilized bentonite sample, C_i = 48 mg Cd^{2+}/L, T = 20°C, pH = 5.4.

A closer inspection of the curves led to the conclusion that in the first 5 minutes removal efficiency increase up to around 50 and 60% for 4 and 6 g respectively, while with a further increase of the bentonite quantity the removal efficiency reached 100% (10 g). Equilibrium was reached around 90 minutes for bentonite quantities between 2 and 8 g, while in case of 10 g, the whole quantity of cadmium from solution was removed in the first 5 minutes. Maximum removal efficiencies vary from 87.50 to 100% (Fig. 1b). We can draw the conclusion than an increase of the bentonite quantity over 8 g is not justified for a heavy metal concentration in wastewater around 50 mg Cd^{2+}/L.

Influence of the temperature and pH over the cadmium removal efficiency is presented in Fig. 2. It can be concluded that an increase of the temperature led to a slight decrease of the process efficiency, fact that can be attributed to the beginning of the desorption process that is favored by higher temperatures. When the removal process was realized in acidic (pH = 2.2) or basic (pH = 9.4) environments the process efficiency also decreased.

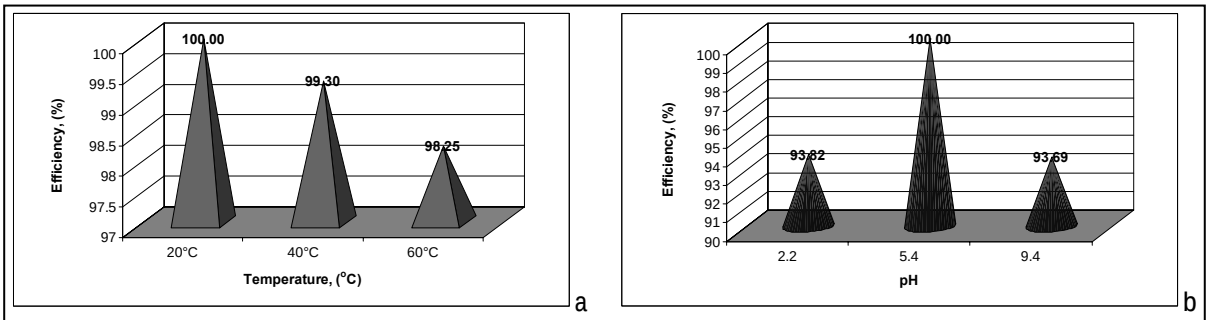


Fig. 2. Influence of the temperature (a) and pH (b) over the cadmium maximum removal efficiency on immobilized bentonite sample (10 g), C_i = 48 mg Cd^{2+}/L, pH = 5.4 (fig. a), T = 20°C (fig. b).

REFERENCES

1. www.osha.gov - Cadmium - Occupational Safety Health Administration (OSHA).
2. www.epa.usa - Environmental Protection Agency (EPA), Public Health Statement.
3. Hu H. - Heavy metal poisoning, in Fauci A.S., Braunwald E., Isselbacher K.J., Wilson J.D, Martin J.B, Kasper D.L., Hauser S.L., Longo D.L. (eds)., Harrison's Principles of Internal medicine, 14th edition, New York, McGraw-Hill, 1998, 2564-2569.
4. Williams F., Robertson R., Roworth M. - Detailed profile of 25 major organic and inorganic substances, 1st edition, Scottish Centre for Infection and Environmental Health, Glasgow (SCIEH), 1999.
5. Baldwin D.R., Marshall W.J. - Heavy metal poisoning and its laboratory investigation, Annal. Clinical Biochem., 1999, 36, 267-300.
6. Timbrell J.A. - Introduction to toxicology, 2nd edition, London, Taylor and Francis, 1995, 76-77.
7. Goldwater L.J., Clarkson T.W. - Metallic contaminants and human health, 1st edition, New York, Academic Press, 1972, 97-124.
8. Schiewer S., Fourest E, Chong K.H, Volesky B. – Ion change biosorption by dried saeweed: experiments and model predictions, Biohydrometallurgical Processing, 1995, 2, 219-228.
9. Zhao M., Duncan J.R. - Use of formaldehyde cross-linced *Saccharomyces cerevisiae* in column bioreactors for removal of metals from aqueous solutions, Biotech. Letters, 1997, 19, 953-955.

IN VITRO CHEMOSENSITIVITY TESTING OF TUMOR CELLS TREATED WITH PLATIN-BASED DRUGS

Eva FISCHER-FODOR*, Rares BUIGA, Doris PELAU, Cosmin LISENCU, Piroska VIRAG, Corina TATOMIR, Olga SORITAU

Ion Chiricuta Cancer Institute - Comprehensive Cancer Center, 34-36 Republicii St., Cluj Napoca 400015, Romania
**fischer.eva@iocn.ro*

ABSTRACT

In the present paper we examined the in vitro chemosensitivity of tumoral cells treated with metal-based antineoplastic drugs in order to optimize the cancer patient's chemotherapy. Biopsies were prevealed from ovary cancer patients and primary cell cultures were processed. We tested on these tumoral cultures the antiproliferative effect of carboplatin, a standard anticancer drug; measurements were made with the CellScan scanning static cytometer, which allow repetitive spectroscopic measurements in living cells.

The detection of cell viability and apoptosis is possible based on the changes which occur in the cytoplasm matrix of tumoral cells treated by cytotoxic compounds. The measurement of fluorescence modifications as result of this phenomenon is possible using the CellScan system. The potential of this technology to detect the in vitro effects of the inhibitory molecules on tumor cells was demonstrated, making this method a valuable tool for chemosensitivity tests. Using polarization and intensity measurements we can develop a prediction marker of in vivo drug effect, and this will conduct to the improvement and personalization of the therapy.

Key words: carboplatin, static cytometry, chemosensitivity, ovarian carcinoma

INTRODUCTION

The platinum drugs represent a distinctive and significant class of antitumor compounds. Alone or in combination with other chemotherapeutic agents they are essential in the treatment of a variety of solid tumors. Carboplatin (CBDCA) or cis-diamminecyclobutanedicarboxylato platinum (II) is an efficient, stable and pharmacokinetically predictable antineoplastic drug with milder hematologic toxicity and it is a standard treatment for patients with diagnosed epithelial ovarian carcinoma (Pujade-Lauraine E. et al, 2000). This drug's cytotoxicity is based on platinum-DNA adducts formation, similarly to other platinum derivates. Carboplatin diffuses rapidly into tissues after infusion, it is noticeably stable in plasma and it is excreted predominantly by the kidneys. Carboplatin is reconstituted in chloride-free solutions and

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 116 - 121, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

administered over 30 minutes as a rapid intravenous infusion with dosages up to 20 mg/min/ml. Dose adaptation according to renal function decreases drug induced thrombopenia and has allowed carboplatin to be used widely and safely. Still side effects like nausea, vomiting, nephrotoxicity, and neurotoxicity occur as consequence of the therapy; therefore it is very important to anticipate the efficiency of the drug for each patient (DeVita V. T. et al, 2007).

The incidence of ovarian cancer is regretfully increasing in our country, and most of the patients are diagnosed in advanced clinically stages. In this point, the therapeutic intervention with the right drug is crucial to obtain a good survival rate and the reminiscence of the disease. A big obstacle in cancer treatment, including ovary cancer, is the heterogeneity of tumor response to chemotherapy. The major limitation to the successful treatment of solid tumors with platinum-based chemotherapy is the emergence of drug-resistant tumor cells.

An in vitro chemosensitivity assay refers to any laboratory analysis that is achieved specifically to evaluate if tumor growth is inhibited by various compounds. The assay guided therapy is in the clear benefit of the patient, but in present this is not widely used in clinical practice because of various technical problems encountered with this kind of assays, including the requirement of a high technical skill level, the large number of required tumor cells, and the long duration. In order to improve the treatment efficacy, we developed a new test to elucidate the individual response to platin-based drugs.

The method is based on static cytometry which was adapted for this purpose (Chaitchik S. et al, 2005). The CellScan system is a laser scanning multiparameter apparatus, equipped with a cell carrier, a special matrix with 10.000 microscopic tronconic apertures and each can incorporate and fix one single living cell (Deutsch M. et al, 1996). This system permits repetitive spectroscopic measurements of individual cells, fluorescence intensity (FI) and fluorescence polarization (FP) of cells can be measured through it (Fixler D. et al, 1997). First utilization of CellScan to assess the antineoplastic drug-induced changes (Schiffenbauer Y. S. et al, 2002) in FI and FP was performed on cell lines, and we adapted the technique for primary tumoral cell cultures prevealed directly from the patient.

MATERIAL AND METHODS

The test was completed on tumor tissue specimens obtained from 18 women with histopathological confirmed ovary malignancies. Tissues were collect following the informed consent of the patients. We performed primary cell cultures from biopsies. Tumor specimens were placed into sterile tubes containing a basic growth medium: Dulbecco's modified Eagle medium (DMEM- Sigma Aldrich) with 10% fetal calf serum and penicillin-streptomycin solution (Sigma Aldrich). Tissues were disaggregated mechanically with a DAKO`s Medimachine, using Filcons and Medicons devices, to obtain single cell suspensions. Cells were centrifuged, washed and plated in 6-well culture plates to let them to adhere. After 24 hours they were adhered to plate surface and drug were added. We used carboplatin (Sindan Pharma) at a final concentration of 1µg/ml.

After 20-24 hour of drug exposure cells were harvested, centrifuged, washed and resuspended in DMEM medium without pH indicator. Cells were stained for 5 minutes with FDA at a final concentration of 2,4 μM, loaded onto cell carrier and washed twice with phosphate buffered saline solution (PBS) supplemented with Mg^{2}+ and Ca^{2+}. Samples were immediately measured and analyzed with the cytometer. Rh123 (Sigma Aldrich) was added to cell suspension to a final concentration of 1 μg/ml and incubated for 30 minutes at 37ºC. The cells were washed twice with cold PBS, loaded on cell carrier and measurements were performed.

RESULTS AND DISCUSSION

We evaluated the FP and FI values for each tumor cell culture using the CellScan system software which include biostatistics calculation. For every case, both FDA and RH123 dying was used. Preclinical evidence suggests that FDA positive test in combination with RH123 positive results predict a clear sensitivity to drugs.

By static cytometry measurements we obtained a relatively narrow range of polarization values, but a large variety of intensity values, because intensity reflects the cell size. The morphology of the cells is heterogeneous inside the same population, and it presents very important differences between two tumors, even if they are in the same histological class.

In treated primary tumoral cultures we detect in 10 cases a platin-induced hyperpolarization of FDA stained cells versus untreated reference cells (Table 1). The ovary primary tumoral populationsT3, T5, T6, T7, T9, T12 and metastatic T15, T16, T17 and T18 cells displayed an increase of polarization values; this denote a cytotoxic effect of carboplatin. Except two cultures (T7 and T10) this tendency is underlined with a decrease of fluorescence intensity values of FDA stained cells. In T16 metastatic population polarization value is equal with those of reference cells, and more, FI value decrease against control, so we can conclude that carboplatin chemosensitization of tumoral cells occur in this case too. FDA fluorescence intensity decrease also for T1, T2, T13 and T14, without being doubled by polarization increase.

Rhodamine marked cells shows increase of polarization in 13 cases: T1, T2, T3, T4, T7, T8, T9, T10, T11, T12, T13, T17 and T18, which indicates the induction of early apoptotic processes in treated cells (Table 2). Moreover, among these cell cultures we found 10 which simultaneously display FI value reduction too.

For tumors T3, T5, T6, T9, T12, T16, T17 and T18 it can be predicted a significant inhibitory effect against tumoral cells. It is an important aspect that among these tumors 4 are metastasis, usually more aggressive cells, and the carboplatin proved his effectiveness in these cases too. In primary T3, T9, and T12, T17 and T18 metastatic tumoral cells it is accomplished an assembly of conditions which emerge to confirmation of antiproliferative effect and cytotoxicity of carboplatin, and we could anticipate that in these cases *in vivo* treatment of patient will reach a maximum efficiency.

.

Table 1. Fluorescence and polarization values of tumoral cells treated with carboplatin. Assessment made using FDA stain.

Tumoral primary cell culture	**FDA**			
Measurements [a.u]	**FP_{contr}**	**FP_{Pt}**	**FI_{contr}**	**FI_{Pt}**
T 1 ovary	0,379	0,374	33743	32407↓
T 2 ovary	0,250	0,247	99389	44233↓
T 3 ovary	**0,264**	**0,275**	72654	67860↓
T 4 ovary	0,358	0,352	26283	39968
T 5 ovary	**0,256**	**0,260**	42147	31870↓
T 6 ovary	**0,316**	**0,341**	45855	28547↓
T 7 ovary	**0,423**	**0,434**	20176	23714
T 8 ovary	0,236	0,225	124771	166144
T 9 ovary	**0,386**	**0,400**	28485	17610↓
T 10 ovary	**0,365**	**0,378**	35039	39001
T 11 ovary	0,334	0,329	25635	32637
T 12 ovary	**0,442**	**0,449**	8892	8349↓
T 13 omental metastasis	0,416	0,407	36087	31620↓
T 14 omental metastasis	0,276	0,241	26582	23369↓
T 15 omental metastasis	0,398	0,377	19093	33478
T 16 abdominal metastasis	**0,234**	**0,234**	257858	200827↓
T 17 abdominal metastasis	**0,272**	**0,345**	92282	23454↓
T 18 omental metastasis	**0,375**	**0,439**	33800	16646↓

Several other chemosensitivity tests are developed (Kommoss F. et al, 2005), such as clonogenic assays on primary cell cultures derived from biopsies (Engblom P. et al, 1999), collagen gel droplet embedded culture drug test (Higashiyama M. et al, 2008) or adenosine triphosphate-based chemotherapy response assay (Moon Y. W. et al, 2007). This one, experimented and implemented by us is much simpler, rapid, need fewer cells and require lower costs, and can be applied easily in a clinical laboratory

Table 2. Fluorescence and polarization values of tumoral cells treated with carboplatin, evaluated through rhodamine staining.

Tumoral primary cell culture	**Rh123**			
Measurements [a.u]	**FP_{contr}**	**FP_{Pt}**	**FI_{contr}**	**FI_{Pt}**
T 1 ovary	**0,385**	**0,407**	73257	55278↓
T 2 ovary	**0,359**	**0,387**	254626	243803↓
T 3 ovary	**0,402**	**0,404**	278370	199288↓
T 4 ovary	**0,358**	**0,367**	199958	190434↓
T 5 ovary	0,401	0,388	263144	267802
T 6 ovary	0,404	0,387	127992	79601↓
T 7 ovary	**0,371**	**0,420**	95552	45543↓
T 8 ovary	**0,373**	**0,381**	148206	297163
T 9 ovary	**0,420**	**0,443**	30886	24571↓
T 10 ovary	**0,427**	**0,430**	72104	52797↓
T 11 ovary	**0,299**	**0,354**	68556	81282
T 12 ovary	**0,423**	**0,425**	7305	3149↓
T 13 omental metastasis	**0,398**	**0,412**	82534	79508↓
T 14 omental metastasis	0,421	0,397	458294	380460↓
T 15 omental metastasis	0,407	0,391	47356	47413
T 16 abdominal metastasis	0,388	0,374	270388	287089↓
T 17 abdominal metastasis	**0,380**	**0,403**	24238	53857
T 18 omental metastasis	**0,378**	**0,427**	45544	41378↓

CONCLUSIONS

The static cytometric assay may be helpful in the prediction of the response to chemotherapy especially in ovarian cancer, localization with high risk of relapse after therapy. The *in vitro* chemosensitivity tests should be promoted to select a better chemotherapy response of the ovarian cancer patients while avoiding the outward side effects of ineffective drugs. Our results sustain effectively the clinical application of the CellScan test.

REFERENCES

1. Pujade-Lauraine E., Guastalla J.P., Vincent P. Carboplatin in cancer of the ovary, Bull. Cancer, 2000, 87, 40-7.
2. DeVita V. T., Hellman S., Rosenberg S. A.- Cancer: Principles & Practice of Oncology, Chapter 15: Pharmacology of Cancer Chemotherapy
   7th Edition, Lippincott, Williams & Wilkins, 2005, 341-369.
3. Chaitchik S., Soritau O., Fischer E., Pelau D., Lazar L., Schiffenbauer Y.S.- CellScan technology for in vitro prediction of tumor response to chemotherapeutic agents, J. Clin. Oncol., 2005, 23, 2125.
4. Deutsch M., Ron I., Weinreb A., Tirosh R., Chaitchik S.- Lymphocyte fluorescence polarization measurements with the CellScan system: application to the SCM cancer test, Cytometry, 1996, 23, 159-165.
5. Fixler D., Tirosh R., Eisenthal A., Marder O., Irlin Y., Lalchuk S., Deutsch M.- Monitoring of effector and target cell stimulation during conjugation by fluorescence polarization, Biol. Cell, 1997, 89, 443-452.
6. Schiffenbauer Y. S., Trubnyikov E., Zacharia B., Gerbat S., Rehavi Z., Berke G., Chaitchik S.- Tumor sensitivity to anti-cancer drugs predicted by changes in fluorescence intensity and polarization in vitro, Anticancer Res., 2002, 22, 2663-2670.
7. Kommoss F., Kommoss S., Schmidt D., Trunk M. J., Pfisterer J., du Bois A.- Survival benefit for patients with advanced-stage transitional cell carcinomas vs. other subtypes of ovarian carcinoma after chemotherapy with platinum and paclitaxel, Gynecol Oncol., 2005, 97, 195-9.
8. Engblom P., Rantanen V., Kulmala J., Grènman S.- Carboplatin-paclitaxel- and carboplatin-docetaxel-induced cytotoxic effect in epithelial ovarian carcinoma in vitro, Cancer, 1999, 86, 2066-73.
9. Higashiyama M., Oda K., Okami J., Maeda J., Kodama K., Takami K., Morinaga K., Takano T., Kobayashi H.- In vitro-chemosensitivity test using the collagen gel droplet embedded culture drug test (CD-DST) for malignant pleural mesothelioma: possibility of clinical application, Ann. Thorac. Cardiovasc. Surg. 2008, 14, 355-62.
10. Moon Y. W., Choi S. H., Kim Y. T., Sohn J. H., Chang J., Kim S. K., Park M. S., Chung K. Y., Lee H. J., Kim J. H- Adenosine triphosphate-based chemotherapy response assay (ATP-CRA)-guided platinum-based 2-drug chemotherapy for unresectable nonsmall-cell lung cancer, Cancer, 2007, 109,1829-35.

BLOOD SUBSTITUTES: CAN WE DO WITHOUT HEMOGLOBIN?

Augustin MOT, Alina ROMAN, Radu SILAGHI-DUMITRESCU
Department of Chemistry and Chemical Engineering, University of Babes-Bolyai, Cluj-Napoca
** E-mail: rsilaghi@chem.ubbcluj.ro*

ABSTRACT

In the present paper we propose hemerythrin as an alternative basis to hemoglobin for blood substitutes, underlying its low reactivity concerning the oxidative and nitrosative stress aspects.

Key words: blood substitutes, hemoglobin, hemerythrin

Artificial oxygen carriers or blood substitutes are used to carry oxygen and increase the fluid volume in the human cardiovascular system in case of emergencies. There are two large approaches within the research field of blood substitutes, a biomimetic and an abiotic one. The first one deals with the development of effective oxygen carriers by mimicking the natural way of oxygen delivery in tissues (chemical modified hemoglobin) while the last approach refers to synthetic compounds which are able to deliver oxygen to the tissues (PFCs and synthetic Fe^{2+}porphyrin-based materials).[1-9]

Despite of some advantageous characteristic of synthetic PFCs such as chemical inertness, capability of dissolving large amounts of oxygen, non immunogenic response, they present a linear oxygen dissociation curve in contrast with the sigmoid dissociation curve of hemoglobin thus implying release of some important amount of oxygen prior to the arrival to the vital tissues. [8,9]

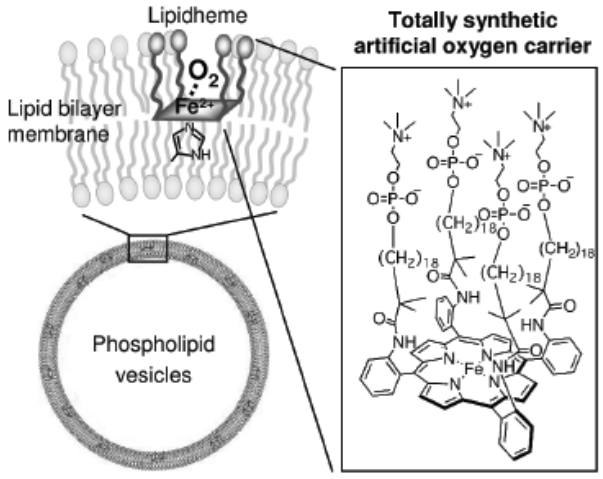


Figure 1 *Phospholipidheme vesicles as totally synthetic artificial oxygen carriers (Tsuchida et al.)*

The second type of totally synthetic oxygen carriers are synthetic Fe^{2+}porphyrin-based materials, they were build in order to avoid the dimerization of heme and reduce the autooxidation by forming a hydrophobic environment in water,

„Metal Elements in Environment, Medicine and Biology", Tome VIIII, pp. 122-125. Publishing House "Eurobit" Timişoara, 2008
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2008, December 5-6, Timişoara, Roumania

this being possible by synthesizing an amphiphilic Fe^{2+} porphyrin embedded into a bilayer of a phospholipid vesicle (Figure 1).

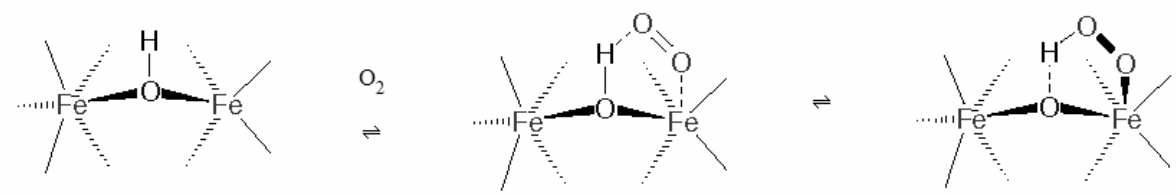
The cell-free hemoglobin (Hb) undergoes slow autooxidation forming ferrihemoglobin and superoxide. The formed superoxide can further react with another oxyhemoglobin molecule forming hydrogen peroxide which can lead to ferrylhemoglobin, hydroxyl and peroxynitrite radical which are highly reactive. The redox reactivity of hemoglobin is described by the following equations:

$$HbFe^{2+}O_2 \rightarrow HbFe^{3+} + O_2^{\bullet -}$$
$$O_2^{\bullet -} + HbFe^{2+}O_2 + 2H^+ \rightarrow HbFe^{3+} + H_2O_2 + O_2$$
$$H_2O_2 + HbFe^{2+}O_2 \rightarrow HbFe^{3+} + OH^{\bullet} + OH^- + O_2$$
$$OH^{\bullet} + HbFe^{2+}O_2 \rightarrow HbFe^{3+} + OH^- + O_2$$
$$HbFe^{3+} + H_2O_2 \rightarrow HbFe^{4+}{=}O + H_2O$$
$$HbFe^{2+}O_2 + NO \rightarrow HbFe^{3+} + NO^{3-}$$
$$O_2^{\bullet -} + NO \rightarrow ONOO^-$$

The highly reactive species are proved to be involved in numerous pathologies (1-7) and the ferrihemoglobin is no longer able to bind oxygen, these are severe problems which still need scientific effort to be reduced if not totally avoid (8, 9). The above reactions also facilitate the release of the heme from the protein matrix undergoing proton-driven oxidation or μ-oxo dimer formation (10). Furthermore, the the ferric-superoxide character explains the high reactivity of oxyhemoglobin towards nitric oxide, the reaction being diffusion controlled and leads methemoglobin and nitrate (11, 12).

Hemocyanin binds oxygen by an active site formed by two copper atoms, but it interacts with nitric oxide being single or double oxidized at the active site (13) and gives a high immunogenic response in human circulatory system, thus, does not appear a proper alternative to hemoglobin for blood substitutes.

Hemerythrin (Hr) is another oxygen carrier which binds oxygen by the use of a non-heme diiron center. The putative hemerythrin based blood substitutes may have numerous advantages over hemoglobin and putative hemocyanine based blood substitutes. Thus, hemerythrins such as that from *Phascolopsis gouldii* are ~108 kDa octamers, which is much larger with respect to 64 kDa hemoglobin tetramer, and which should decrease the level of extravasation and excretion. DeoxyHr reversibly binds oxygen, the diferrous site being two-electron oxidized while the oxygen is two-electron reduced, and binding at the pentacoordinate iron atom as a hydroperoxide ligand (Scheme 1) (14). This reversible process does not imply any highly reactive species such as superoxide in the case of hemoglobin.



$[Fe^{II}\text{-}OH\text{-}Fe^{II}]$ $[Fe^{II}\text{-}OH\text{-}Fe^{III}]O_2$ $[Fe^{III}\text{-}O\text{-}Fe^{III}]OOH$

Scheme 1

In contrast with haemoglobin, hemerythrin is much less problematic when it comes to discuss its reactivity towards oxidants such as nitric oxide, hydrogen peroxide and nitrite (15). There is no direct evidence for interaction between nitric

oxide with the oxyHr while the oxyHb present a nearly diffusion-controlled reaction with NO (16). While metHb form the highly reactive ferryl form when it interacts with hydrogen peroxide, the metHr does not form any radical or high valent species when it comes in contact with hydrogen peroxide. Unlike deoxyHb which has high affinity for NO, deoxyHr has several magnitudes order lower affinity for NO under anaerobic conditions fact which implies low probability for deoxyHr to scavenge NO under physiological conditions. In addition, in contract with deoxyHb which present a high affinity for carbon monoxide, deoxyHr does not present a reaction with carbon monoxide.

In order to avoid the immunogenic response of pure Hr, a proper methodology could be to line the surface of native protein with PEG units using an amino reactive reagent. As illustrated by SDS-PAGE in Figure 2, this approach is possible since Hr possesses eleven lysine residues on the surface of each subunit of the *P. gouldii* Hr octamer.

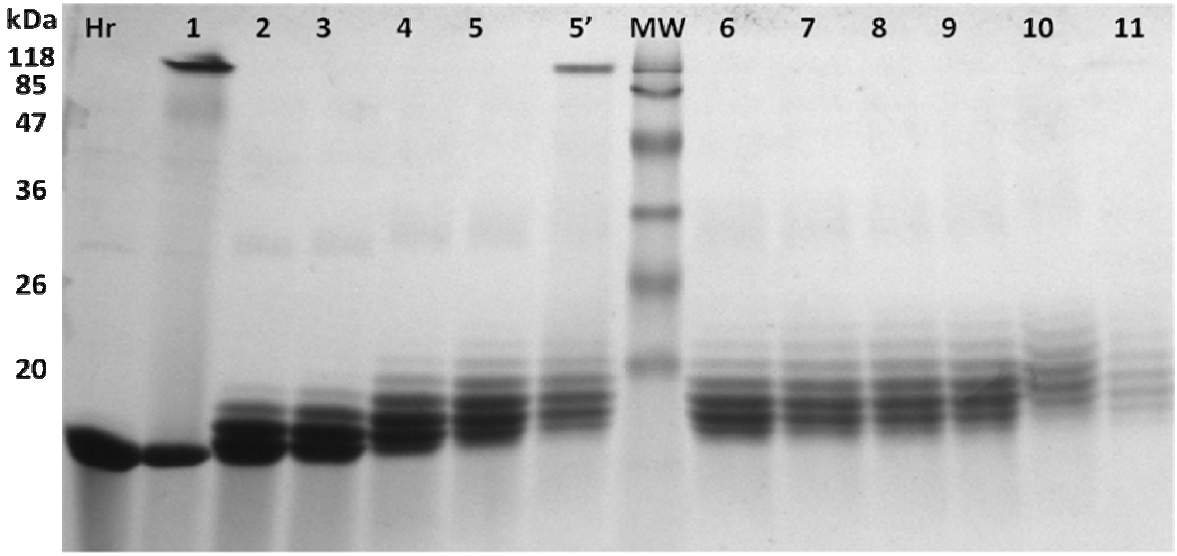


Figure 2 *SDS-PAGE gel of a PEGylated Hr at several reaction mole ratios, ranging from 0.2 – 200.*

Crosslinking of native Hr with bifunctional reagents as glutaraldehyde or o-ATP may be necessary retaining its stability in case of further chemical modification. The autooxidation and the oxygen affinity can be controlled by the degree of chemical modification (for instance the number of PEG units on the surface of each protein subunit) or the degree of polymerization.

Acknowledgements: the work shown here has been supported by the Romanian Ministry for Education and Research (ID565/2007).

REFERENCES

1. Reeder, B. J.;Grey, M.;Silaghi-Dumitrescu, R. L.;Svistunenko, D. A.;Bulow, L.;Cooper, C. E.,Wilson, M. T. Tyrosine residues as redox cofactors in human hemoglobin: implications for engineering nontoxic blood substitutes. *J Biol Chem*, 2008, *283*, 30780-7

2. Reeder, B. J.;Svistunenko, D. A.;Sharpe, M. A.,Wilson, M. T. Characteristics and Mechanism of Formation of Peroxide-Induced Heme to Protein Cross-Linking in Myoglobin. *Biochemistry*, 2002, *41*, 367-375
3. Reeder, B. J.;Svistunenko, D. A.;Cooper, C. E.,Wilson, M. T. The radical and redox chemistry of myoglobin and hemoglobin: from *in vitro* studies to human pathology. *Antioxidant & Redox Signaling*, 2004, *6*, 954-966
4. Reeder, B. J.;Sharpe, M. A.;Kay, A. D.;Kerr, M.;Moore, K.,Wilson, M. T. Toxicity of myoglobin and haemoglobin: oxidative stress in patients with rhabdomyolysis and subarachnoid haemorrhage. *Biochem. Soc. Trans.*, 2002, *30*, 745-748
5. Vollaard, N. B.;Reeder, B. J.;Shearman, J. P.;Menu, P.;Wilson, M. T.,Cooper, C. E. A new sensitive assay reveals that hemoglobin is oxidatively modified in vivo. *Free Radic Biol Med*, 2005, *39*, 1216-28
6. Vollaard, N. B.;Shearman, J. P.,Cooper, C. E. Exercise-induced oxidative stress:myths, realities and physiological relevance. *Sports Med*, 2005, *35*, 1045-62
7. Cooper, C. E.;Silaghi-Dumitrescu, R., Rukengwa, M., Alayash, A. I.,Buehler, P. W. Peroxidase activity of hemoglobin towards ascorbate and urate: a synergistic protective strategy against toxicity of Hemoglobin-Based Oxygen Carriers (HBOC). *Biochim Biophys Acta*, 2008, *1784*, 1415-20
8. Tsuchida E., Sou K., Nakagawa A., Sakai H., Komatsu T., Kobayashi K. - Artificial oxygen carriers, hemoglobin vesicles and albumin-hemes, based on bioconjugate chemistry, Bioconjugate Chem, 2009, 20, 1419-1440;
9. Reiss J.G, Oxygen Carriers ("Blood Substitutes") Raison d'Etre, Chemistry, and Some Physiology *Chem. Rev.* 2001, 101, 2797-2919
10. Jones, R. D., Summerville, A., and Basolo, F. Synthetic oxygen carriers related to biological systems. *Chem. Re*v. *79*, 1979, 139–179.
11. Herold, S. Kinetic and spectroscopic characterization of an intermediate peroxynitrite complex in the nitrogen monoxide induced oxidation of oxyhemoglobin. *FEBS Lett*, 1998, *439*, 85-8
12. Olson, J. S.;Foley, E. W.;Rogge, C.;Tsai, A. L.;Doyle, M. P.,Lemon, D. D. No scavenging and the hypertensive effect of hemoglobin-based blood substitutes. *Free Radic Biol Med*, 2004, *36*, 685-97
13. Van der Deen H., Hoving, H., Nitrite and nitric oxide treatment of Helix pomatia hemocyanin: single and double oxidation of the active site, *Biochemistry*, 1977, *16*, 3519–3525
14. Kurtz, D. M., Jr. Oxygen-carying proteins: three solutions to a common problem. *Essays in Biochemistry*, 1999, 55-80
15. Kryatov, S. V.;Rybak-Akimova, E. V.,Schindler, S. Kinetics and Mechanisms of Formation and Reactivity of Non-heme Iron Oxygen Intermediates. *Chem Rev*, 2005, *105*, 2175-2226
16. Herold, S.;Exner, M.,Nauser, T. Kinetic and mechanistic studies of the NO*-mediated oxidation of oxymyoglobin and oxyhemoglobin. *Biochemistry*, 2001, *40*, 3385-95

NOVEL MIXED LIGAND CADMIUM(II) THEOPHYLLINATO COMPLEXES WITH POTENTIAL BIOACTIVITY

Béla MIHÁLY*, Edit FORIZS, Ioan SILAGHI-DUMITRESCU

Babes-Bolyai University, Faculty of Chemistry and Chemical Engineering, Str. Kogălniceanu nr. 1, RO-400084, Cluj-Napoca, Romania,

** Email: mb15@chem.ubbcluj.ro*

ABSTRACT

The synthesis, infrared spectra and thermal properties of three mixed-ligand complexes of Cd(II) with N(7)-coordinated theophylline, N-donor (ba=benzylamine) and N,N-donor ligands, like dmen=N,N-dimethyl-ethylenediamine and phen=1,10-phenantroline are reported.

Keywords: biologic activity, cadmium, mixed ligand complexes, theophylline

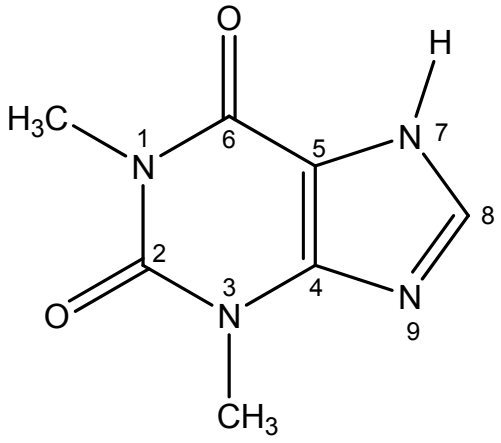
INTRODUCTION

The coordination compounds involving biologically important purine derivatives, like theophylline have been the subject of numerous studies in the last decades, since the metal ions – theophylline interactions may serve as model compounds for the interaction between metal ions and oxopurine bases of nucleic acids. [Birdsall, 1985; Steinkopf et al., 1995]. The most intensively addressed part of guanine molecules in reference to metal–DNA interactions is the N(7)/O(6) region, considered to be the primary site of attachment of platinum antitumor drogs. [Birdsall et al., 1979**;** Birdsall, 1985; Begum et al., 1994] (See Scheme 1 for numbering of atoms)

According to previous studies, theophylline (ThH), 1,3-dimethyl-2,6-dioxo-purine, coordinates as a monodentate ligand in neutral media via N(9), while in basic media it coordinates via N(7). [Umapathy et al., 1985*;* Bombicz et al., 2000; Mihály et al., 2007; Mihály et al., 2009].

Cadmium is a highly toxic, potent carcinogen metal whose carcinogenic molecular mechanism is unclear, probably caused by direct (covalent binding with DNA) or indirect binding (oxidative damage to DNA by increasing the cellular oxidants in the cells, and may also involve the impairment of DNA repair processes) to DNA. [P. Amo-Ochoa et al., 2005] It has been accepted by the International Agency for Research on Cancer as a category 1 (human) carcinogen [Beryllium, Cadmium and Mercury Exposures int he Glass Manufacturing Industry, Vol. 58, IARC, International Agency for Research on Cancer, Lyon, 1993].

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 126 - 129, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania



Scheme 1.

Structural studies on metal-nucleobase complexes can provide suitable models for the understanding the role of actions of metal ions in biological systems.

The structure of nucleobases allows the formation of more complex molecular architectures beyond their simple monomeric molecular structure by the formation of new bonds using the available H-bond donor-acceptor positions giving coordination polymers. Therefore, nucleobases can be used as building blocks for formation of supramolecules, and as it is, for obtaining promising nanomaterials.

We are interested to investigate the coordination behaviour of cadmium-theophyllinato complexes, since the literature contains only few references.

MATERIALS AND METHODS

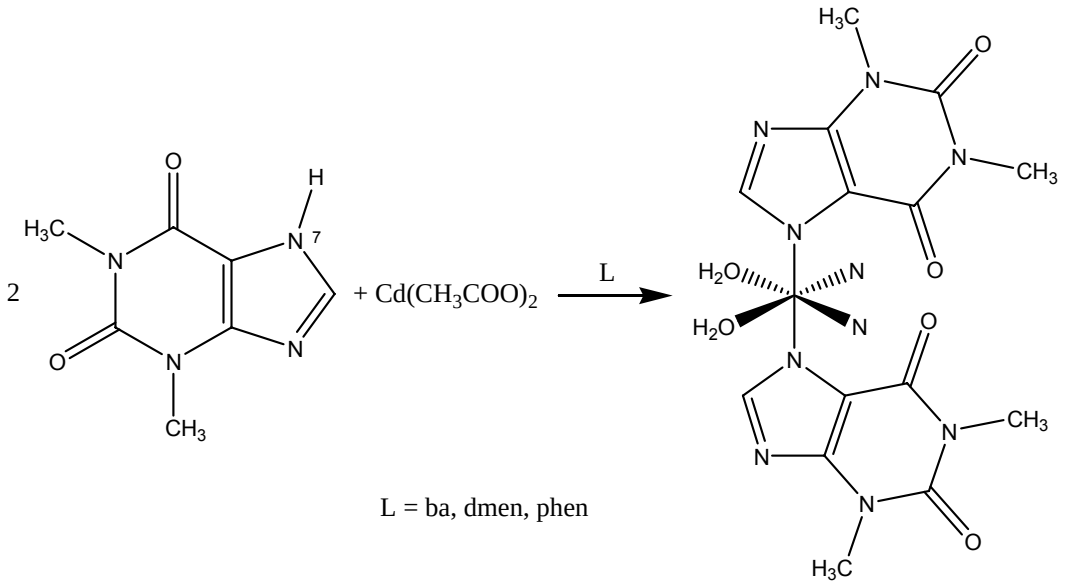
Synthesis of complexes. To a suspension of theophylline in water, the corresponding amine was added. The resulted solution was mixed with $Cd(CH_3COO)_2 \cdot 2H_2O$ in amine–water mixture. The reaction mixture was heated at 50°C, for 20 min, under stirring and stored at room temperature over night. The resulted white powders were collected by filtration, washed with aqueous amine solution and dried.

FTIR spectra were recorded on a Bruker Vector 22 FTIR spectrophotometer in the 4000–600 cm^{-1} range, using KBr pellets.

Thermal decomposition was investigated with an Universal V4.5A TA Instruments derivatograph (and α-Al_2O_3 as reference), at a heating rate of 10°C min^{-1}.

RESULTS AND DISCUSSION

Herein we report our results on the synthesis of some mixed-ligand complexes of Cd(II) containing N(7)-coordinated theophylline (Scheme 2) and N,N-donor coligands (dmen = N,N-dimethyl-ethylenediamine, phen=o-phenantroline). The prepared compounds, $[Cd(tp)_2(ba)_2 (H_2O)_2]$ (**1**), $[Cd(tp)_2(dmen)(H_2O)_2]$ (**2**) and $[Cd(tp)_2(phen)(H_2O)_2]$ (**3**), have been characterized by infrared spectroscopy and thermal analysis.



Scheme 2.

Most of the coordination polymers are insoluble materials, and in some cases degradate rapidly in solution and decompose upon heating. [F. Zamora et al., 2009]

Infrared spectra

The characteristic vibration bands of theophylline and complexes with their assignments are given in Table 1.

The FTIR spectrum of $[Cd(tp)_2(ba)_2(H_2O)_2]$ (**1**) show the two ν(C=O) stretching vibrations of coordinated theophylline at lower frequencies than those in free theophylline, due to the deprotonation at N(7) atom. The presence of symmetric and asymmetric $ν(NH_2)$ stretching vibrations can be assigned to the coordinated benzylamine. The spectrum shows a broad band in the 3200−3450 cm^{-1} region due to the presence of hydrogen bonded water molecules.

$[Cd(tp)_2(dmen)(H_2O)_2]$ (**2**): the existence of peaks around 3150 cm^{-1} confirm that the coordination of amine accur, as expected. The stretching vibration of OH groups are present also in the spectra, proving the existence of water in the crystal. The ν(C=O) vibrations are moved to lower frequencies due to the coordination (27 and 39 cm^{-1}, respectively).

$[Cd(tp)_2(phen)(H_2O)_2]$ (**3**): ν(NH) stretching misses in the infrared spectrum due to the deprotonation of theophylline at N(7) atom. The ν(C=O) vibrations shows a 29−37 cm^{-1} negative shift, proving that the coordination occur, while the presence of the ν(OH) vibrations can be assumed to the formation of H-bonds involving the water molecules.

Table 1.

Assignment of vibrations (cm^{-1}) for theophylline and the synthesized compounds

Compound	**ν(OH)**	**ν(NH)**	**ν(C=O)**	**ν(C=N)**	**δ(CH_3)**
Theophylline (th)	−	3120m	1714s 1667s	1566s	1444m
$[Cd(tp)_2(ba)_2(H_2O)_2]$ **1**	3447m 3422m	3324m 3264m	1681s 1619s	1550s	1430m
$[Cd(tp)_2(dmen)(H_2O)_2]$ **2**	3447m 3422m	3154m 3126m	1687s 1628s	1540s	1418m
$[Cd(tp)_2(phen)(H_2O)_2]$ **3**	3419w	−	1685vs 1630vs	1531m	1428m 1416m

s=strong; m=medium; w=weak; v=very

Thermal studies

Thermal decomposition studies on anhydrous theophylline shows a single weight loss, starting from 275°C to 380°C, without the formation of any solid residue. The DSC curve presents two endothermic effects (the first centered at 270, corresponding to the fusion of the sample, and the second at 330°C, when the sample decompose).

In the case of theophyllinato complexes, three processes occur: dehydration, deammination and pyrolytic decomposition. The first step is associated with the elimination of water. The second step indicate an expected endothermic behavior (observed from the DSC curves). The last step is given by the pyrolytic processes, attributed to the combustion of the organic matter (showing exothermic effects in DSC curves), while a residue of CdO is formed.

The thermogravimetric curves of compound **1** are similar to those of Cu(II) analogues (Bombicz et al., 2000).

Compound **2** starts to lose water at 80°C (only 0.2%, considered physically adsorbed water), continuing with the release of water molecules in the range 90−155°C (loss of 3.98%, calcd. 2.94%). This process is followed by the decomposition of the diamine molecule, 170−350°C (loss of 15.3%, calcd. 17.45%). The final decomposition product is CdO (exp. 21.9%; calcd. 20.93%)

The TG and DTG curves shows that compound **3** is stable up to 150°C, above this temperature two water molecules are lost (ca. 4.82%, calcd. 5.24%). This process is followed by the pyrolysis of organic material up to 500°C. The final decomposition product is CdO (exp. solid residue 19.31%; calcd. 17.4%)

REFERENCES

1. Amo-Ochoa P., Rodríguez-Tapiador M.I., Alexandre S.S., Pastor C., Zamora F. - Structural models for the interaction of Cd(II) with DNA: *trans*-$[Cd(9\text{-}RGH\text{-}N7)_2(H_2O)_4]^{2+}$, J. Inorg. Biochem., 2005, 99, 1540-1547.
2. Begum N.S., Manohar H. - Synthesis and x-ray crystal structure of a Cu^{II}-theophylline complex: $[Cu(theo)_2(H_2O)_3]\cdot 2H_2O$, *Polyhedron*, 1994, 13, 307-312.
3. Beryllium, Cadmium and Mercury Exposures int he Glass Manufacturing Industry, Vol. 58, IARC, International Agency for Research on Cancer, Lyon, 1993, 119.
4. Birdsall W.J., Zitzman M.S. - Divalent metal complexes of purines. Preparation of copper(II) complexes of theophylline with primary amines, J. Inorg. Nucl. Chem., 1979, 41, 116-117.
5. Birdsall W. - Complexes of Theophylline with Divalent Metal Ions, Inorg. Chim. Acta, 1985, 99, 59-62.
6. Bombicz P., Madarász J., Forizs E., Czugler M., Pokol G., Gal S., Kalman A. - Crystal structure and thermal characterization of a theophyllinato Cu(II) complex with benzylamine, Z. Kristallogr., 2000, 215, 317−321.
7. Bombicz P., Madarász J., Forizs E., Foch I. - Structure and thermal behavior of (SPY-5-12)-(2-aminoethanol-N)(2-aminoethanol-N,O)-bis(theophyllinato)copper(II) dihydrate: a model for DNA-metal interactions, Polyhedron, 1997, 16, 3601-3607.
8. Mihály B., Forizs E., Silaghi-Dumitrescu I. - Synthesis and characterization of some mixed ligand zinc(II) complexes of theophylline, Studia Univ. Babes-Bolyai, 2007, 4, 111-116.
9. Mihály B., Forizs E., Silaghi-Dumitrescu I. - (Diethylenetriamine)bis(theophyllinato)zinc(II) dihydrate, Acta Cryst., 2009, E65, m579.
10. Steinkopf S., Garoufis A., Nerdal W., Sletten E. - Sequence-Selective Metal Ion Binding to DNA Oligomers, Acta Chem. Scand., 1995, 49, 495.
11. Umapathy P., Shaikh R.A. - Synthesis and spectral studies on divalent metal complexes of theophylline, J. Indian Chem. Soc., 1985, 62, 103-105.
12. Zamora F., Amo-Ochoa M.P., Sanz Miguel P.J., Castillo O. - From metal-nucleobase chemistry towards molecular wires, Inorg. Chim. Acta, 2009, 362, 691-706.

A novel mechanism for platinum-based drugs: cisplatin and related compounds as pro-oxidants in blood

Vicentiu TACIUC, Cristina BISCHIN, Radu SILAGHI-DUMITRESCU

Department of Chemistry and Chemical Engineering, "Babes-Bolyai" University, 11 Arany Janos str, Cluj-Napoca RO-400028, Romania

e-mail : rsilaghi@chem.ubbcluj.ro

ABSTRACT

The most important drugs used in cancer treatment are cisplatin, carboplatin and oxaliplatin. These drugs are platinum-based, with organic and inorganic-type ligands. Previous mass-spectrometric studies have shown that these drugs bind to hemoglobin; however, consequences of this binding on reactivity were not shown. In fact, currently available data suggests that the medically-relevant chemical properties of hemoglobin are in no significant way affected by Pt-based drugs, within the concentration ranges attainable under treatment of a pacient. Here, it is shown that, contrary to common belief, cisplatin can have a distinct pro-oxidant effect on hemoglobin, and that carboplatin has a similar effect but with a different magnitude. We speculate that the stress imposed by Pt-based drugs on hemoglobin should be responsible for at least part of the side-effects of this drug. Furthermore, we formulate a hypothesis according to which the therapeutic effect of the drug may be related to the oxidative stress induced by it in the blood.

Key words: hemoglobin, cisplatin, carboplatin, oxaliplatin, auto oxidation

INTRODUCTION

One of the most important drugs used to treat various types of cancers (testicular tumors, malignant melanoma), including sarcomas (osteogenic sarcoma), carcinomas and germ cell tumors, is *cisplatin*. Cisplatin is a yellow crystalline solid that is slightly soluble in cold water and insoluble in most common solvents except *N,N*dimethylformamide.[1, 2] It has a simple inorganic structure [$H_6Cl_2N_2Pt$] compared with all other drugs which have an organic structure. Cisplatin has two isomer forms, cis-form and trans-form. In aqueous solution, cisplatin slowly changes to the *trans*-form. The last isomer form has a low pharmacological activity.[3,4] The drug is administered by a single infusion or by several infusions over 2–5 days, as multiple intraperitoneal injections.[5,6] These drugs react in vivo, binding to and causing crosslinkind of DNA. One of the important roles of those drugs is caused apoptosis (death cell).

After administration, one of the chloride ligands is displaced by water, process termed aquation. The water ligand is displaced, allowing the platinum atom to coordinate to a basic site in DNA. After that, crosslinking of two DNA bases occurs via displacement of the other chloride ligand. Cisplatin crosslinks DNA in several different ways, interfering with cell division by mitosis. The damaged DNA elicits DNA

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 130 - 134, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

repair mechanisms, which in turn activate apoptosis when repair proves impossible.[4]

Cisplatin can be used in combination with other drugs (Paclitaxel, Vinblastin and Xeloda) in cancer treatment.[7]

Two other widely-used platinum derivates feature organic ligands to the metal: *oxaliplatin* [$C_8H_{14}N_2O_4Pt$] and *carboplatin* [$C_6H_{14}N_2O_4Pt$]. Both drugs have a lowered toxicity than cisplatin, allowing them to be used in higher dosage; both have a kinetically slower leaving group, and are also less nephrotoxic than cisplatin. The limiting toxicity of oxaliplatin is peripheral sensory neuropathy, also seen with cisplatin. [8] Because the drugs have different profiles, the targets and mechanisms of action for these drugs are different; thus, oxaliplatin can be used at on the patients who have developed resistance to cisplatin. Comparative with cisplatin, oxaliplatin has dicarboxylate ligand replacing the chloride ligands, the ligands can be displaced by nucleophilic (electron-rich) atoms to form strong bonds with covalent characteristics, its molecule is much bigger and its reactivity is much lower than cisplatin. Comparative with cisplatin, carboplatin is better retained in the body, so that its effect is longer lasting. Its toxicity is lower than cisplatin or radiation therapy. In cancer treatment, oxaliplatin by itself had no stronger activity, because that, this drug is used in combination with Fluorouracil and Folinic Acid (a combination known as FolFOx). In combination with 5-fluorouracil, is the only platinum drug that is effective against colorectal cancer, if a surgery was made before, the treatment with drugs is not recommended. [9] [10] [11] [12]

The adverse effects of these drugs are as enumerated: nausea, progressive peripheral sensory neuropathy, fatigue, vomiting, alopecia, hematological suppression, because of that is indicated to used adequate hydration and dieresis to prevent renal damage. [10]

Cisplatin-protein binding has been proposed to explain the adverse effects of treatment; it is thus known that the binding of cisplatin to proteins reduces the urinary excretion of platinum and causes the deposit of platinum in tissues. [6] [10]

One mode to study cisplatin- protein binding is made with atomic absorbance spectrometry (AAS), but this method was not able to differentiate the binding of cisplatin with specific proteins, and could not provide detailed information about the binding site in the protein. [6]

Other methods to study cisplatin–protein binding are to use *high performance liquid chromatography (HPLC)* and to detect the unbound cisplatin is used inductively coupled plasma mass spectrometry (ICPMS). Using both machine, enabled the hemoglobin can be study using *nanospray tandem mass spectrometry*. In researches was used a neutral aqueous solution of hemoglobin with 20% methanol in water and acidified with dilute formic acid to pH 6 and 2.[6] The mass spectrum was shifted towards higher charge states, suggesting that cisplatin in the form of Pt(NH3)2 (229 Da) was bound to the protein and that two protons were lost, with evidence for platinum binding to cysteine and proline residues. This binding was dependent on the concentration of cisplatin; importantly, cisplatin–Hb complexes were shown to be formed using clinically relevant concentrations of cisplatin and Hb.[6]

The interaction of oxaliplatin and carboplatin with hemoglobin was also studied, with similar results; heme release was a noted side effect of platinum binding. The studies were made with nanoelectrospray ionization quadrupole time-of-flight mass spectrometry (nanoESI-QTOF-MS) and size-exclusion highperformance liquid chromatography/inductively coupled plasma mass spectrometry (HPLC/ICPMS).[9]

MATERIALS AND METHODS

The UV-vis spectra for solution assays were carried out on a Cary50 UV-vis spectrophotometer (Varian, Middleburg, the Netherlands).

We have used a bovine hemoglobin that was purified following a variation of the general protocol of Antonini and Brunori (see also paper by Deac et al, in this volume).

Stock solution (1mM) of drugs was prepared using *phosphate buffered saline* (PBS) and the drugs in certain amounts in function of the drugs concentration. Absorbances of hemoglobin+drug solutions in PBS were measured at various concentrations of the drugs (0μM – 1000 μM), before and after incubation of the solution at 37 °C for 4h. We have used the values of absorbance and we have made a graphic in function of concentration of the drugs and absorbance $\left(\frac{A_{f620}-A_{f577}}{A_{i620}-A_{i577}}\right)$, where A_{f620} - final absorbance at 620 nm wavelength, A_{f577} - final absorbance at 577 nm wavelength, A_{i620} - initial absorbance at 620 nm wavelength, A_{i577} - final absorbance at 577 nm.

RESULTS AND DISCUSSION

Hemoglobin has the capacity to auto oxidize, i.e. go from the Fe^{2+}-dioxygen adduct (named oxyhemoglobin) to Fe^{3+} (named methemoglobin), with liberation of the dioxygenic ligand in the form of superoxide. This process can be monitored easily via UV-vis spectroscopy, in the form of a decrease in absorbance at 577 nm (wavelength characteristic to the oxy form), and an increase 620 nm (wavelength characteristic of the met form). Furthermore, the very intense Soret band moves from 415 nm (oxyhemoglobin) towards 405 nm (methemoglobin). Figure 1 illustrates these changes, and the way they can be affected by the presence of cisplatin, after 4 hours of incubation at 37 ºC, in PBS.

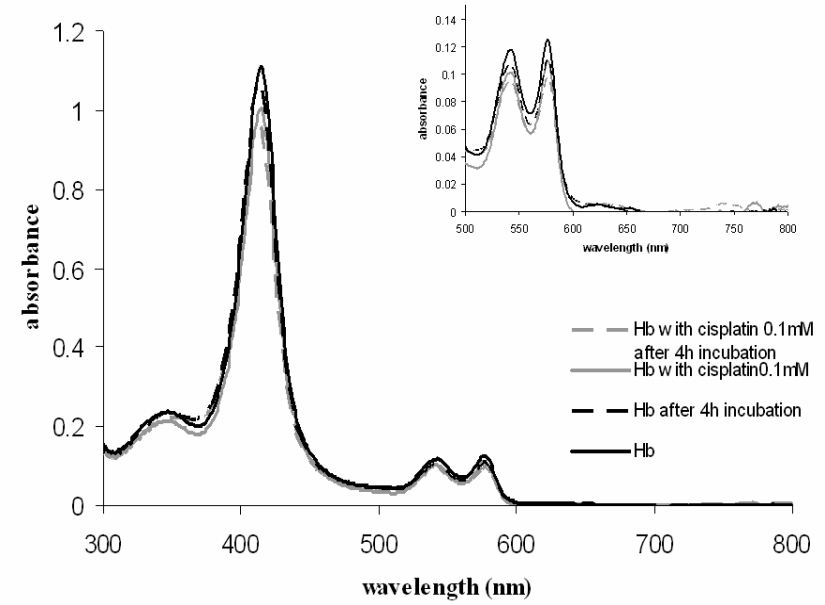


Figure 1. Influence of cisplatin on the oxy-hemoglobin UV VIS spectrum.

Figure 2 shows the degree to which hemoglobin autooxidizes, under conditions such as shown in Figure 1. The ratio shown on the y axis, defined in the Methods section, should take a value of 1 for a non-oxidizable material and 0 for a

material that fully oxidizes during the 4 hours of incubation. It can be seen that cisplatin has a dose-dependent pro-oxidant effect, so much so that at ~500 μM it can induce oxidation of 30% of the hemoglobin. At higher concentrations a saturation phenomenon is observed, suggesting well-defined binding sites as opposed to a random distructive effect of the drug on the protein.

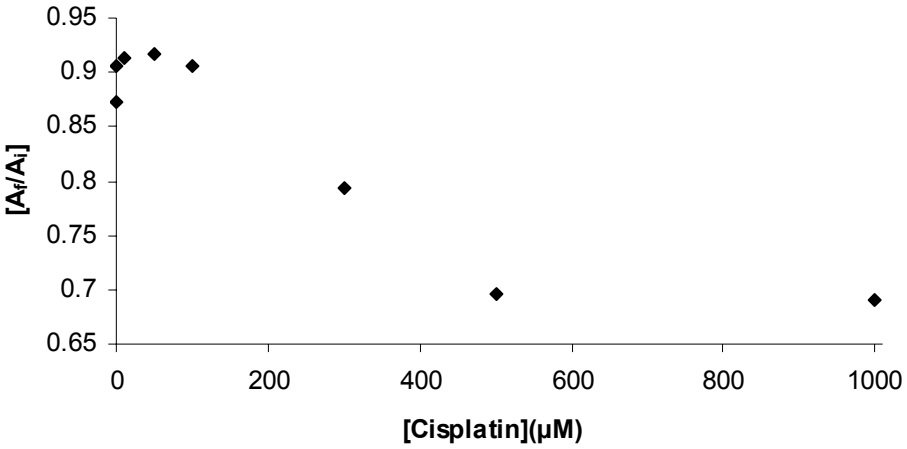


Figure 2. Influence of cisplatin concentration on hemoglobin autooxidation (normalized autooxidation fractions are defined as shown in the Methods section), and essentially describe the fraction of hemoglobin still in oxy form after 4 hours of incubation at 37 ºC in PBS.

Figure 3 shows that carboplatin has a similar effect to cisplatin, both in terms of pro-oxidant effect and in terms of saturation behaviour. The maximum degree of autooxidation attained by the sample is higher with ciplatin compared to carboplatin; another difference is seen at lower Pt concentrations, where carboplatin appears to display prooxidant effects at lower concentrations than cisplatin

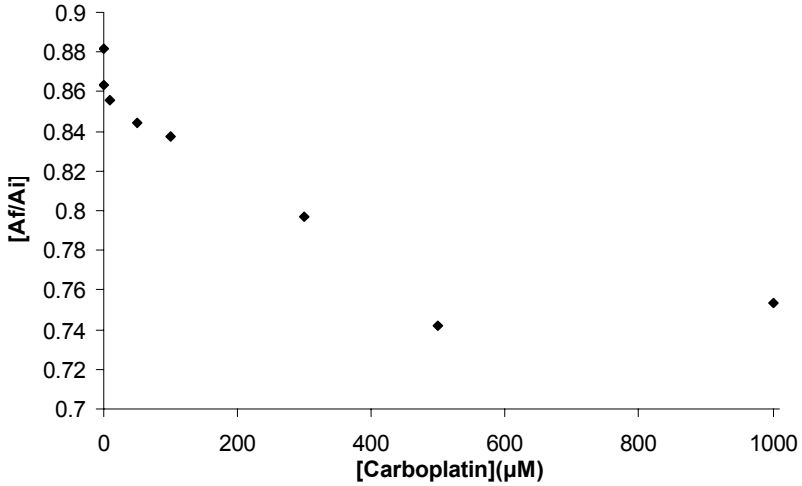


Figure 3 Influence of carboplatin concentration on hemoglobin autooxidation, and essentially describe the fraction of hemoglobin still in oxy form after 4 hours of incubation at 37 ºC in PBS.

.

In conclusion, cisplatin and carboplatin appear to both display pro-oxidant effects on hemoglobin; this effect is seen even at concentrations attainable during

administration of the drug and as such may hold medical relevance. A pro-oxidant in the blood may elicit multiple responses. However, in a first instance, the effect of such a pro-oxidant action is expected to be severe free-radical-type oxidative stress, especially on kidneys – well in line with what is known experimentally/medically about the side-effects of this class of drugs, and suggesting that our studies may have uncovered a new mechanism for these side-effects. Another hypothesis, which we intend to investigate as well in the future, is that the flux of free radicals brought about by Pt compounds via hemoglobin autooxidation may be involved, directly or indirectly, in the therapeutic action of these drugs.

Acknowledgements: the work shown here has been supported by the Romanian Ministry for Education and Research (ID565/2007).

REFERENCES

1. Cisplatin: CAS No. 15663-27-1. Fifth Annual Report on Carcinogens, 1989, eleventh edition,
2. Fouad A. A., Mohamed A. M., Gomaa W. - Protective effect of carnosine against cisplatin-induced nephrotoxicity in mice, Environmental Toxicology and Pharmacology, 2007, 25, 292-297
3. Trzaska, S. Cisplatin - Chemical & Engineering News, 2005, 83,
4. Lippert B. Cisplatin: chemistry and biochemistry of a leading anticancer, Verlag Helvetica Chemica Acta, Zürich, 1999, pp563, pp8-15
5. Tsujitani S., Fukuda K., Saito H., Kondo A., Ikeguchi M., Maeta M., Kaibara N., - The administration of hypotonic intraperitoneal cisplatin during operation as a treatment for the peritoneal dissemination of gastric cancer, Surgery, 2002, 131, S98-S104
6. Mandal R.,Teixeira C., Li X. F., - Studies of cisplatin and hemoglobin interactions using nanospray mass spectrometry and liquid chromatography with inductively- coupled plasma mass spectrometry, The Analyst, 2003, 629 - 634
7. Clipca, I. - Chimioterapia combinata cu cisplatin in tratamentul cancerului mamar metastatic rezistent la antracicline. 2009, 54- 85
8. Roat-Malone R. M., - Bioinorganic chemistry: a short course, John Wiley & Sons Inc, New York, 2003, pp348, pp290- 291
9. Mandal R., Sawyer M. B., Li X. F. - Mass spectrometry study of hemoglobin-oxaliplatin complexes in colorectal cancer patients and potential association with chemotherapeutic responses. Rapid Commun. Mass Spectrom., 2006, 20, 2533-2538
10. Peng J., Mandal R.,Sawyer M.,Li X. F. - Characterization of Intact Hemoglobin and Oxaliplatin Interaction by Nanoelectrospray Ionization Tandem Mass Spectrometry, Clinical Chemistry, 2005, 51, 2274-2281
11. Correale P.,Messinese, S., Marsili S.,Caraglia M., Piccolomini A., Petrioli A., Ceciarini F., Micheli L., Nencini C., Neri A., Vuolo G., Guarnieri A., Abbruzzese A., Prete S. D., Giorgi G., Francini G., A novel biweekly multidrug regimen of gemcitabine, oxaliplatin, 5-fluorouracil (5-FU), and folinic acid (FA) in pretreated patients with advanced colorectal carcinoma, British Journal of Cancer, 2004, 90, 1710–1714
12. Culy C. R., Clemett D., Wiseman L. R., Oxaliplatin : A review of its pharmacological properties and clinical efficacy in metastatic colorectal cancer and its potential in other malignancies. Drugs, 2000, 60, 895 - 924 (143 ref)

THE EFFECT OF SELF-ASSEMBLIES BASED ON NANOSTRUCTURED PHOSPHATES AND COLLAGEN MIXTURES ON CELL CULTURES

Gheorghe TOMOAIA[1], Olga SORITAU[2], Maria TOMOAIA-COTISEL[3,*], Lacrimioara-Bianca POP[3], Alexandru POP[3], Aurora MOCANU[3], Ossi HOROVITZ[3] and Liviu-Dorel BOBOS[3]

[1]Iuliu Hatieganu University of Medicine and Pharmacy, Orthopaedics and Traumatology Department, 47 Traian Mosoiu Str., 400132 Cluj-Napoca, Romania
[2]Oncologic Institute of Cluj-Napoca, 34-36 Republicii Str., 400015 Cluj-Napoca, Romania
[3]Babes-Bolyai University, Faculty of Chemistry and Chemical Engineering, Physical Chemistry Department, 11 Arany J. Str., 400028 Cluj-Napoca, Romania

* E-mail : mcotisel@gmail.com

ABSTRACT

Nanostructured calcium phosphate (CP) with or without various SiO_2 contents of controlled porosity and crystalinity as well as composite biomaterials formed of calcium phosphate powders mixed with collagen type I (COL), at different CP/COL weight ratios were prepared and structural characterized using SEM, TEM and AFM. The observed data show that the several prepared biocomposites present a rather uniform globular structure, controlled porosity, and high crystalinity in the nano and micro meter range. The inorganic nano powder incorporation within collagen matrix leads to biocomposites with good mechanical properties, which can be controlled by the CP/COL/SiO_2 weight ratios. By AFM investigations on the surface of the composite biomaterials were strongly evidenced the collagen fibers mineralized with inorganic powders at different mineralization degrees. The AFM images represent a convincing indication that the nano- or microparticles of CP particularly with SiO_2 are well covered by the natural protein, collagen type I, fibers. The AFM data are in substantial agreement with TEM and SEM observations. This work has demonstrated that calcium phosphates/collagen/SiO_2 composites present a micro-structure, which shows a nanostructure connected by bridges with controlled porosity and good mechanical properties. These biocomposites show good biocompatibility and an excellent biological response on cell cultures. The data indicate that these biocomposites could have multiple medical applications in orthopedics coated metal implants and bone tissue engineering.

Key words: collagen layers, inorganic powder, composite biomaterials, AFM

INTRODUCTION

Bone defects caused by cancer or by trauma are very common and the bone tissue engineering was put forward under intensive investigations. Thus, many biomaterials have been studied to repair bone tissue defects and bone functionality (Yovana et al., 2007) or to cover the implants used in orthopedic and dental surgery (Sopyan et al., 2007).

During the last several decades, important research on ceramic materials, such as calcium phosphates, has indicated that these inorganic powders are rather

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 135 - 140, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

appropriate as bone substitutes due to their bioactive, biodegradable, biocompatible and osteoconductive characteristics. These inorganic materials are not toxic and when they are introduced in vivo they do not induce antigenic responses, but they are brittle and difficult to process into fine nanocrystals. Thus, the crystal nanostructure and porosity of these inorganic materials are not yet optimally established and the nano crystals are difficult to obtain.

Bone exhibits normal hydroxyapatite crystals with needle or rod like shape well arranged within the polymeric matrix of collagen type I. These inorganic nanoparticles formed in physiological environment have a better response when compared with synthetic calcium phosphates with larger particle size. To prepare fine inorganic nanoparticles (nano powders) many chemical processing routes have been employed, including hydrothermal reactions, micro-emulsion reactions, and chemical precipitation, which is the most used alternative, but more research is needed to clarify their controlled synthesis for a particular purpose.

On the other hand, collagen is a rather widely used biomaterial. The advantages of using collagen or collagen products are their very low antigenicity and excellent biocompatibility. They associate easily with various biologically active compounds, such as calcium phosphates.

The reconstitution of collagen fibers from aqueous solutions is of great interest (Tomoaia-Cotisel, 2006; Tomoaia et al., 2007; Tomoaia et al., 2008) for understanding the collagen applications such as regenerative, healing and hydrating layers at the molecular level. The disadvantage of collagen matrices is that these matrices present relatively weak mechanical properties due to their high porosity, which limit their use for bone tissue engineering.

In this work, we optimized the process of fabrication of composite biomaterials based on nanostructured calcium phosphates with or without SiO_2 content and collagen type I. The highly porous crystalline inorganic materials were synthesized and structural characterized. The nano- and micro-structure and the characteristics of the new collagen composite materials were determined and their effect on the cell cultures was evaluated.

MATERIALS AND METHODS

Calcium phosphate nanoparticles (CP) of high crystalinity and acicular shape are produced in the absence and in the presence of an anti-sintering agent. CP nanoparticles are synthesized by a substantial number of methods to produce powders with controlled structures and improved properties. Particularly, calcium phosphate nanoparticles were prepared using the chemical synthesis from a calcium salt and an ammonium phosphate in the presence of a surfactant, with the major role of controlling the size of inorganic nanoparticles in the aqueous dispersion. Various other ingredients are also used for controlling pH conditions. The resulted dispersions were hydrothermally processed for 24 hours at a temperature of 75°C.

Then, porous CP with different silica (e.g., 1%, 5%, 10% and 20 % SiO_2) content was synthesized in two different controlled conditions. The resulted porous mixed CP and SiO_2 powders are of high crystalinity having a rather uniform globular size and interconnected porosity.

The ten powders, namely five: CP and CP1, CP2, CP3 and CP4 obtained without and the other five CP' and CP'1, CP'2, CP'3 and CP'4 obtained with the anti-sintering compound were previously characterized using FTIR, X-ray diffractions and SEM. Moreover, BET and porosity were used to analyze their micro-structure and TEM and AFM to analyze their nanostructures.

The results showed that pure NP and NP' powders are of high crystalinity and needle like shape in the nanometer range. On the other hand, the inorganic porous composites CP and CP1, CP2, CP3 and CP4 as well as CP' and CP'1, CP'2, CP'3 and CP'4 powders, in the presence and in the absence of anti-sintering compound, respectively, were obtained as globular shape.

In parallel, a thorough study was carried out to determine the nanoscale structure of collagen type I fibrils with the use of atomic force microscopy (AFM) and scanning electron microscopy (SEM).

RESULTS AND DISCUSSION

The organization of proteins at solid surfaces is of increasing importance in applications like implant biocompatibility, cell adhesion and growth, and biomaterials design. Among proteins, type I collagen became an interesting model compound, due to its auto-associative properties. Type I collagen (Fig. 1) is the major fibrillar protein in the extracellular matrix and in connective tissues. It is abundant in bone, cartilages, ligaments, tendons and skin.

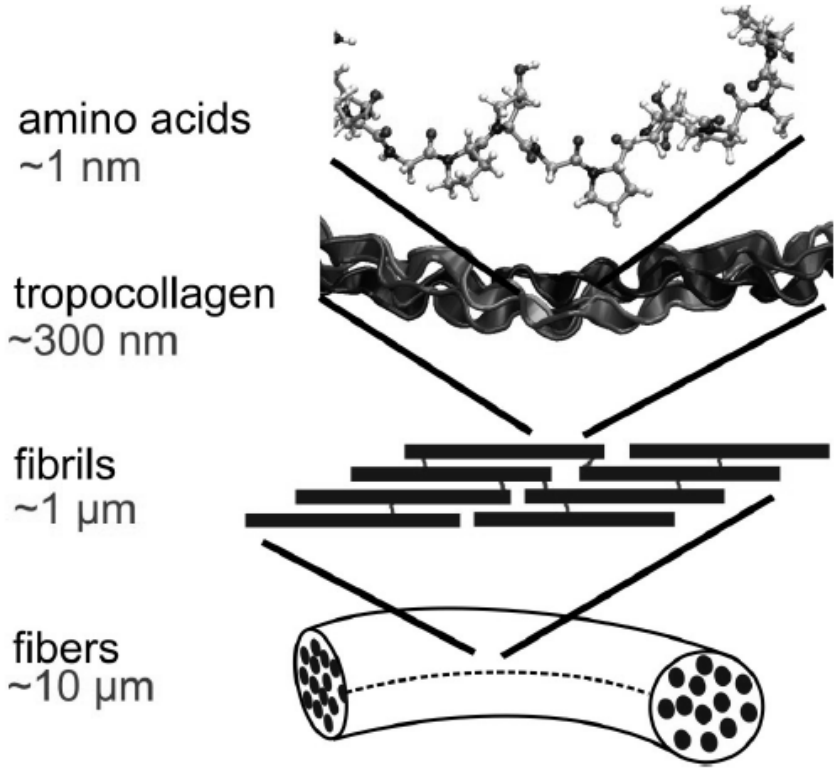


Fig. 1. Model for collagen organization and structure from amino acids to collagen fibers**.**

Type 1, collagen fibrils and fibers were re-assembled from acidic aqueous solutions in the absence (Fig. 2) and the presence of different inorganic materials developed in this work(see, for example Fig. 3). The organization of collagen molecules was imaged with JEOL AFM, operating in tapping mode, and with JEOL SEM. The collagen assemblies were engineered by deposition method (e.g., casting method) and evaporation from aqueous solutions on different solid supports: glass, mica or silicon.

The surface morphology of collagen self-assemblies shows the structure of the collagen fibrils and fibers in the absence (Fig. 2) and the presence of inorganic phase (i.e., collagen (20%), CP (70%), and SiO_2 (10%), in Fig. 3). These AFM observations indicate that the image resolution is good even for the said composite biomaterial, which is stabilized through interactions between collagen molecules and inorganic phase particularly in the presence of SiO_2 (e.g. Fig. 3).

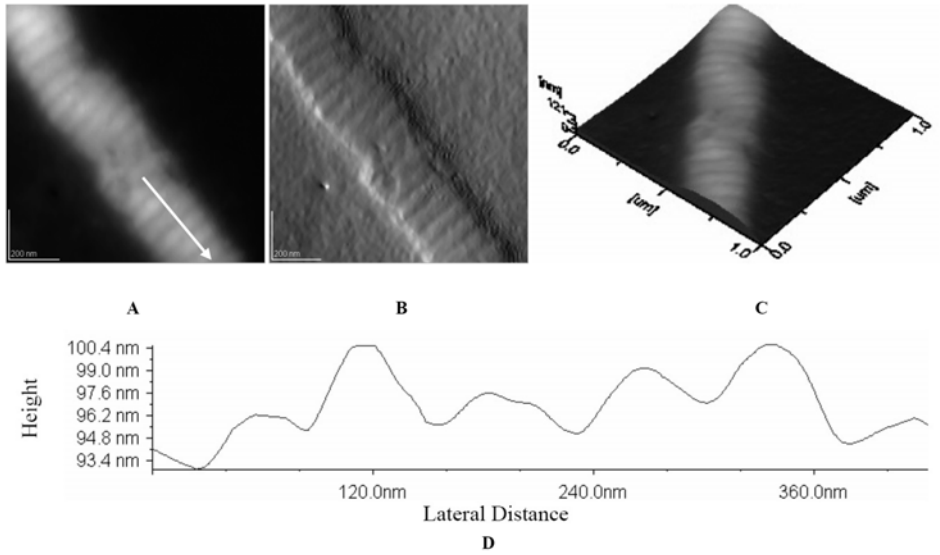


Fig. 2. Collagen fiber on glass, re-assembled from acidic solution of collagen. Scanned area : 1 μm x 1 μm.A) 2D-topography; B) amplitude image; C) 3D – view of panel A; D) cross section along the arrow in panel A.

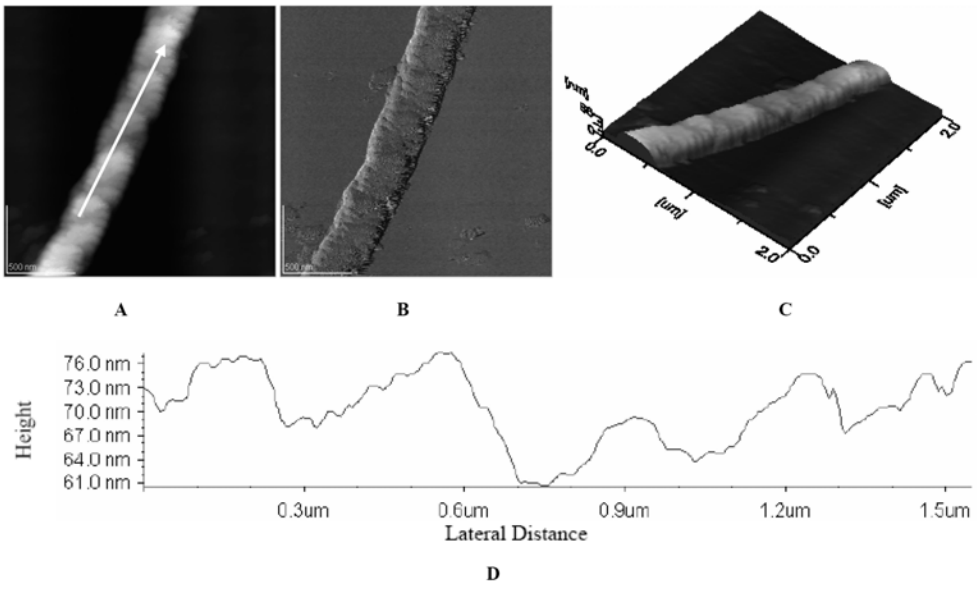


Fig. 2. Biomaterial based on collagen fiber and inorganic phase (e.g., collagen (20%), CP (70%), and SiO_2 (10%)) on glass. Scanned area: 2 μm x 2 μm. A) 2D-topography; B) amplitude image; C) 3D – view of panel A; D) cross section along the arrow in panel A.

The AFM images are in substantial agreement with SEM and TEM observations. The image analysis provided fruitful information about collagen fibrils and fibers by developing and employing different sample preparations and imaging conditions. Collagen assemblies imaged with AFM exhibited a transverse banding pattern with a period of about 70 ± 10 nm in substantial agreement with theoretical modeling (Fig. 1 and Fig. 2).

The nodule structure of collagen fiber shows the different mass density across the fibrils of collagen, especially in the presence of inorganic phase given in Fig. 3, where the transverse banding pattern has a period of about 100 nm to 200 nm. This periodic pattern rather coincides with the size of inorganic particles. The inorganic material probably enhances the mechanical stability, elasticity and resistance of collagen fibrils through its binding process and molecular interactions to the collagen molecules.

The structural investigations revealed the banding of collagen fibrils in the presence of inorganic phase. Type 1 collagen co-assembles with inorganic materials to form rather ordered films on glass, silicon and mica substrates, as visualized by AFM and SEM. The inorganic materials appear to lead to supramolecular collagen structures, with a remarkable nano- or micro- scale order, that mimics natural collagen assemblies. Direct incorporation of the inorganic phase into the collagen assemblies represents a step toward rational design of nanostructured biomaterials for a wide range of applications in synthetic biology and medicine, including the design of new bone substitutes and novel drug delivery systems.

Since natural bone is a 3D composite made up of collagen fibers threading through crystalline apatite, to simulate the natural bone tissue, we develop porous crystalline calcium phosphate particles with or without SiO_2, which were incorporated into collagen resulting in the new composite biomaterials. The silicated calcium phosphates with various SiO_2 contents showed a better behavior when mixed with collagen, due to the fact that silicates adsorb organic compounds, such as collagen.

A common theory is that silica and the silicates have the capacity for adsorbing organic molecules, such as collagen, and aligning them on the surface. The adsorption phenomenon on silica surfaces is due to electrostatic forces, van der Waals forces and hydrogen bonds. Our findings are in substantial agreement with the recent observations that silica participates participate actively in the formation process of the bone tissues even stimulating the collagen synthesis (Chirita, 2008).

Further, the optical microscopy, scanning electron microscopy and confocal microscopy were used to evaluate the behavior of osteoblasts, like MG-63 cells, cultured on scaffolds made of the different mixed inorganic powder/collagen self-assemblies for several days. The surface of the biocomposite scaffolds was deeply analyzed by AFM and SEM investigations. Results showed a good adhesion, growth and proliferation of osteoblasts on the surface of the different biocomposite scaffolds. Even more, it appears that the osteoblasts can build bridges between adjacent micro- or nano-spheres of composite material forming spheres-cells clusters with all types of investigated biomaterials developed in this work.

REFERENCES

1. Yovana, A., Mateus, P., Barrias, C.C., Ribeiro, C., Ferraz, M.P., Monteiro, F.J. – Comparative study of nanohydroxyapatite microspheres for medical applications, J. Biomed. Mater Res., 2008, 86A, 483-493.

2. Sopyan, I., Mel, M., Ramesh, S., Khalid, K.A. – Porous hydroxyapatite for artificial bone applications, Science and Technology of Advanced Materials, 2007, 8, 116-123.
3. Tomoaia-Cotisel, M. - The nanostructure formation of the globular seed storage protein on different solid surfaces studied by atomic force microscopy, in *Convergence of Micro-Nano-Biotechnologies*, Series in *Micro and Nanoengineering*, ***Volume* 9**, *Editors*: Maria Zaharescu, Emil Burzo, Lucia Dumitru, Irina Kleps and Dan Dascalu, Romanian Academy Press, Bucharest, **2006**, pp. 147 - 161.
4. Tomoaia, Gh., Pop-Toader, V.-D., Mocanu, A., Horovitz, O., Bobos, L.-D., Tomoaia-Cotisel, M. - Supramolecular organization and nano structuration of collagen and anti cancer drugs, Studia, Univ. Babes-Bolyai, Chem., 2007, 52 (4), 137-151.
5. Tomoaia, Gh., Tomoaia-Cotisel, M., Mocanu, A., Horovitz, O., Bobos, L.-D., Crisan, M., Petean, I. - Supramolecular organization of collagen and anti-cancer drugs, Journal of Optoelectronics and Advanced Materials, 2008, 10 (4), 961-964 (2008).
6. Chirita, M. – Mechanical properties of collagen biomimetic films formed in the presence of calcium, silica and chitosan, Journal of Biomic Engineering, 2008, 5, 149-158.

STUDY REGARDING THE CHARACTERISTICS AND HEAVY METALS CONTENT IN THE SLUDGE IN THE MUNICIPAL WASTEWATER TREATMENT PLANTS IN THE COUNTY OF SUCEAVA.

Valeria Diţoiu, Nina Holban*

APM Suceava, „Stefan cel Mare" University of Suceava

**E-mail : ninah@seap.usv.ro*

ABSTRACT

The paper proposes to present a few general characteristics of the sludge from the municipal wastewater treatment plant, and according to them, the possibility to use them in various fields, such as: their use as soil conditioner, as construction material or as a fuel through joint incineration. The paper presents the sludge characteristics that come from the wastewater treatment plants in some cities from Suceava County in order to establish the possibility to use them as a soil conditioner.

Key words: treatment, sludge, particular weight, heavy metals, humidity, filterability, effluent.

INTRODUCTION

Following the sewage treatment processes of the municipal waste waters the result consists of sludge and treated waters.

From a technological point of view, the sludge is considered to be the final stage in the wastewater treatment, where metabolic and / or raw materials are comprised next to the intermediary and finite products of some industrial activities.

From a physical point of view the sludge resulted from the waste waters treatment are considered complex colloidal systems, with an heterogeneous composition, with a content of colloidal particles (d<1 μm), dispersed particles (d<1-100 μm), suspension material, etc, with a semi-solid material aspect, the organic polymers of biologic origin and with a high content of water become "unwanted" secondary results that concentrate the eliminated pollutants from the waste waters and can become dangerous for the environment.

A wastewater treatment plant can be considered efficient once the effluent frames within the limits imposed by the regulations in force to all the physical-chemical and biological indicators for it to be discharged in a certain tank and if the extracted sludge was efficiently digested for its final valorization, without endangering the quality of the environmental factors.

Given such conditions, the technological scheme of a wastewater plant should consist of two technological circuits: the technological circuit of the waste water, whose purpose is the compliance with the effluents quality requirements, before their evacuation in the natural receiver and the sludge technological circuit, whose purpose is given by the diminishing of the sludge quantity and their transformation into raw

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 141 - 147, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

materials as less dangerous as possible both for human kind and environment or even into economically valorization substances (agriculture, energy, construction materials, etc)

The sludge sewage procedures are multiple and varied. There are no universally used formulas; each objective needs to be studied according to its particular conditions.

The processing technology of the sludge must be subordinated to their final valorization and evacuation, and the utilization of a certain procedure of sludge processing involves a thorough knowledge of the sludge characteristics that are under sewage treatment processes and the performances used in the processing procedures.

THE CHARACTERISTICS OF SLUDGE FROM MUNICIPAL TREATMENT PLANTS

The main types of sludge developed in wastewater treatment procedures, considering their degree of processing in the flow sheet, are: primary sludge, produced in the mechanical wastewater treatment stage; secondary sludge, produced in the biological wastewater treatment stage; mixed sludge, resulted from the mix of primary sludge and following the secondary settling and precipitation sludge, resulted from the physical-chemical wastewater treatment by adding neutralizing, precipitation or coagulation-flocculation agents.

The amounts retained in various stages of the municipal wastewater treatment process are different from one source to another. The approximated sludge flows, produced in an urban wastewater treatment plant, are calculated as specific flows per resident/day, assuming a water consumption level of 150 l/res. day, and thus the daily specific sludge flow is about 7 dm^3 / res / day.

For the categorization of sludge, a series of general values is used (humidity, specific weight, pH, mineral-volatile proportion, calorific value, etc.) as well as a series of specific values (fertilizing matters, detergents, heavy metals, oils and fats, etc.), which vary depending on the characteristics of the sewage wastewater.

- *The color and smell of sludge* from the wastewater treatment process provide the first data on the condition of the sludge and they vary depending on their origin, in the following manner: fresh sludge in the primary settling tanks are light-grey or yellowish and their smell is barely discernible; anaerobic digested *sludge* are brown to black, they smell like resin and their aspect is granular; biological *sludge* in the secondary settling tanks following biologic filters are brown and smell like humus; with activated sludge following the aeration tanks, the color varies from dark yellow, grey-brown to dark brown depending on the main bacterial species, an the smell of raw activated, well aerated sludge is a faint smell of humus, while sludge produces by the mechanical-chemical treatment has a muddy aspect, and its color depends on the coagulating agent used.
- *Sludge humidity* is an important feature for the adoption of the treatment processes (fermentation, dewatering, centrifugation, etc.), and for its transportation, and broadly varies depending on sludge type (mineral or organic), on its origin (primary, secondary, precipitation, etc.), thus: raw materials retained on strainers and sieves have a humidity of about 60%; fresh primary sludge: 95-97%; activated excess sludge: 98-99.5%, and precipitation sludge: 92-95,%. The main element for the treatment of sludge is the diminution of the amount of sludge depending on water discharge and the change in the sludge structure.
- *Sludge specific weight (S)* depends on the specific weight of the solid matters contained, on their humidity and on sludge origin.

For sludge coming from urban wastewater treatment, the value S varies in the range 1.001- 1.004 t/m^3, thus: untreated primary sludge has a specific weight in the range 1.004 -1.010 t/m^3, post-thickening sludge has a value of approximately 1.003 t/m^3 and excess activated sludge 1.001 t/m^3. Roughly calculated, considering that sludge mean humidity exceeds 90%, we may accept a sludge specific weight equal to that of water.

- *Sludge calorific value* varies depending on its organic matter (volatile substances) contained.

Primary sludge, containing approximately 70% (of the dry substance) organic matter, have a higher calorific value (approx 4300 kcal/kg) as compared to fermented sludge that contain approx 40% organic matter (approx 2050kal/kg calorific value).

- *pH value* must be controlled in the methane fermentation stage: throughout the sludge methane fermentation process, pH range between 7 and 7.5 pH units; a pH value < 6 indicated the beginning of sludge fermentation, but values exceeding 8.5 pH units are not allowed in the sludge fermentation process.
- *Organic matters* (volatile fractions of the dry substance, determined by sludge calcinations at a temperature of 550^{0}C) and *inorganic matters* have the following value: for *primary sludge* (containing 70-97% water) 3-5% solid substance, of which approx. 70% the volatile fraction, while the remaining 30% represents the mineral portion, whereas for *fermented sludge*, the volatile fraction diminishes to 40-50% of the solid substance, and the mineral portion increased to 50-60%.

The values mineral substance (M) and volatile substance (V) in the dry substance form a criterion for the classification of sludge and a criterion for the selection of the treatment procedures, thus: putrescent organic sludge (with a mineral/volatile value of M/V<1) must first be stabilized by a biological procedure (by aerobic or anaerobic fermentation); inorganic sludge (with a value of M/V>1) may be treated directly by physical-chemical processes (solidification, removal of useful elements, etc.)

- *Fertilizing substances: in* municipal (or similar) sludge, there are important amounts of nitrogen and phosphor, which facilitates its agricultural use or its use as a soil-conditioning agent.

Considering Babbit and Bauman, the mineral components for household sludge are rendered in table 1.

Table 1. Nutrient amounts in urban sludge (as compared to the dry substance)

Component	**Primary sludge (ppm)**	**Fermented sludge (ppm)**	**Activated sludge (ppm)**
Total nitrogen	45000	22500	62000
Phosphates P_2O_5	22500	11000	25000
Potassium, K_2O	5000	5000	7500
Aluminum, Al_2O_3	21000	43000	32000
Chlorides, Cl^-	5000	5000	5000
Calcium, CaO	27000	57000	17000
Magnesium, MgO	6000	10000	14000
Sodium, Na_2O	8000	15000	10000

- *Heavy metals in sludge: sewage sludge* contains small amounts of heavy metals, but when household and industrial wastewaters are treated jointly, this may lead, depending on the industrial branch, to the increase of the heavy metals contents above the limits allowed.

- *The biological and bacteriological characteristics* of sludge display biological traits similar to wastewater undergoing treatment.

Fresh sludge may contain pathogenic microorganisms, eggs and helminths that could be a threat to the environment and to the human being. By anaerobic fermentation, pathogenic bacteria (Salmonella, Koch Bacilli, Escherichia coli, etc.) and helminth eggs are destroyed, however fermented sludge must be pasteurized at a temperature of 80-90^{0}C or by lime treatment, before using it for agricultural purposes.

By sludge composting, when its humification occurs, disinfection of it also takes place.

SLUDGE TREATMENT PROCEDURES

Sludge treatment procedures target the diminution of humidity, respectively of the amount of sludge, as well as the increase of their economic value.

Sludge in urban treatment plants is generally difficult to dewater; a diminution of the water content by gravitational force (gravitational sedimentation) is possible up to a 6% dry substance content. The methods and simple separation techniques, such as the injection of flocculating substances, for instance polyelectrolyte, metal salts, etc., are enough to bring produce a 12% dry substance content in sludge, and in the natural de-watering layers, found in glass-roofed bays, approx. 50% dry substance is reached.

Using settling centrifugal tanks, chamber or conveyor-type presses, 18-40% dry substance may be obtained, but this requires high consumption of power.

Because of the price of the consumables and because of the energetic inefficiency of a sludge dewatering process, the recommendation is to gauge the sludge tank so as to contain the amount produces in approx. 90 days, following which it is drained and spread on fields as a fertilizer.

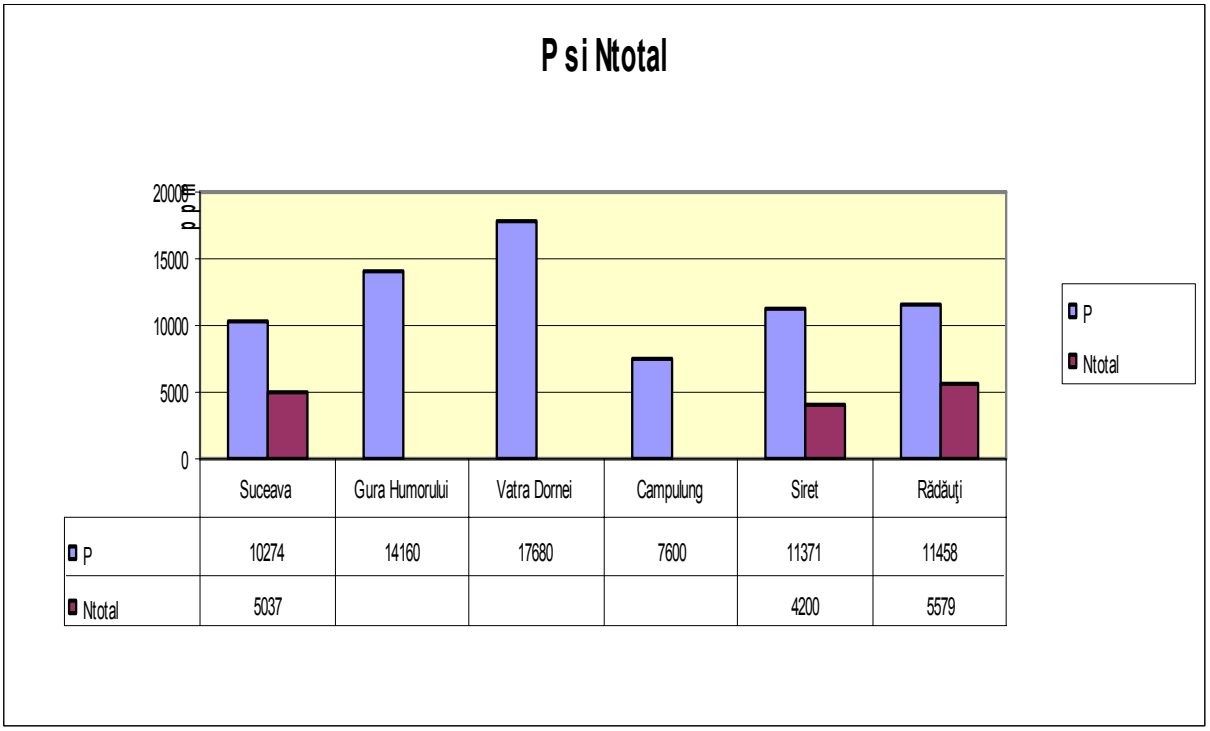
Among the administration methods for the sludge from treatment plants, depending on their characteristics, we can count: agricultural use as fertilizer, use in landscaping, use in incineration and joint incineration, anaerobic fermentation (in methane tanks and biogas production) and storage.

PHYSICAL-CHEMICAL CHARACTERISTICS OF SLUDGE FORM URBAN TREATMENT PLANTS IN THE COUNTY OF SUCEAVA.

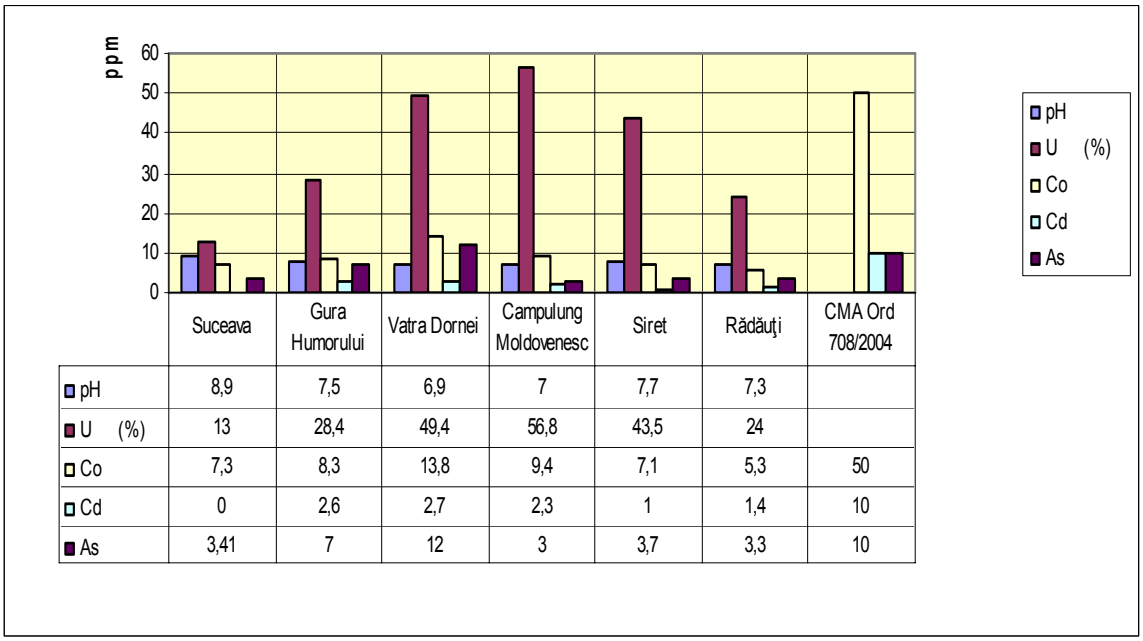
EU Directive no. 86/278/EEC concerning environmental and, particularly, soil protection, when sludge from treatment plants is used agriculturally, is translated in Romania by the joint ministry Order of MMGA (Ministry for the Environment) and MAPDR (Ministry for Agriculture, Forests, and Rural Development) no. 344/708/ 2004, with reference to the approval of the technical standards for environmental and, particularly, soil protection when sludge are used agriculturally.

Figures 1 and 2 render the physical-chemical traits of sludge from the sludge beds (where natural dewatering takes place) of the treatment plants in the towns Suceava, Siret, Radauţi, Gura Humorului, Vatra Dornei and Câmpulung Moldovenesc in Suceava county, and the evaluation against the limits admitted in the Order 344/2004 in order to facilitate the use of sludge for agricultural purposes. The samples were collected during summertime, years 2005-2007, and the physical-chemical studies were performed in the Suceava APM (Agency for Environmental Protection) laboratory, using standardized methods for all values. Metals were calculated using the atomic absorption spectro-photometric method.

The analysis of the measurements made shows that the corresponding sludge has a phosphor amount similar to the values indicated in the literature, so that they may be used as agricultural fertilizers. Exception is given by the sludge samples in the towns Vatra Dornei and Gura Humorului, which showed excess in the chrome, arsenic and nickel values, with an inhibitor function of the sludge anaerobic and aerobic fermentation process (probably because of the development of mining activities in the respective areas).

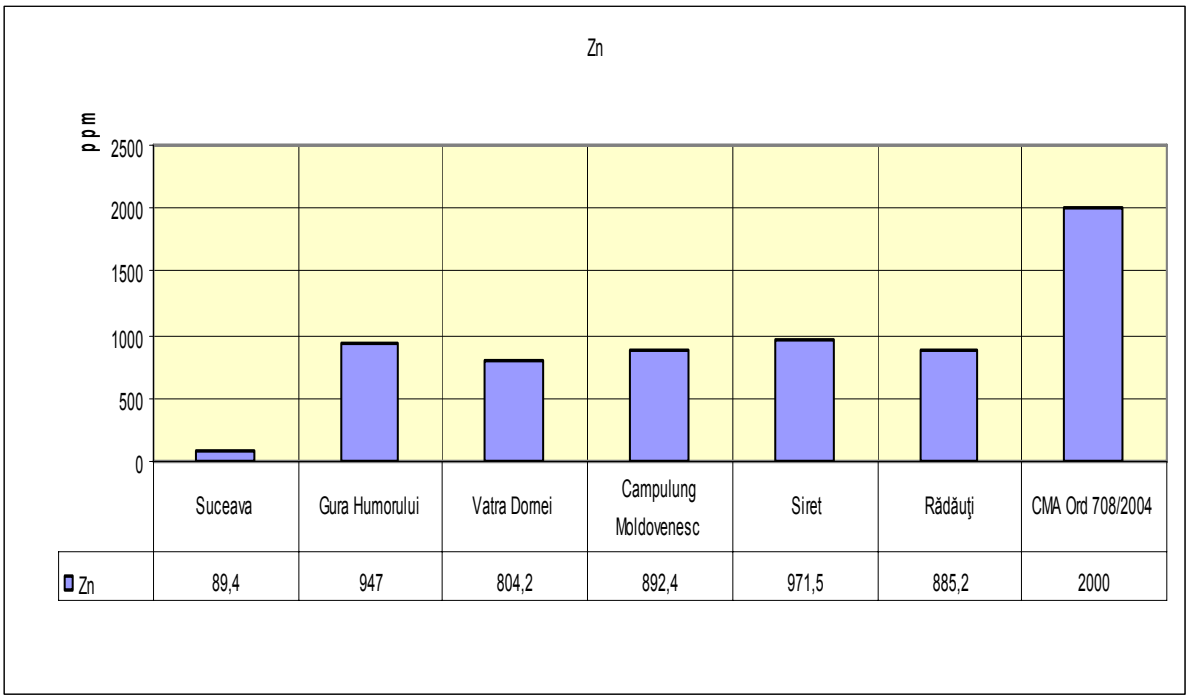


	Suceava	Gura Humorului	Vatra Dornei	Campulung	Siret	Rădăuţi
P	10274	14160	17680	7600	11371	11458
Ntotal	5037				4200	5579



	Suceava	Gura Humorului	Vatra Dornei	Campulung Moldovenesc	Siret	Rădăuţi	CMA Ord 708/2004
pH	8,9	7,5	6,9	7	7,7	7,3	
U (%)	13	28,4	49,4	56,8	43,5	24	
Co	7,3	8,3	13,8	9,4	7,1	5,3	50
Cd	0	2,6	2,7	2,3	1	1,4	10
As	3,41	7	12	3	3,7	3,3	10

Fig. 1. P and N_{total} values and respectively: pH, humidity and heavy metals (Co, Cd and As) in naturally dewatered sludge in the urban treatment plants of Suceava county.



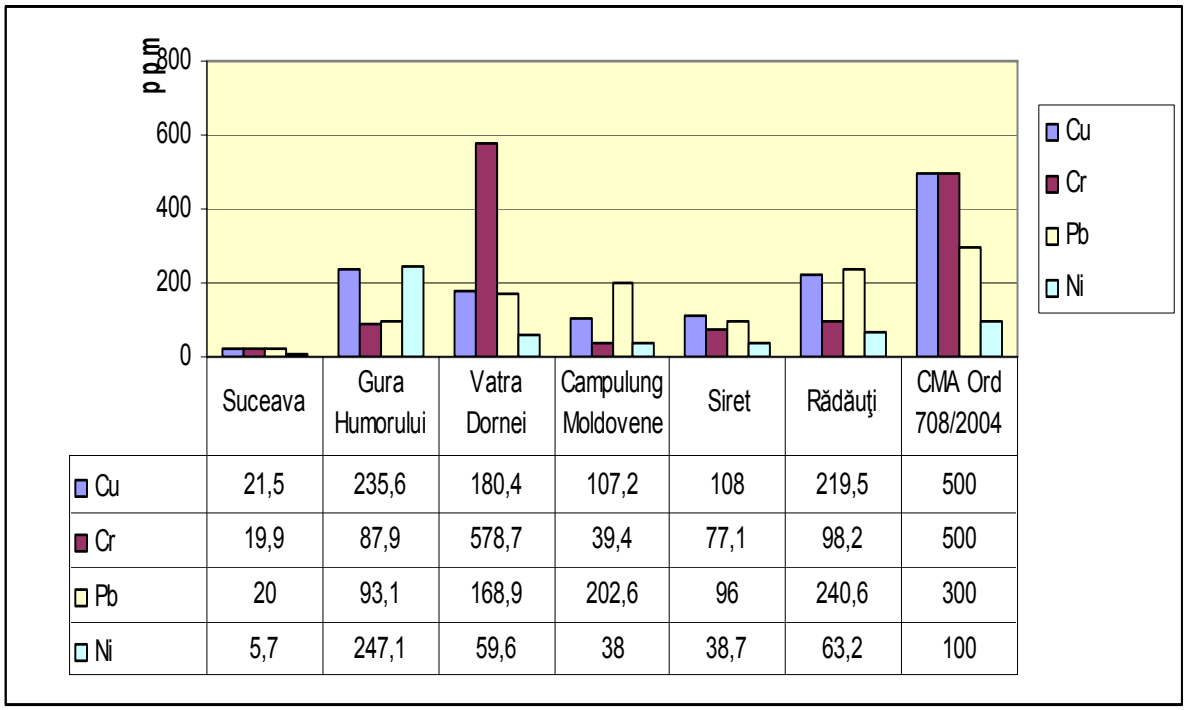


Fig. 2. Values for the indices: Zn and respectively Cu, Cr, Pb, Ni in naturally dewatered sludge

As environmental protection measures, concerning the administration of sludge from the urban treatment plants, we may count:

- prevention of illegal sludge depositing on the soil;
- prevention of sludge draining in superficial waters;
- supporting the use of non-contaminated sludge as agricultural fertilizer;
- performing sludge dewatering and pre-treatment in view of its joint incineration;
- using advanced technologies for sludge treatment in view of its subsequent exploitation.

REFERENCES

1. Dima M. (1998), Urban wastewater treatment, Junimea Publishing House, Iaşi
2. Diţoiu, Valeria, Holban, Nina, (2001), Waste Management, National Session with the participation of the International and Technical-Scientific Communication « Economic and Social Relaunch - Brăila 2001», AGIR Publishing House, page 119-121, ISBN 973-810-51-4, Bucharest.
3. Rojanschi V. ş.a. (1996)(1996), The book of the operator from the wastewater treatment plants, Bucharest.
4. Vaicum L.M.(1981) Wastewater treatment with active sludge, Academy Publishing House, Bucharest.
5. Negulescu, M., (1987), Wastewater treatment plants, Volume I, Technical Publishing House, Bucharest.
6. *** www.hydrop.pub.ro/pu7.plf- Unit processes for the treatment of sludge resulted from liquid industrial effluents, Chair of Hydraulic and Hydraulic Machines - Polytechnic University of Bucharest..
7. *** APM Suceava – Statistical yearbook regarding the environment quality.
8. *** EU Directive UE no. 86/278/EEC on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture.
9. *** MMGA and MAPDR mutual Ministry Order no. 344/708/ 2004, regarding the approval of the technical environment protection regulations and in particular of the soil, when sewage sludge is used in agriculture.

INTERACTION BETWEEN BIOCOMPOSITES AND Co ALLOYS FOR DENTAL CROWNS

Marioara MOLDOVAN[1], Cristina PREJMEREAN[1], M.TRIF[1], Laura SILAGHI DUMITRESCU[1], Olga MUSAT[2], Doina PRODAN[1], Codruta SAROSI[1], Gabriel FURTOS[1], Ioan ROMAN[3], Stanca BOBOIA[1]

[1]Babes Bolyai University - Raluca Ripan Chemistry Research Institute, 30 Fantanele, 400294, Cluj-Napoca, Romania
[2]SC Remed Prodimpex srl, 208A Bd. Basarabia, 022131, Bucharest, Romania
[3]"S.C. METAV C-D" Society, Bucharest, Romania

Abstract. By broadening of the physical-chemical link at the alloy/polymeric crown interfaces it was possible to made dental prosthetic devices for a single tooth (single crowns) with apparent improving physics-chemical and biochemical properties compared to the classical one.

The purpose of the present study is to evidence and to evaluate the efficiency of the alloy/ceramic and polymeric composite surface link, by SEM investigation. The study shows an intermediate layer whose thickness is about average 45 μm, at Co3-ceramic interfaces, as proof of a very good adherence of ceramic and metal support; also the biocomposites based on HAP achieve good adhesion compared to the related biocomposites based on cobalt alloy.

Introduction

Today, the ceramo-metal restoration is perhaps the most common fixed dental prosthetic restoration used. However, despite the many advantages of using porcelain as a dental restorative material, it has some significant drawbacks. By utilizing an underlying alloy substructure the porcelain is reinforced in such a way that it is also possible to apply these ceramo-metal restorations in the posterior area of the mouth, and fabricate entire bridges in alloy-reinforced porcelain. Developments and improvements in polymerizable resins and composite resins have enabled these materials to become increasingly attractive as alternative materials for porcelain. However, the mechanical properties of these materials do not allow their use for sole formation of entire permanent fixed restorations. Instead, they are usually either applied as a facial veneering material onto a cast crown and bridge replacement alloy structure, or they are used in combination with a thin, underlying, reinforcing alloy substructure.

The dental resin flow composites could be used for cosmetical covers, for plating of metalic components and for corection of the prothetic restauration in tehnical dentistry.

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 148 - 153, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

Materials and methods

Samples of cobalt based alloys Co1, Co2, Co3 (based on Co and Cr Mo and Ta) covered one of them with ceramics (with ZnO 25%) and another with experimental polymeric composite based on mixture of: urethanic monomers UDMA (1,6-bis (methacryloxy-2-ethoxycarbonyl amino)-2,4,4-trimethylhexane), Bis-GMA (2,2-bis [4-(2-hydroxy-3-methacryloyloxypropoxy) phenyl] propane), TEGDMA (triethylene glycol dimethacrylate) with inorganic filler consist of HAP and/or glass with zinc corresponding (Table 1). The polymeric composite was applied on alloy with and without primer- 3-methacryloyloxypropyl-1-trimethoxysilane (A-174).

Table 1: Composition of experimental composite wt[%]

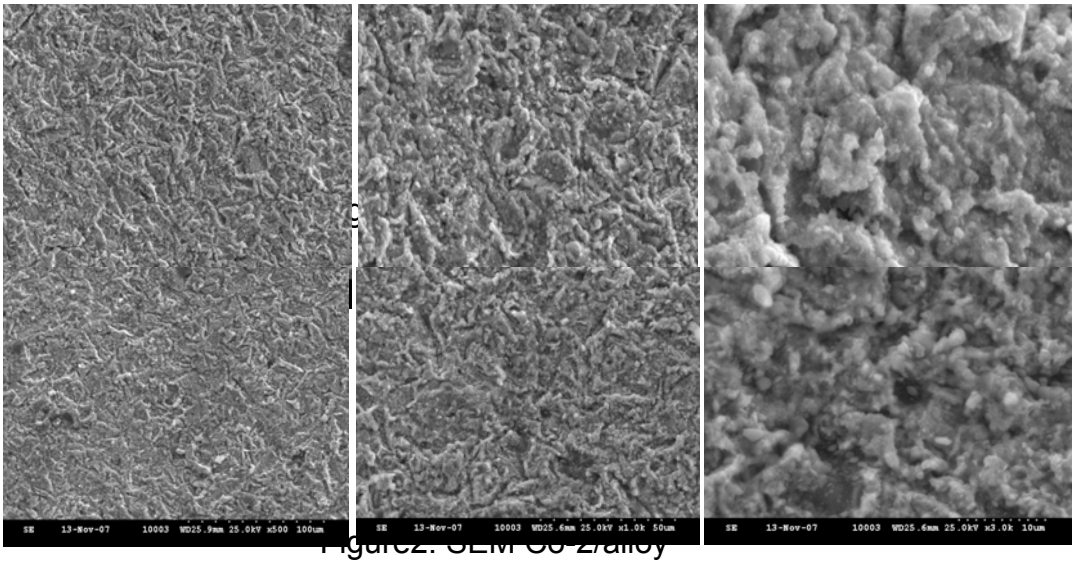
Composite	**Urethanic monomer**	**Bis-GMA**	**TEGDMA**	**Filler**
C12	-	29,4	12,6	58
C13	14,7	14	12	60
C14	14,7	14,7	12,6	58
C20	14	14,7	12,6	58

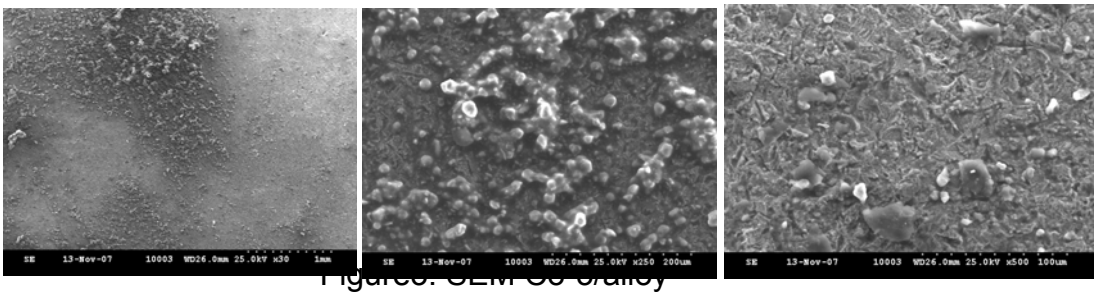
C12; C13; C14 – **based on Hidroxylapatite** (HaP)
C20- **based on glass with Zn** (40% SiO_2; 30% ZnO; 10% Al_2O_3; 10% B_2O_3; 5% CaO; 5% Na_2O melting temperature: 1350^0C

Microstructure of the alloy samples and the appearance of cobalt oxide layer formed on samples of metal from the same alloy, also microlayer acrosaj interface for ceramic dental/cobalt alloys were analysed with SEM (Hitachi S-2600 N); for interface composite /metal were analized with SEM (Quanta 130,FEI Company)

Results and discussion

The microstructure of these samples consist of a network of carbides interdendritical arranged in the matrix of solid solution on the basis of cobalt. It was highlighted two different types of carbides, microcomposition, some very rich in content of tantalum and moderate in chromium and molybdenum and others in poorer tantalum. In all three cases, the matrix is more than rich in formations of chromium carbide. SEM micrograph for the same alloy samples were highlighted the appearance of cobalt oxide layer formed on samples of metal from the same alloy, after the surgery oxidation: Thus oxide layer with a specific irregular relief; oxide particles of a micronics submicronics size (0.9-3 μ m) with spherical form was included into a discontinuous film (Co-1) (Figure 1) At Co-2 sample (Figure 2) the oxide layer is a rugged, it can be seen numerous formations island of different shapes and sizes, which included micronics and submicronics oxide particles which size and diameter are between 0.2 and 5 μ m At (Co-3) sample (Figure 3) the oxide layer is non-uniform, on his land were a number of conglomerates with particulate large (5 - 25 μ m), which are linked together by a binder. The layer of oxide has the same appearance and on the irregular surface appear isolated particles from different shapes and sizes with a diameter located in the range 10 to 30 μm.





A SEM study for the adherence of ceramic layer on three metal supports is shown in figure 4, 5 and 6

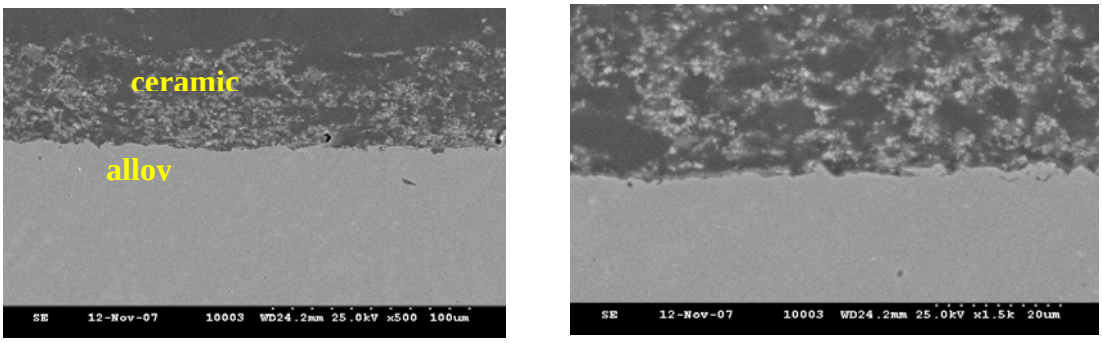


Figure 4. SEM Co-1 alloy/ceramic interference

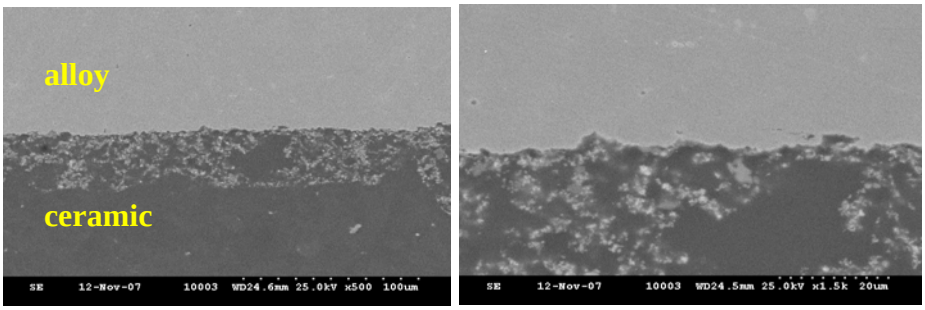


Figure 5. SEM Co-2 alloy/ceramic interference

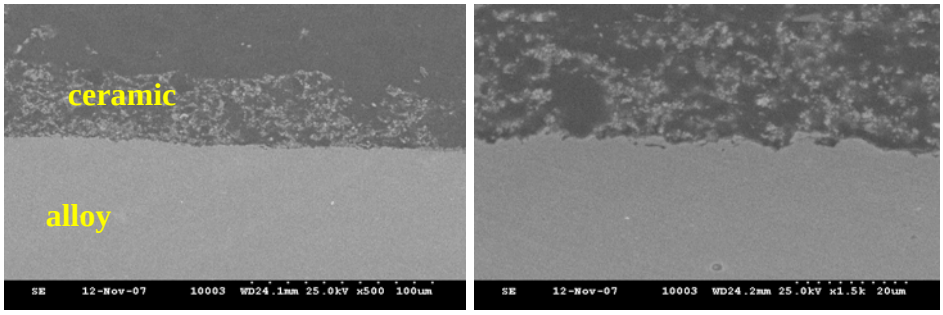


Figure 6. Co-3 alloy/ceramic interference

At the threshold between Co-1/ceramic appears an intermediate layer with a thickness of about average 60 μm, continues and non-uniform as thick (Figure 4). At the threshold between Co2-/ceramic the two materials an intermediate non-uniform layer is formed, with a thickness of about 40 μm. It can be seen that layer is intimately related on the imperfections of metallic support surface (Figure5). Intermediate layer, whose thickness is about average 45 μm, is well highlighted at Co3-ceramic in both figures. The two images are evidenced a very good adherence of ceramic and metal support (Figure6). Adherence between alloy and polymeric composite with hydroxylapatite was shown in following figure 7.

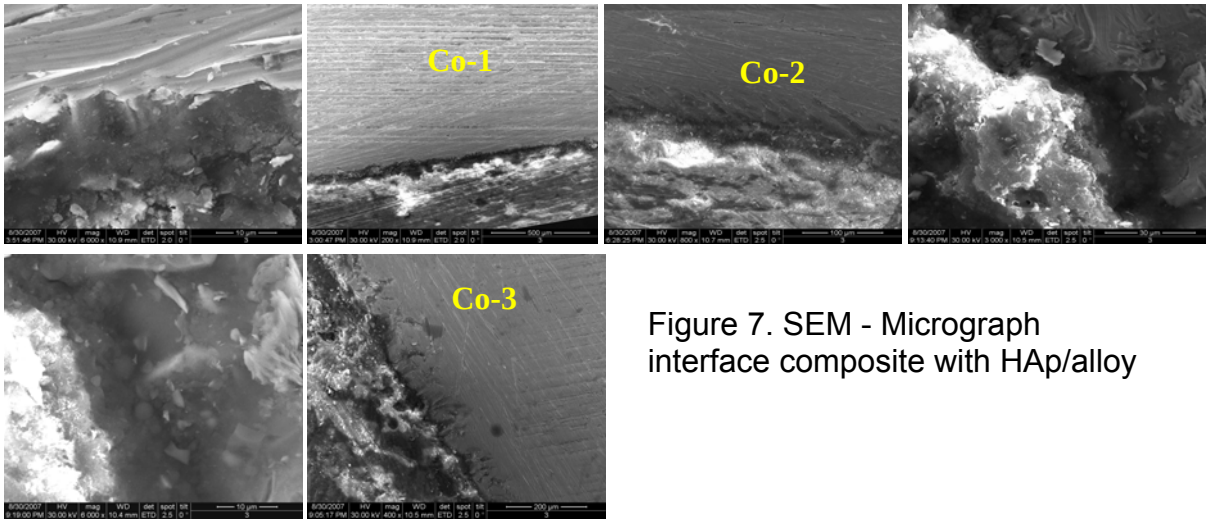


Figure 7. SEM - Micrograph interface composite with HAp/alloy

The obtained micrographs indicate a good adhesion in the case of the composite with 60 % of HAP and Co-3 alloy. Good results were obtained for C2 composite with Co-2 alloy. Co-1 alloy was not satisfactory behaved with none of the

composite. It was possible that not be made a properly surface treatment, or to be adsorbed of the impurities on the alloy surface, which made that the effective wetting area to be limited resulting defects of the composite-alloy interface. Study results reveal that exist relationship between composition, microstructure and adhesive properties of the light cured polymer composites layer coated on the metal alloy

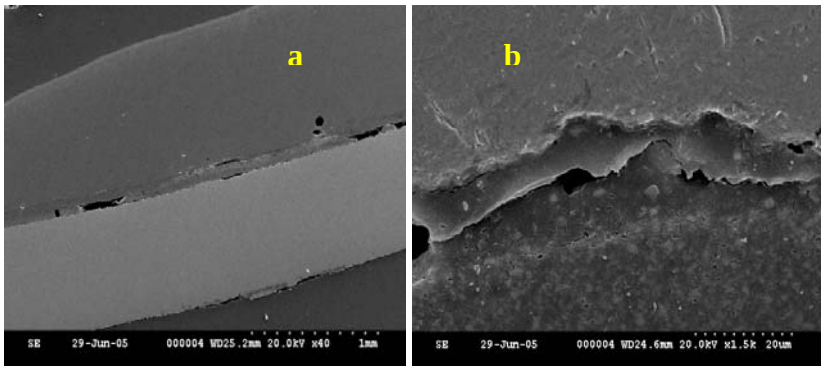


Figure 8. SEM - Micrograph interface composite C20/alloy

The composite's (C20) adherence to the metal is very good. An aspect regarding the separation limit between the two layers is presented, the metallic support being placed in the upper part of the figure. In addition the appearance of an oxide layer with a different coloration and structure from the rest of the layer was observed, right next to the support, and it may have an "fastening" layer role

Conclusion

Regarding alloy ceramic interface, studies with electron microscopy have allowed an assessment of the quality layer adherence of ceramic and metallic oxide formed. Adherence between metallic support and ceramic layer is very good throughout the entire length of support. Common to all three samples (with the submission of ceramics) is the presence of an intermediate layer between the visible metal support and ceramics submission. Oxide layers of the first two trials shows no significant differences regarding the design, quality and rugged topography. In the case of CrCo-3 proof, the oxide layer presents different aspects compared with the other two samples, consisting in differences that occurrences in the islands, agglomeration (conglomerates) covered with a film.It was been found that the direct applications of composite resins, on the surface alloy without primer using do not ensure interfacial linking of the two materials. A low rate of detachment of C20**a** experimental composite from alloy surface treated with primer 1 (figure 8b) was observed on the SEM micrographs. A corresponding primer using (figure 8a) improves adaptation to the alloy surface of resins composite; chemical links has been realized to the interface composite -metal alloy. It is estimated that composite filled with the hydroxyapatite and dental alloys basis of Cobalt may be feasible for a biocompatibilization of the dental devices made of these alloys.

Acknowledgments: Project PNII no: 71-057/2007

References

1. K.F.Leinfelder, Current status of dentin adhesive systems. Alpha Omegan, 91, 4, 1998;

2. GS. Scuster, CA. Lefebvre, JC. Wataha, SN.White, Biocompatibility of posterior restorative materials, J Calif Dent Assoc. 24, 17-31,1996;3. H. Schweikl, G.Schmalz, Toxicity parameters for cytotoxicity testing of dental materials in two different mammalian cell lines. Eur J Oral Sci 104:292-9, 1996;
4. J.Malacarne, R.M.Carvalho, M.F.de Goes, N.Svizero, D.H.Pashley, F.R.Tay, C.K.Yiu, M.Rocha de Oliveira Carrilho, Water sorption/solubility of dental adhesive resins, Dental Materials, p.973-980, 22, 2006;
6. G.C. Lopes, P.C. Cardoso, L.C.C.Vieira, L.N.Baratieri, K.Rampinelli, G.Costa, Shear bond strength of acetone-based one-bottle adhesive systems, Braz Dent J, p.39-43, 17(1), 2006;
7. V.G. Arias, I.T.Campos, L.A.F. Pimenta, Microleakage Study of Three Adhesive Systems Braz Dent J, p.194-198, 15(3), 2004.

FAST MEASUREMENT METHOD OF LEAD ISOTOPIC RATIO IN METEORITIC MATERIAL USING QUADRUPOLE ICP-MS

Claudiu TĂNĂSELIA*[1], Stanko ILIK-POPOV[2], Dana POP[3], Bela ABRAHAM[1], Cecilia ROMAN[1], Emil Cordoş[1], Tiberiu Frenţiu[4], Trajče STAFILOV[2], Leontin DAVID[5]

[1]INCDO-INOE2000 Research Institute for Analytical Instrumentation (ICIA), Donath 67, 400293 Cluj-Napoca, Romania
[2]Institute of Chemistry, Faculty of Science, POB 162, 1000, Skopje, Republic of Macedonia
[3]Museum of Mineralogy, Babeş-Bolyai University, Kogălniceanu 1, 400084 Cluj-Napoca, Romania
[4]Faculty of Chemistry and Chemical Engineering, Babes-Bolyai University, 11 Arany Janos, 400028, Cluj-Napoca, Romania
[5]Faculty of Physics, Babeş-Bolyai University, Kogălniceanu 1, 400084, Cluj-Napoca, Romania

E-mail: claudiu.tanaselia@icia.ro

ABSTRACT

Isotopic ratio measurement is a very important tool for geologists and analytical chemist, as it can be used for dating meteorite samples and also trace a particular pollution agent, since no common chemical or physical processes can tamper with isotopic ratios of a certain element, while natural processes that affects this ratio are well understood and can be very well predicted. ICP-MS instruments offers fast analysis time and relatively simple sample preparation and it also has good isotopic ratio capabilities, although performance of TIMS or SF-ICP-MS cannot be met. Lead isotopic ratio of meteoritic sample was measured in this work using a fast measurement method, with NIST 981 standard reference material used for calibration and control.

Keywords: isotopic ratio, lead, meteorite

INTRODUCTION

Mocs meteorite, classified as L5-6 chondrite fell on 3rd February 1882, in Transylvania region over an area of several dozen squared kilometers, near Mocs village (now Mociu). Since its fall, scientific investigation was performed on the meteorite, since fragments from it was distributed in museums all over the world. However, no Pb isotopic data was reported in literature for this specific meteorite. Pb isotopic data is important for determining the age and possibly the origin of the meteoritic fragment. Meteoritic control samples material from an undocumented fall was also considered for this study.

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 154 - 156, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

MATERIALS AND METHODS

Only ultrapure acids were used for all sample preparation. Ultrapure water produced in laboratory, using a Millipore Milli-Q system, was used for sample initial washing and dilutions. For all measurements, Perkin Elmer Elan DRC II ICP-MS model was used. Dynamic Reaction Cell was used in rf-only mode (vented, no reaction gas). Detector dead-time value was set to 55 ns. Instrument check was performed every working day with a solution containing 1 ppb In, 1 ppb Ce, 10 ppb Ba and 1 ppb. Oxides levels and double ionized levels were kept under 3%, background for both low and high mass was under 1 cps and all the other parameters were chosen considering the best signal/noise ratio. Pulse mode eas chosen for the detector, since it offers higher sensitivity.

The meteorite samples were weighted and each sample was grinded and turned into fine powder. The dissociation was done in a Teflon cup on a sand box. The Teflon was cleaned with *aqua regia*. The temperature of the sand box was kept constant (T = 90ºC). A modified method of four steps was used. Every step consists of combination of strong acids. HNO_3 was used in the first step. The vaporizing should continue until approximately 2ml of sample remains in an opened Teflon cup. Strong acids have been used next (HCl and HNO_3) with combination of ultrapure water. The last step is the most significant one, using a combination of HNO_3, HF, HCl and H_2SO_4 acids. Following the dissociation, the solution was filtered into flask of 25ml.

RESULTS AND DISCUSSION

Calibration with NIST 981 standard reference material was done every 5 samples. The Mocs meteoritic fragment, received from the collection of Mineralogical Museum of Cluj-Napoca, had the crust removed and was split in three parts that were digested separately. The same procedure was applied for some possible meteorite control samples (not classified or registered). Quantitative analysis revealed 0.888 mg/kg concentration of Pb in Mocs samples and 0.574 mg/kg in control samples. The obtained solutions for Mocs sample, NIST 981 SRM and control sample were read in three consecutive days.

Only ^{206}Pb, ^{207}Pb and ^{208}Pb isotopes were considered for this study. ^{204}Pb was not considered, due to its low abundance and ^{204}Hg traces found in the sample (isobaric interference) that would introduce a significant error source in the measurement. The measured isotopic ratios averages for NIST SRM, Mocs meteorite and control samples are reported in Table I. The proposed method was tested using a NIST 981 standard reference material and offers good results for lead determination. Some built-ins limitation would not yield performance figures as the traditional TIMS or MC-SF-ICP-MS would, but considering instrument's high availability, fast sample preparation and analysis time, and low costs (when compared with MC-SF-ICP-MS for instance), single collector, quadrupole based ICP-MS offers decent performance in elemental isotopic determinations.

Table I. Measured averages (± SD)

	NIST 981		Mocs fragment	Control sample
	Measured	Certified		
$^{207}Pb/^{206}Pb$	0.9142±0.0005	0.9146±0.0003	0.8656±0.0084	0.8463±0.0031
$^{208}Pb/^{206}Pb$	2.1683±0.0015	2.1681±0.0008	2.1011±0.0156	2.0630±0.0112

REFERENCES

1. Mason B., Wiik H.B., 1961. The composition of the Ottawa, Chateau-Renard, Mocs and New Concord Meteorites, American Museum Novitates, 2092:1-25

2. Hintenberger H., Jochum K.P., Seufert M., 1973. The concentration of the heavy metals in the four new antartic meteorites Yamato (a), (b), (c) and (d) and in Orgueil, Murray, Allende, Abee, Allegan Mocs and Johnstown, Earth and Planetary Science Letters 20:391-394

3. Miura Y., Iancu G. Iancu G., Yanai K., Haramura H., 1995. Reexamination of Mocs and Tauti chondritic meterotites: Classification with shock degree, Proceedings NIPR Symposium of Antarctic Meteorites, 8:153-166

4. Monna F., Loizeau, J.L., Thomas B.A., Gueguen C. and Favarger P.Y., 1998. Pb and Sr isotope measurements by inductively coupled plasma mass spectrometer: efficient time management for precision improvement. *Spectrochimica Acta Part B*, 53:1317-1333

5. Tănăselia C., Miclean M., Roman C., Cordoş E. and David L., 2008. Optimization of operating parameters of a quadrupole inductively coupled plasma mass spectrometer used in the determination of lead isotopic ratio. *Studia Universitatis Babeş-Bolyai Chemia*, LIII, 4:123-129

6. Tănăselia C., Miclean M., Roman C., Cordoş E. and David L., 2008. Determination of lead isotopic ratio in organic and soil materials using a quadrupole mass spectrometry method with fast inductively coupled plasma. *Optoelectronics and Advance Materials – Rapid Communications*, vol 2, 5:299-302

DETERMINATION OF DISSOLVED METALS IN WATER SAMPLES BY DIFFUSION GRADIENTS IN THIN FILMS (DGT) METHOD

Marin ŞENILĂ[1]*, Lăcrimioara ŞENILĂ[1], Tiberiu FRENŢIU[2], Michaela PONTA[2], Emil CORDOŞ[1], Luminiţa SILAGHI-DUMITRESCU[2]

[1]INCDO-INOE Research Institute for Analytical Instrumentation, 67 Donath, 400293, Cluj-Napoca, Romania
[2]Faculty of Chemistry and Chemical Engineering, Babes-Bolyai University, 11 Arany Janos, 400028, Cluj-Napoca, Romania

** E-mail : marinsen@yahoo.com*

ABSTRACT

The diffusive gradients in thin films (DGT) technique has invented in the mid 1990s and has rapidly become one of the most promising in situ sampling techniques for trace metal measurement in natural waters.

The method, based on diffusive gradients in thin films, offer the possibility of in situ preconcentration of metals content, by accumulation in a resin layer. Also, the method offer the possibility of speciation in function of form in that the metals exist in water: dissolved, in colloids or in suspended matter.

We evaluated the application of DGT as a tool to speciation metals in river waters affected by mining activity. Total metals, dissolved metals and labile-DGT metals concentration from two rivers with different degree of pollution with metals, Crisul Alb and Certej, were determined by ICP-MS, after preliminary preparation.

Key words: DGT, trace metals, dissolved metals, bioavailability, speciation

INTRODUCTION

Biological responses of organisms often have been shown to be related to the free-ion activity of a metal ion or to the concentration of labile metal species in solution. For these reason, speciation of trace metals is very important to evaluating their bioavailability and mobility. DGT method can be used for many different purposes, including: in situ measurements, monitoring (time averaged concentrations), speciation (labile inorganic and/or organic species), bioavailability (effective concentration) (Davison at al 1994, Gimpel et al 2003, Tusseau et al 2003, Zhang et al 1995, Zhang et al 2000, Zhang et al 2001, Scally et al, 2003).

DGT - diffusive gradients in thin-film technique comprises a layer of hydrogel (as thin film) overlying a layer of immobilised binding agent (ion-exchange resin) (Senila et al, 2008).

One of the attractive features of the DGT technique is that it does not measure all trace metal species present in natural waters. Only species that can pass trough the hydrogel film are accumulated in the binding phase (usually free metals,

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 157 - 161, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

inorganic metals and part of organic metals) and are measured as “DGT metals” (Li et al, 2005).

The device is deployed in the water for a fixed period of time and than the mass of accumulated trace metals in the binding layer is measured. Knowing the diffusion layer thickness, the mean concentration in solution during deployment can be calculated using Fick’s first law of diffusion.

In practice the DGT device is deployed for a fixed time, t. On retrieval the resin-gel layer is peeled off and the mass of the accumulated ions in this layer is measured. The ions in the resin-layer are eluted with a known volume, Ve, of solution 1M HNO_3, in the case of metals bound to Chelex. The concentrations of ions in the eluent, Ce, are than measured by a spectrometric technique. The ratio of the eluted to bound metal is known as the elution factor, fe. Values of fe of 0.8 have been reported.

The concentration in the bulk solution can be calculated from the known values of tin tickness, coefficient of diffusion and exposure area, the measured deployment time, t, and accumulated mass of metal.

We evaluated the application of DGT as a tool to speciation metals in river waters affected by mining activity. The *in situ* measurements were performed in June 2007, by placing the DGT devices for two days in Certej and Crisul Alb river water. Unfiltered, filtered and DGT water samples were collected from five sampling points in Crisul Alb and three sampling points in Certej river.

MATERIALS AND METHODS

All reagent used were of analytical grade (suprapure nitric acid). Ultarpure water was used obtained using a Millipore system. DGT devices were purchased from DGT Research Ltd (Lancaster, UK).

Instrumental determinations were done using an ICP-MS ELAN DRC II, Perkin-Elmer with reaction cell for reducing the interferences.

Clean bottles (washed with nitric acid 0.2 M) were used to collect water samples. For total metals determinations, unfiltered water was sampled and acidulated immediately on the field (pH<2) using 2-3 drops of concentrate nitric acid suprapure. The samples were digested in laboratory using the digestion unit.

For total dissolved metals determinations, the water samples were filtered on the field using syringes with filter holder (0.45 micron) and acidulated to pH<2.

DGT labile metal species were measured by deploying the DGT devices in the river water for metals accumulation. After two days, the devices were retrieved and the resins containing the retained metals were placed into plastic tubs and 10 ml nitric acid 1 M was added for metals elution.

RESULTS AND DISCUSSION

The principle of method was verified in the lab using a synthetic solution with 0.2 µg l^{-1} from each metal that was measured in the field. Good recoveries ranged between 0.85 - 1.0 were obtained for all metals.

The influence of acidic pH around 4 on the binding of trace metals by DGT technique was investigated in the lab, in order to check if these techniques can be used in waters strongly influenced by acid mine drainage (AMD), with low pH. A decreasing of recovery degrees bellow pH 4 was observed. However, at pH around 4 or higher good recoveries were obtained.

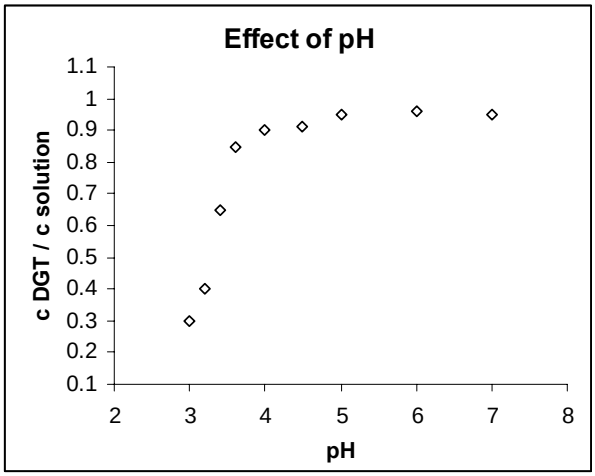


Fig. 1. Effect of pH on DGT measurement assessed by the ratio of Cd concentrations measured by DGT to the theoretic concentration from solution.

The distributions of Cu, Zn, Ni and Mn in DGT-labile form, in colloids and in particulates in Crisul Alb and are presented in figure 2.

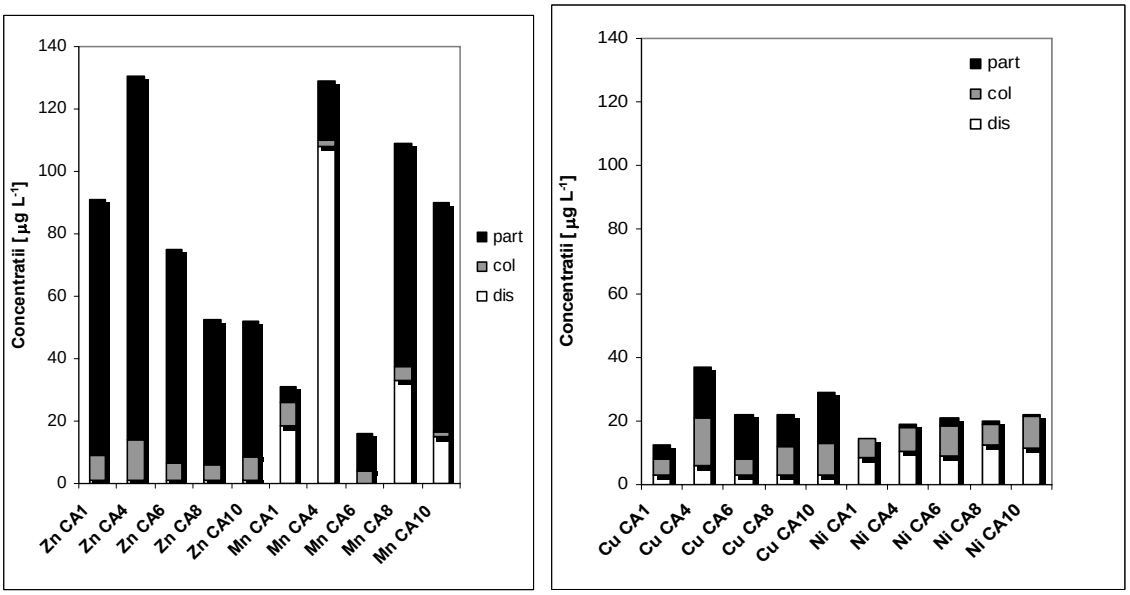


Figure 2. Metals distribution in dissolved form, in colloids and in particulate in Crisul Alb river (dis= DGT; col= filtrate – DGT; part = total - filtrate (0.45µm))

For copper, dissolved fractions amount to 10-22 % of the total concentrations. The colloid fractions cover 25-40 % and particulates comprehend 35-65 %. It may be interesting to note, that at the slightly impacted sites, CA4, CA6, CA8 and CA10, the sum of the dissolved and colloidal fractions, the conventional defined dissolved fraction, exceed the European quality standard for Cu, whereas the DGT dissolved values fall below. In the case of nickel (Ni), 40-65 % of the total contents are in the dissolved form, 30-45 % exist as colloids and only 3-10 % as particles. Only a small part of Zn is found in dissolved form (1-2%), the biggest fraction is in the particulate fraction (83-90%) and 7-15% being colloids.

The distributions of Cu, Zn, Ni and Mn in DGT-labile form, in colloids and in particulates in Certej river and are presented in figure 3.

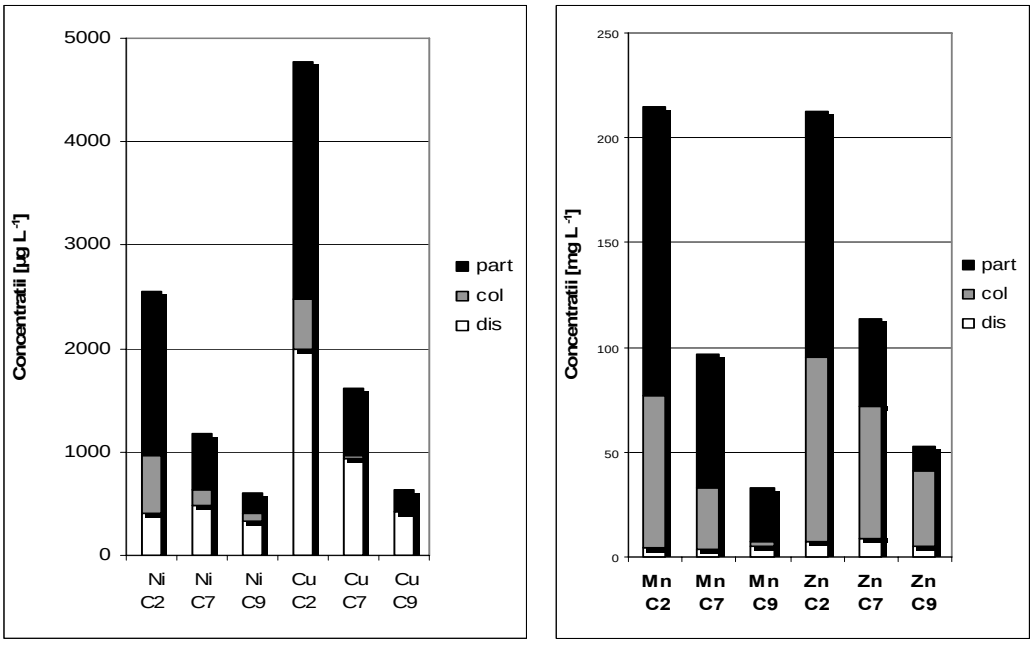


Figure 3. Metals distribution in dissolved form, in colloids and in particulate in Certej river (dis= DGT; col= filtrate – DGT; part = total - filtrate (0.45µm))

The influences of acid mine waters are evident in Certej River that is strongly affected by the outflows of underground mines. Very high concentrations of manganese, zinc, copper and nickel were found in river water. All heavy metal concentrations go far beyond the European quality standards in rivers.

Only a small part from total concentration of Mn is in dissolved form (bellow 5%), an important amount is in particulates. In case of Fe, around 50% from total metals content are in dissolved form, and a low part is in particulates. Similar distribution as in case of Mn has Zn, a small part being in dissolved form.

REFERENCES

1. Davison W., and Zhang H. - In situ speciation measurements of trace components in natural waters using thin-film gels, Nature, 1994, 367, 546-548
2. Gimpel J., Zhang H., Davison W., In-situ trace metal speciation in lake surface waters using DGT, dialysis and filtration, Env. Sci. Technol., 2003, 37, 138-146
3. Li W., Zhao H., Teasdale P. R., John R., Wang F. - Metal speciation measurement by diffusive gradients in thin films technique with different binding phases, Analytica Chimica Acta, 2005, 533, 193–202
4. Scally S, Davison W, Zhang H - In situ measurements of dissociation kinetics and labilities of metal complexes in synthetic solutions using DGT, Env. Sci. Technol., 2003, 37, 1379-1384
5. Senila M., Levei E., Roman C., Senila L., Abraham B., Emlil C. - Estimation of metals bioavailability in river waters by in situ speciation using the technique of diffusion gradients in thin films (DGT), Studia Universitatis Babes-Bolyai Chemia, 2008, 53, 65-70

6. Tusseau-Vuillemin M., Gilbin R., Taillefert M. - A dynamic numerical model to characterize labile metal complexes collected with diffusion gradient in thin films devices Env. Sci. Technol., 2003, 37, 1645-1652
7. Zhang H., Davison W. - In situ speciation measuements. Using diffusive gradients in thin films (DGT) to determine inorganically and organically complexed metals, Pure Appl. Chem., 2001, 73, 9-15
8. Zhang H., Davison W. - Direct In Situ Measurements of Labile Inorganic and Organically Bound Metal Species in Synthetic Solutions and Natural Waters Using DGT, Anal. Chem., 2000, 72, 4447-4457
9. Zhang H., Zhao F. J., Sun B., Davison B., McGrath S. P. - A new method to measure effective soil solution concentration predicts Cu availability to plants, Env. Sci. Technol., 2001, 35, 2602-2607

PLANT UPTAKE FACTORS FOR 55 ELEMENTS IN A RURAL MINING AREA, NW ROMANIA

Mirela MICLEAN *, Marin ŞENILĂ, Cecilia ROMAN, Tiberiu FRENŢIU, Emil CORDOŞ

INCDO-INOE 2000, Research Institute for Analytical Instrumentation. RO-400293, Cluj-Napoca, Donath 67.
** E-mail : mirelamiclean@yahoo.com*

ABSTRACT

In this study, plant uptake factors for 55 elements (Ag, As, Au, Ba, Bi, Cd, Ce, Co, Cs, Cu, Dy, Er, Eu, Fe, Ga, Gd, Ge, Hf, Hg, Ho, In, Ir, La, Lu, Mn, Mo, Nb, Nd, Ni, Os, Pb, Pr, Rb, Re, Rh, Ru, Sb, Sc, Sm, Sn, Sr, Ta, Tb, Te, Th, Ti, Tl, Tm, U, V, Y, Yb, Zn, Zr and W) were calculated for vegetables (potato and carrot) collected in private gardens, near sedimentation ponds, in Baia Mare area, NW Romania. The concentrations of those elements in vegetable and adjacent soil samples were determined using ICP-MS, after appropriate digestion methods.

Key words: trace, ultratrace elements, plant uptake factor, ICP-MS.

INTRODUCTION

The soil is the main source of major, minor, trace and ultratrace elements for plants, as nutrients or pollutants. Through trophic chain, the soil is also a source of these elements for human organism. Elements transfer from soil to plants is a part of their cycle in nature. This process is very complex and is governed by many natural or anthropical factors (Kabata-Pendias and Mukherjee, 2007).

Metabolic role of each element in plants is in relationship with different processes: element's absorption, its transport in plants, its concentration and species, its deficiency and toxicity, ionic competition and different interactions between elements. Some trace elements – Cu, Fe, Mn, Mo, Zn – are constituents of some enzymes and play an important role in plants metabolism (Alloway, 1995).

Generally, the environment and especially the nutrition are the major factors that induce many maladies, including cancer. Epidemiological studies incriminate trace and ultratrace elements in toxicology and oncogenesis.

The most deleterious elements for biosphere are: Ag, Au, Cd, Cr, Cu, Hg, Pb, Sb, Tl and Zn, and less toxic are: Ga, La, Nb, Sr, Ta and Zr.

For human organism, the trace elements divide in: essential (B, Br, Co, Cl, Cu, Cr, F, Fe, I, Li, Mn, Mo, Se, Si, V and Zn), possible essential (Al, Ba, Ge, Ni, Rb, Sn, Sr, Ti) and non essential (Ag, Au, Be, Bi, Cd, Cs, Hf, Hg, In, Ir, Pb, Sb, rare earth elements, Ta, Te, Tl, U, Y, Zr, and the most toxic are Be, Bi, Cd, Hg, Pb, Tl) (Kabata-Pendias and Mukherjee, 2007).

In this study, plant uptake factors for 55 elements (Ag, As, Au, Ba, Bi, Cd, Ce, Co, Cs, Cu, Dy, Er, Eu, Fe, Ga, Gd, Ge, Hf, Hg, Ho, In, Ir, La, Lu, Mn, Mo, Nb, Nd, Ni, Os, Pb, Pr, Rb, Re, Rh, Ru, Sb, Sc, Sm, Sn, Sr, Ta, Tb, Te, Th, Ti, Tl, Tm, U, V, Y, Yb, Zn, Zr and W) were calculated, by determining the concentrations of those elements in vegetable (potato and carrot) and adjacent soil.

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 162 - 165, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

MATERIALS AND METHODS

In the Baia Mare area, North-Western Romania, around an industrial complex involved in mining, metallurgical and chemical activities, the environment and particularly the soils are polluted due to the acid rains and heavy metal emissions (Lacatusu et al., 2001).

The investigated rural area is situated near three sedimentation ponds. The strong winds from the sedimentation ponds area displace fine particulates, containing heavy metals, from ponds' walls and deposited in adjacent area, contaminating the air, soil and vegetation. Also, residual water infiltration from the sedimentation pond with high heavy metal contents has a serious contribution to the pollutant dispersion in soil and ground waters, reaching also the food chain, since the residents from rural adjacent areas cultivate the vegetables and the animals feed in their own gardens. Considering all these aspects, it is important to monitor the heavy metal pollution in soils and vegetables from this area (Mihaly-Cozmuta et al., 2005, Cordos et al., 2006). The soil and vegetable samples were collected from the gardens in the localities: Tăuţii Măgherăuşi, Săsar, Bozânta Mare. The sampling area of soils and vegetables are represented in Figure 1.

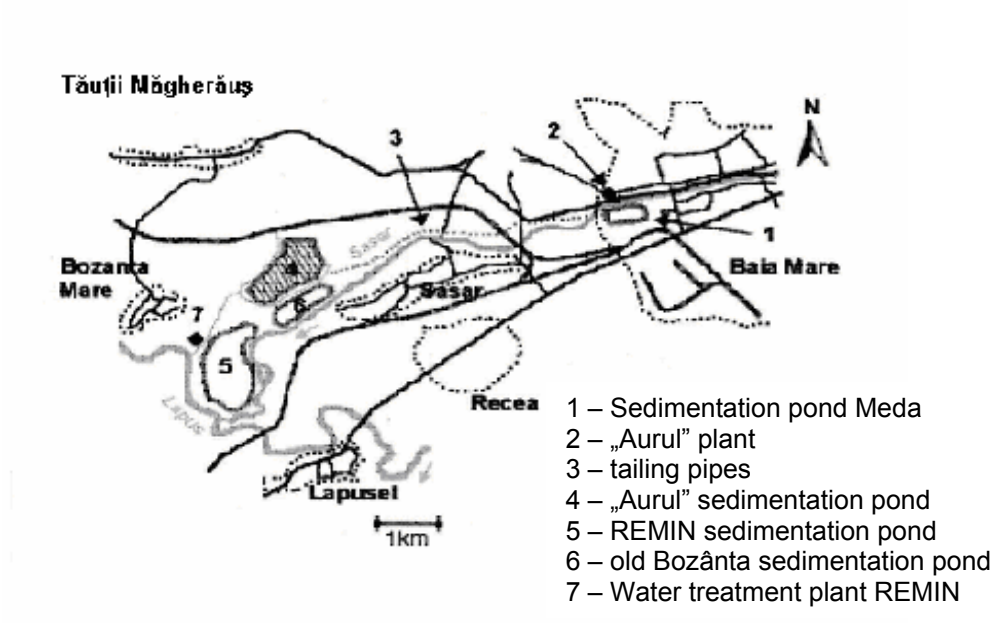


Fig. 1. The sampling area of soils and vegetables

A number of 8 potatoes, 8 carrots and 16 soil samples (the adjacent soil where vegetables are grown) were collected in the rural area, in October 2008, from the gardens belonging to the houses in the vicinity of sedimentation ponds. Soil was collected in the root zone of vegetable sample. Thus, soil and vegetable are complementarily sampled.

Soil samples were collected at 0-20 cm depth and stored in polyethylene bags for transport to laboratory. The soil samples were air-dried, mechanically ground and sieved. To determine the total metal content, fraction below 2 mm was digested in *aqua regia* (HCl 37.5% and HNO_3 65%), during 16 hours at room temperature and then, 2 hours, at reflux conditions. The extract was analyzed by inductively coupled

plasma mass spectrometer ICP-MS using a Perkin-Elemer Sciex ICP-MS, ELAN DRC II, Toronto, Canada.

The plant samples were washed in distilled water, dried at 105 °C for 24 h and grounded to obtain a homogenized powder. The samples were then digested in HNO_3 in microwave oven, using a method in three stages of pressure and times. Metals concentrations were measured using ICP-MS. The quantification was performed using an external calibration with multielemental Merck standard solution.

All chemicals used were of high-purity reagent grade. Throughout all analytical work, ultrapure water (Millipore, 18.2 MΩ/cm) was used.

ICP-MS is a powerful analytical technique for multielemental analysis of trace and ultratrace elements in biological samples with complex matrix. ICP-MS offer a rapid analysis, with excellent detection limits. The 55 elements were determined using Total Quant, a semi-quantitative method.

RESULTS AND DISCUSSION

The mean concentrations obtained for soil samples were within values reported by Kabata-Pendias and Mukherjee, 2007 for 17 major and minor elements in soils collected around the world.

The obtained mean concentrations of elements extracted in *aqua regia* from soil samples were lower than those obtained by Takeda et al., 2004, in soil samples collected in Japan, except for Zn, Ag, Cd, Sb, Bi, Th and U.

The obtained concentrations for the elements in vegetable samples varied between 0.01 µg/kg (for Ru, Lu, Hf, Re, in some samples) and 9.22 g/kg (for Fe), 10 orders of magnitude.

The concentrations of elements in studied vegetables were very variable and depended on vegetable species and their habitat (Ichihashi et al., 1992, Wyttenbach et al., 1998). In vegetable samples, concentrations of Ag, Ir and Os were below detection limits.

The mean elements concentrations in potato samples were higher than those obtained by Bibak et al., 1988, except for Au, Ge, Nb, Sb, Sn şi Te.

The mean concentrations obtained for studied elements were compared with those reported by Djingova et al., 2001 for mean concentrations of Ce, Dy, Er, Eu, Ga, Gd, Ho, La, Lu, Nd, Pr, Sm, Tb, Te, Th, Tl, U, Y, Yb in poplar's leaves (*Populus nigra*), collected in different areas of Bulgaria. The mean concentrations of investigated elements in potato samples were within values obtained by Djingova et al., 2001. In carrot samples, the mean concentrations obtained for Ce, Eu, Ga, Pr, Y were higher than those reported by Djingova et al., (2001) and the concentrations for Tb and Th were lower than in poplar's leaves.

The mean concentrations of lanthanides determined in soil and vegetable samples collected in studied rural area were lower than those reported by Ichihashi et al., 1992, in *Phytolacca americana* plants and adjacent soil, collected in Shikoku Island, in Japan.

The obtained concentrations of rare earth elements in soil and vegetable samples were highly variable, the lowest values were for Lu, and the highest for Ce.

The relationship between contaminant concentrations in soil and edible plant material is highly specific to the plant species. The relationship between contaminant concentration in edible produce and the concentration in soil is described using Plant Uptake Factor (PUF), which is defined as follows:

PUF = Concentration in edible portion of plant (mg/kg) / Concentration in soil (mg/kg)

The PUF values quantify the relative differences in bioavailability of metals to plants and identify the efficiency of a plant species to accumulate a given metal (Kachenko et al., 2004).

For rare earth elements, the mean values for plant uptake factors are in a large range of values: (3.28-14.00) x 10^{-4} for potatoes and (36.16-130.0) x 10^{-4} for carrots. For potato samples, values obtained for PUF were lower than those obtained for carrots.

The obtained values for PUF of lanthanides were compared with those obtained by Rogan et al., 2006 for rice samples, collected in Eastern Macedonia. For potatoes, the values for PUF were comparable with those for rice, and for carrots were 20 times higher than those for rice samples.

REFERENCES

1. Alloway, B.J. - Soil processes and the behaviour of heavy metals, B.J. Alloway Eds., Heavy metals in soil, Blackie, Glasgow, 1995, 35-51.
2. Bibak, A., Sturup, S., Gundersen, V. - Levels of 44 elements in organically grown potatoes *(Solarium tuberosum,* Sava), Riso National Laboratory, Roskilde, 1998, Riso-R-1060 (EN), ISBN 87-550-2403-3, ISSN 0106-2840, Information Service Department, Risø, 1998 http://www.risoe.dk/rispubl/PBK/pbkpdf/ris-r-1060.pdf (online 5.10.2009).
3. Cordos, E. A., Frentiu, T., Ponta, M., Marginean, I., Abraham, B., Roman, C. - Distribution study of inorganic arsenic (III) and (V) species in soil and their mobility in the area of Baia Mare, Romania, Chem. Spec. Bioavail., 2006, 1, 11-25.
4. Djingova, R., Ivanova, J., Korhammer, S., Markert, B. - Distribution of lanthanoids, Be, Bi, Ga, Te, Tl, Th and U on the territory of Bulgaria using *Populus nigra* „Italica" as an indicator, Sci. Total Environ., 2001, 280, 85-91.
5. Ichihashi, H., Morita, H., Tatsukawa, R. - Rare earth elements in naturally grown plants in relation to their variation in soils, Environ. Pollut., 1992, 76, 157-162.
6. Kabata-Pendias, A., Mukherjee, A. B. - Trace elements from soil to human, Springer-Verlag Berlin Heidelberg, 2007, 23-66.
7. Kachenko, A., Singh, B. - Heavy metals contamination of home grown vegetables near smelters in NSW. SuperSoil 2004: 3rd Australian New Zealand Soils Conference, 5-9 December 2004, University of Sydney, Australia.
8. Lacatusu, R., Dumitru, M., Risnoveanu, I., Ciobanu, C., Lungu, M., Carstea, S., Kovacsovics, B., Baciu, C. - Soil pollution by acid rains and heavy metals in Zlatna region, Romania, 2001, p. 817-821, In D. E. Stott, R. H. Mohtar and G. C Steinhardt (eds.). Sustaining the Global Farm. Selected papers from the 10th International Soil Conservation Organisation Meeting, May 24-29, 1999 at Purdue University and the USA-ARS National Soil Erosion Research Laboratory.
9. Mihaly-Cozmuta, A., Mihaly-Cozmuta, L., Viman, V., Vatca, G., Varga, C. - Spectrometric methods used to determine heavy metals and total cyanides in accidental polluted soils, Am. J. Appl. Sci. 2005, 2(1), 358-362.
10. Rogan, N., Serafimovski, T., Dolenec, M., Tasev, G., Dolenec, T. – The distribution of rare earth elements in paddy soil and rice seeds from Kocani Field (eastern Macedonia), Mat. Geoenviron., 2006, 53, 433-444.
11. Takeda, A., Kimura, K., Yamasaki, S. - Analysis of 57 elements in Japanese soils, with special reference to soil group and agricultural use, Geoderma, 2004,12, 291-307.
12. Wyttenbach, A., Furrer, V., Schleppi, P., Tobler, L. - Rare earth elements in soil and in soil-grown plants, Plant Soil, 1998, 199, 267-273.

GLUTARALDEHYDE DERIVATIZATION OF HEMOGLOBIN: A POTENTIAL BLOOD SUBSTITUTE

Violeta-Florina DEAC, Anamaria TODEA, Radu SILAGHI-DUMITRESCU

Department of Chemistry and Chemical Engineering, "Babes-Bolyai" University, 11 Arany Janos str, Cluj-Napoca RO-400028, Romania

e-mail : rsilaghi@chem.ubbcluj.ro

ABSTRACT

A protocol for glutaraldehyde derivatization of bovine hemoglobin is presented, which yields a reasonably stable polymer, with reduced affinity towards peroxide, increased affinity towards ascorbate, all of which would be advantageous within the framework of our working hypothesis, that a large molecular weight, increased reactivity with reducing agents and decreased reactivity with stress agents, are pre-requisites for a successful hemoglobin-based artificial oxygen carrier ("blood substitute").

Key words: hemoglobin, blood, glutaraldehyde, peroxide, ascorbate

INTRODUCTION

The blood currently used in transfusion present some problems like: stability, availability, antigens, contamination or ethics. [1] Blood substitute known also artificial oxygen carriers, are chemical or biochemical preparations aimed to replaced the donated human blood in transfusions, do not present the previous mentioned disadvantages.[1]

There are three classes of blood substitutes known. The first class of blood substitutes consists of organic compounds of the perfluorocarbon type, which, while not water-miscible, can dissolve molecular oxygen very efficiently and are quite stable from a biochemical point of view. The second class of substitutes use derivatized heme groups for transporting oxygen - following the natural example of hemoglobin, which also uses a heme group for this purpose, forming a reversible bond between the ferrous center and molecular oxygen. The third class of blood substitutes are hemoglobin-based, which constitute the major research direction in the field of artificial blood. The Hb can be obtained from outdated human blood - with limited availability in certain cases, or from bovines - with virtually unlimited availability. Pure Hb would be first option for a blood substitute preparation. This protein is in fact the most important ingredient of the blood but is toxic when is released from the cells in the organism - toxicity due by the change of oxygen affinity but the principal cause is the high reactivity towards oxidative stress agents

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 166 - 173, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

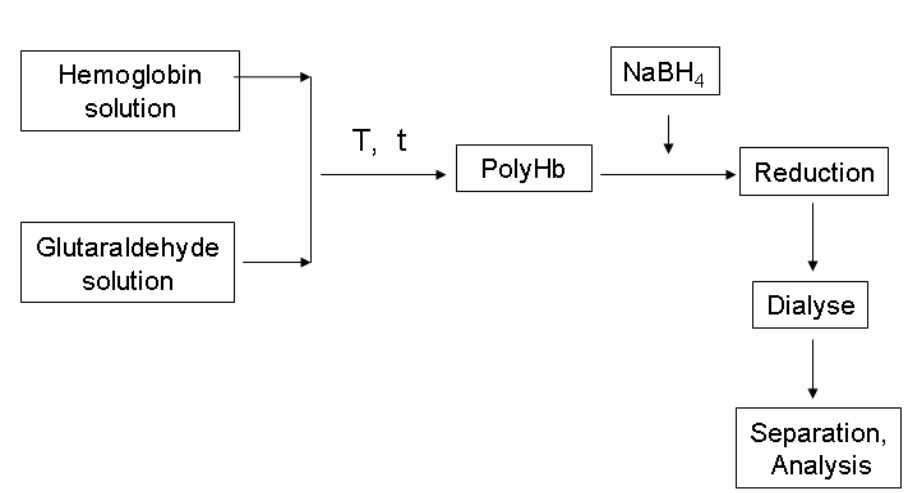
(especially hydrogen peroxide) and nitrosative stress agents (especially nitric oxide, NO) [1,2].

Intermolecular cross-linking or “polymerization” was used for the purpose increasing its size so as to prevent rapid excretion and prolong plasma half-life. Glutaraldehyde polymerization of hemoglobin has been studied before, but to our knowledge there are no published defined protocols for producing a blood substitute via this route.[3-8]

MATERIALS AND METHODS

Hemoglobin Purification. Bovine hemoglobin was purified following a variation of the general protocol of Antonini and Brunori. Bovine blood, freshly drawn on citrate, was centrifuged 15 minutes at 5000 rpm (g) to separate the red blood cells, which were then washed three times with 5 mM phosphate pH 7.4 + 150 mM NaCl. Hemoglobin concentrations in text are given per heme rather than per tetramer.

The polymerization procedure:



PolyHb was obtained by mixing a native bHb solution with glutaraldehyde; the reaction was stopped by addition of $NaBH_4$, which reduces imine bonds to stable amines and also quenches excess carbonyl groups. After quenching the, the reaction mixtures were dialyzed in 20 mM Tris buffer, 150 mM NaCl at pH 7.4 to remove excess $NaBH_4$ and side-products.

SDS-PAGE. The gels for electrophoresis were prepared in our laboratoryby standard protocols. The gels generally consist of acrylamide, bisacrylamide, SDS, Tris-Cl buffer with adjusted pH, ammonium persulfate and TEMED.

Fast protein liquid chromatography (FPLC, GE Healthcare, Suedia) was used to determine the percent of total cross-linked Hb. A Sephacryl S-300 size exclusion column was used with a mobile phase of 20 mM Tris buffer, 150mM NaCl at pH 7.4. The absorbance was monitored at 280 nm.

Ascorbate peroxidase activity was measured monitoring the time course of absorbance at 290 nm (specific for ascorbate), under conditions detailed in Figure legends. At this wavelength there are no significant changes in absorbance assignable directly to protein or hydrogen peroxide during the experiments. The presence of dehydroascorbic acid as primary reaction product was not verified directly, but was inferred by similarity with previously reported data on heme-containing ascorbate peroxidases.[9,10] Plots showing measurements of reaction

rates as a function of substrate concentrations were fit to a simple Michaelis-Menten equation, using a least-squares UV-vis spectra were recorded on Agilent 8453 (Agilent, Inc.) and Cary 50 (Varian, Inc) instruments.

Autooxidation experiments for polyHb in PBS solution, pH 7,4, 37ºC were carried out in the dark for 4 hours. The change in absorbance at 630 nm was used to determine the rate of autoxidation.

RESULTS AND DISCUSSION

Figure 1 shows an analysis by SDS-PAGE electrophoresis of the influence of glutaraldehyde concentration on the degree of polymerisation induced on bovine hemoglobin. A clear trend is seen in the band corresponding to the monomeric structure, whose intensity decreases constantly with the increase in glutaraldehyde concentration. At glutaraldehyde concentrations larger than those shown in Figure 1 the protein starts to precipitate soon after the start of the reaction; this was also accompanied by a change in colour of the hemoglobin, towards a dark brown specific to the mer form, suggesting that a high degree of polymerization may also entail an undesirable (for blood substitutes purposes) oxidation at the iron.

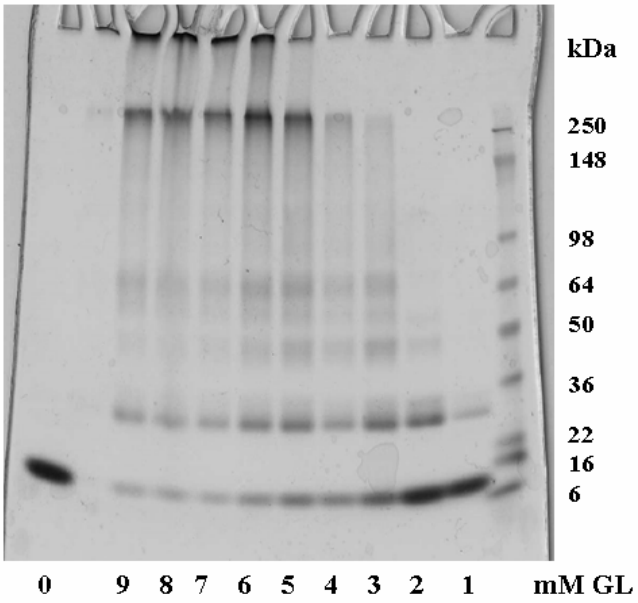


Figure 1. Influence of glutaraldehyde concentration upon polymerisation degree. SDS-PAGE 15%

The influence of hemoglobin concentration on the reaction yields is illustrated in Figure 2 in the form of an SDS-PAGE analysis. Optimal yields are seen at 1 mM Hb in the reaction mixture.

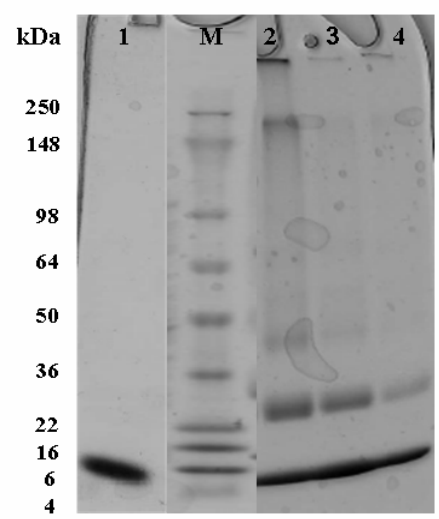


Figure 2. Influence of Hb concentration upon polymerisation degree. SDS-PAGE 15%;
1 - pure Hb, **2** - 1mM Hb, **3** - 0.8mM Hb, **4** -0.6mM Hb;

Figure 3 shows that stirring dramatically improves polymerization yields, as determined by size-exclusion chromatography.

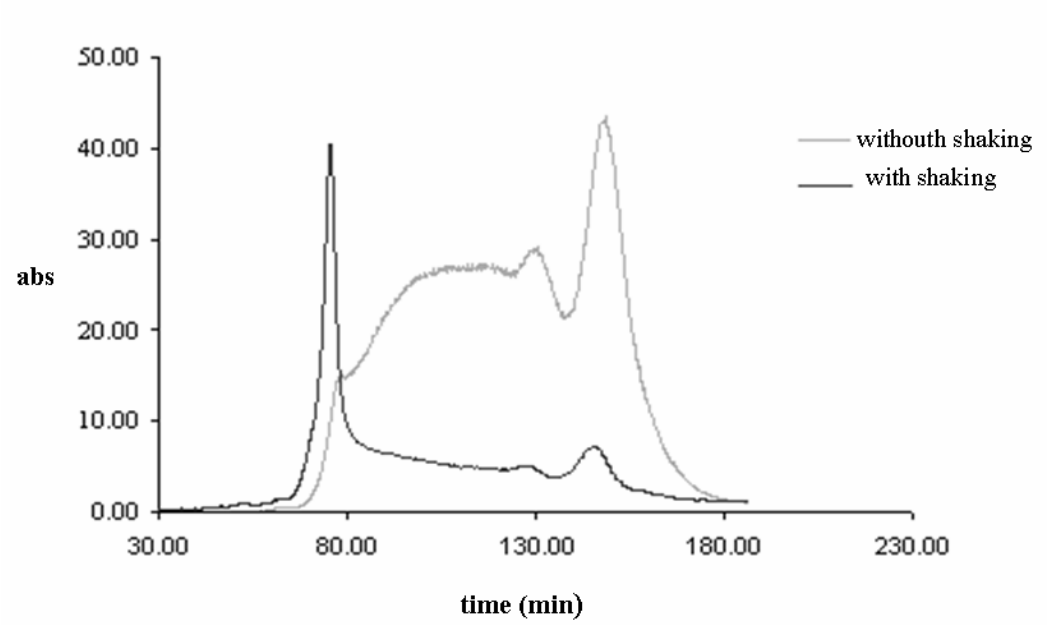


Figure 3. Size excluzion chromatographic profiles of the polymerized Hb with and without shaking.

Figure 4 shows the influence of the reaction time opn the glutaraldehyde+hemoglobin polymerization yield, as measured by size-exclusion chromatography. Longer reaction times appear to increase the yield of the highest-molecular weight polymerised fraction.

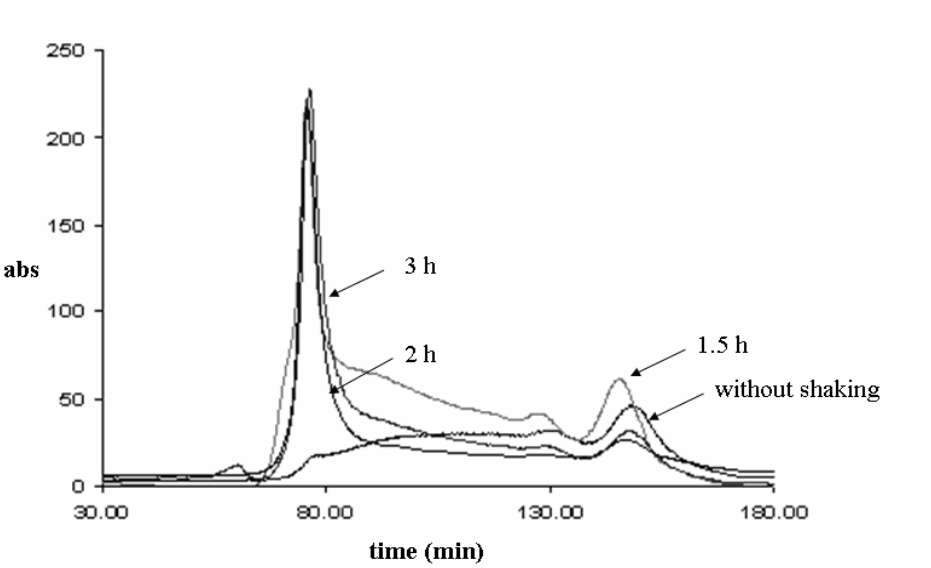
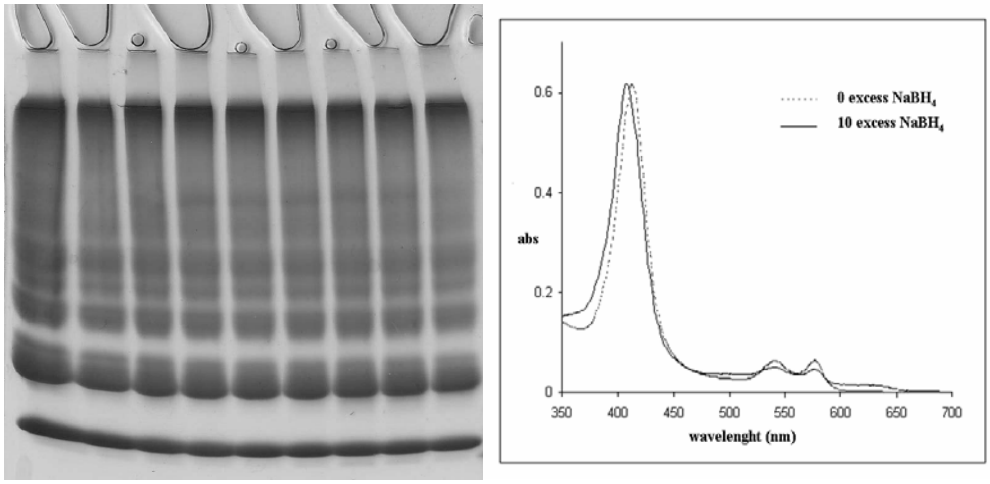


Figure 4. Size excluzion chromatographic profiles of the polymerized Hb at diferent time of reaction.

Quenching of the reaction with sodium borohydride is a redox process, which in principle may be affecting the heme active site, not just the newly-formed lysine-aldehyde bonds; indeed, reactions of the active sites in hemoproteins with borohydride are documented.[11] On the other hand, as borohydride is rapidly consumed in water in an H_2-producing reaction, an excess of borohydride is expected to be needed in order to ensure complete reduction of all mine bonds; incomplete reduction may result in subsequent slow hydrolysis of the crosslinks and a reduction in size of the polymer. For this reason, the influence of the borohydride concentration on the properties of the polymerized product was analyzed as illustrated in Figure 5. Thus, the SDS-PAGE analysis of samples differing simply in the relative excess of borohydride, incubated for 24 hours after quenching and dialysis in order to allow for possible imine hydrolysis, shows no significant differences in molecular weights, suggesting that the reaction proceeds efficiently even without excess borohydride. On the other hand, the UV-vis spectra indicate a paradoxical pro-oxidant effect of the borohydride, which is undesirable for our present purposes.



a)% excess $NaBH_4$ b)

Figure 5. Influence of sodium borohydride upon polymerized degree
a) SDS-PAGE 15% b) UV-vis spectrum

Based on the above-discussed data, a protocol was devised for bovine hemoglobin derivatization, where the hemoglobin concentration was at 1 mM, glutaraldehyde at 6 mM, with the reaction proceeding under stirring for two hours, followed by quenching with borohydride in equimolar amount to the glutaraldehyde. This product was further analyzed in terms of reactivity. Thus, Figure 6 shows UV-vis spectra illustrative for experiments where autooxidation was measured. The autooxidation percentages after a 4-hour incubation were 21.42% for native Hb and 29.24% for polyHb respectively. The slightly increased autooxidation rate in the polymerized product is undesirable, but it is expected that it can be compensated by addition of appropriate antioxidant agents.

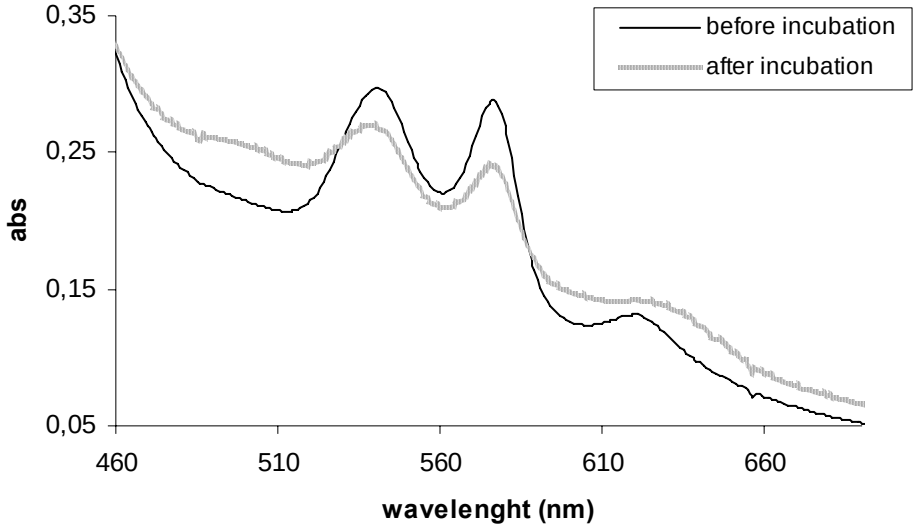


Figure 6. Autooxidation of polymerized bovine Hb releaved by UV-vis spectra

Figure 7 illustrates oxygen binding curves for native and polymerized bovine hemoglobin. The cooperativity effect is seed to disappear (n=1.9 measured here for native bovine hemoglobin vs. n=1 for the polymerized version), with the affinity towards molecular oxygen increasing by a factor of 10.

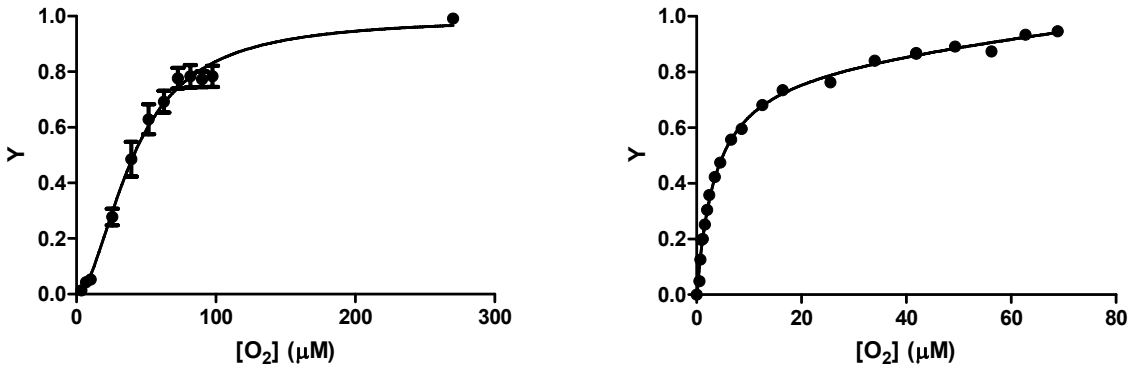


Figure 7. Oxygen saturation curves of a) native bovine Hb and b) polymerized bovine Hb

Figure 8 illustrates the affinity towards peroxide of the native vs. polymerized bovine hemoglobin, as measured via an ascorbate peroxidase assay.[9] A twofold decrese in reactivity towards peroxide appears to be the effect of glutaraldegyde polymerization.

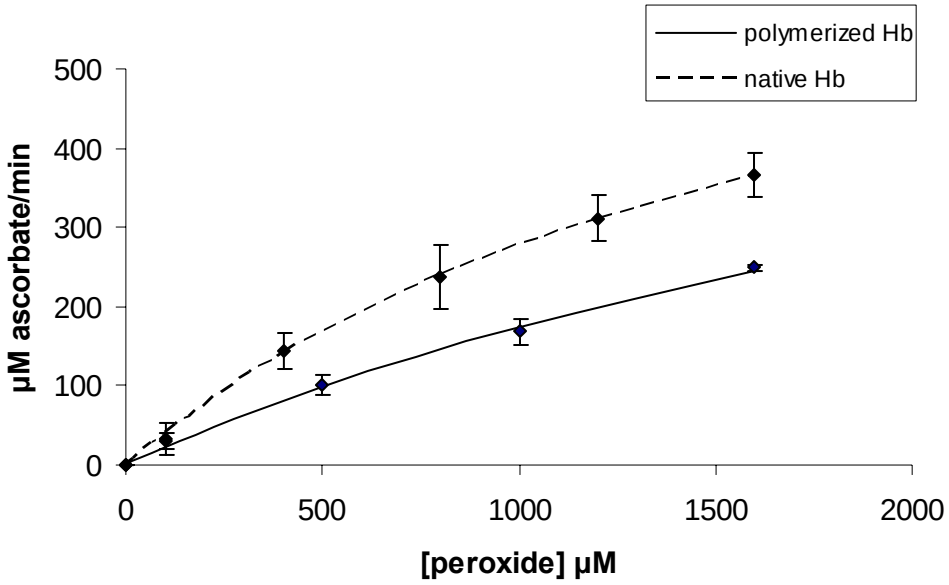


Figure 8. Saturation behaviour with respect to peroxide in the ascorbate peroxidase activity of native and polymerized bovine Hb. Shown is a fit to the Michaelis-Menten equation, suggesting a Km of 1835 µM for native Hb and Km of 3490 µM for polymerized Hb . Conditions: Hb 12 µM, acetate 50 mM pH 5, ascorbate 350 µM.

Figure 9 illustrates, using again ascorbate peroxidase kinetics, an ~5-fold increase in affinity towards peroxide for polymerized hemoglobin compared to the native form.

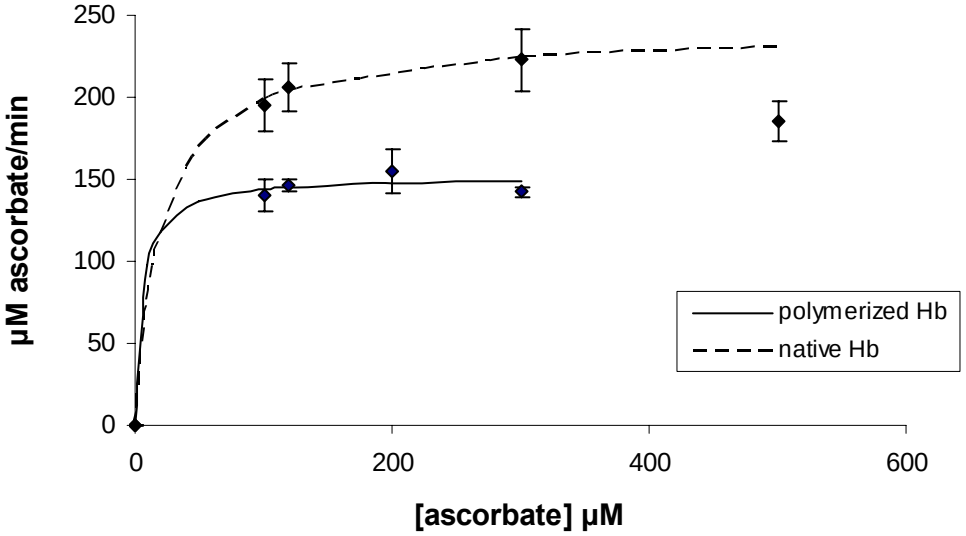


Figure 9. Saturation behaviour with respect to ascorbate in the ascorbate peroxidase activity of native and polymerized bovine Hb. Shown is a fit to the Michaelis-Menten equation, suggesting a Km of 21 µM for native bovine Hb and Km of 5,5 µM for polymerized bovine Hb, implying that bovine Hb is the protein with the highest affinity for ascorbate, of all proteins known to date. Globins thus have Km values lower than those of the bona fide ascorbate peroxidase (389 µM). Conditions: Hb 12 µM, acetate 50 mM pH 5, peroxide 800 µM.

To conclude, glutaraldehyde reticulation of bovine hemoglobin was analyzed, with the purpose of setting up a protocol for production of a polymerized form with decreased reactivity towards oxidizing agents, for potential use as a blood substitute.

Acknowledgements: the work shown here has been supported by the Romanian Ministry for Education and Research (ID565/2007).

References:

1. Alayash, A. I. Oxygen therapeutics: can we tame haemoglobin? *Nat Rev Drug Discov*, **2004**, *3*, 152-9
2. Doherty, D. H.;Doyle, M. P.;Curry, S. R.;Vali, R. J.;Fattor, T. J.;Olson, J. S.,Lemon, D. D. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. *Nat Biotechnol*, **1998**, *16*, 672-6
3. Riess, J. G. Oxygen Carriers ("Blood Substitutes") - Raison d'Etre, Chemistry, and Some Physiology. *Chem. Rev.*, **2001**, *101*, 2797-2919
4. Doyle, M. P.;Apostol, I.,Kerwin, B. A. Glutaraldehyde Modification of Recombinant Human Hemoglobin
Alters Its Hemodynamic Properties. *The Journal of Biological Chemistry*, **1999**, *274*, 2583–2591
5. Eike, J. H.,Palmer, A. F. Effect of Cl- and H+ on the oxygen binding properties of glutaraldehyde-polymerized bovine hemoglobin-based blood substitutes. *Biotechnol Prog*, **2004**, *20*, 1543-9
6. Eike, J. H.,Palmer, A. F. Effect of glutaraldehyde concentration on the physical properties of polymerized hemoglobin-based oxygen carriers. *Biotechnol Prog*, **2004**, *20*, 1225-32
7. Eike, J. H.,Palmer, A. F. Oxidized mono-, di-, tri-, and polysaccharides as potential hemoglobin cross-linking reagents for the synthesis of high oxygen affinity artificial blood substitutes. *Biotechnol Prog*, **2004**, *20*, 953-62
8. Eike, J. H.,Palmer, A. F. Effect of NaBH4 concentration and reaction time on physical properties of glutaraldehyde-polymerized hemoglobin. *Biotechnol Prog*, **2004**, *20*, 946-52
9. Cooper, C. E.;Silaghi-Dumitrescu, R.;Rukengwa, M.;Alayash, A. I.,Buehler, P. W. Peroxidase activity of hemoglobin towards ascorbate and urate: a synergistic protective strategy against toxicity of Hemoglobin-Based Oxygen Carriers (HBOC). *Biochim Biophys Acta*, **2008**, *1784*, 1415-20
10. Lad, L.;Mewies, M.;Basran, J.;Scrutton, N. S.,Raven, E. L. Role of histidine 42 in ascorbate peroxidase. Kinetic analysis of the H42A and H42E variants. *Eur J Biochem*, **2002**, *269*, 3182-92
11. Silaghi-Dumitrescu, R. Nitric Oxide Reduction by Heme-Thiolate Enzymes (P450nor): A Reevaluation of the Mechanism. *Eur. J. Inorg. Chem.*, **2003**, 1048-1052

UNUSUAL METAL OXIDATION STATES IN METALLOPROTEINS AND RELATED COMPLEXES: FROM DEGENERATE ORBITALS TO APOPTOSIS

Radu SILAGHI-DUMITRESCU,[1]* Cristina BISCHIN,[1] Florina DEAC,[1] Zoltan KIS,[1] Augustin MOT,[1] Sergei V. MAKAROV[2]

[1] Department of Chemistry and Chemical Engineering, "Babes-Bolyai" University, 11 Arany Janos str, Cluj-Napoca RO-400028, Romania
[2] State University of Chemistry and Technology, Engels str.,7, Ivanovo, Russia

e-mail : rsilaghi@chem.ubbcluj.ro

ABSTRACT

Recent results from our group on super-reduced (Fe(0), Fe(I)) as well as on high-valent Fe(IV) are reviewed. These include density functional calculations, UV-vis, NMR and EPR spectra, stopped-flow kinetics, and enzyme kinetics. The targets include globins (with relevance for blood substitute analysis, among others), cytochrome c (with possible relevance for apoptosis) and various model compounds.

Key words: iron, ferryl, peroxide, ascorbate, Fe(IV), Fe(V), Fe(I), Fe(0), heme

Unusual valence states for iron in proteins have been known for a long time, as formally Fe(IV) and Fe(V) compounds of heme proteins are among the species eliciting interest since the foundations of bioinorganic chemistry, almost 100 years ago.[1-3] Interest in such states continues to date, as many key reactive intermediates in protein as well as small "model" compound catalysis are known or presumed to entail such unusual metal oxidation states. Examples in this respect are cytochromes P450, chloroperoxidases, peroxidases, catalases, or non-heme dioxygenases (where Fe(IV) and Fe(V) are implicated),[4-11] but also hydrogenases (where Fe(I) has been implicated).[12] Here, recent results from our group on high- as well as low-valent states of iron in proteins and related complexes are reviewed.

Fe(0), Fe(I)

We have recently investigated the interaction of heme-containing proteins (myoglobin, hemoglobin, cytochrome c) as well as of protein-free iron containing macrocycles, with sulfoxylate, a powerful chemical reducing agent of a standard reduction potential of approximately -1.2 V; evidence for carbon dioxide reduction to carbon monoxide was found in these experiments, which may implicate iron forms other than ferrous and ferric.[13] EPR spectra assigned to super-reduced Fe(I) states of the macrocycles were recorded, cf. Figure 1;[13] although the signal obtained upon reduction is rather small, its g-value matches closely those observed much more clearly upon reduction of model compounds, where Fe(I) states have

„Metal Elements in Environment, Medicine and Biology", Tome IX, pp. 174 - 182, Cluj University Press, „Babeş-Bolyai" University, Cluj-Napoca, 2009
Proc. of 9th Int. Symp. of Roumanian Academy - Branch Cluj-Napoca and Branch Timişoara, 2009, October 16-17, Cluj-Napoca, Roumania

been confirmed independently by UV-vis (Figure 1) and electrochemical measurements (data not shown).

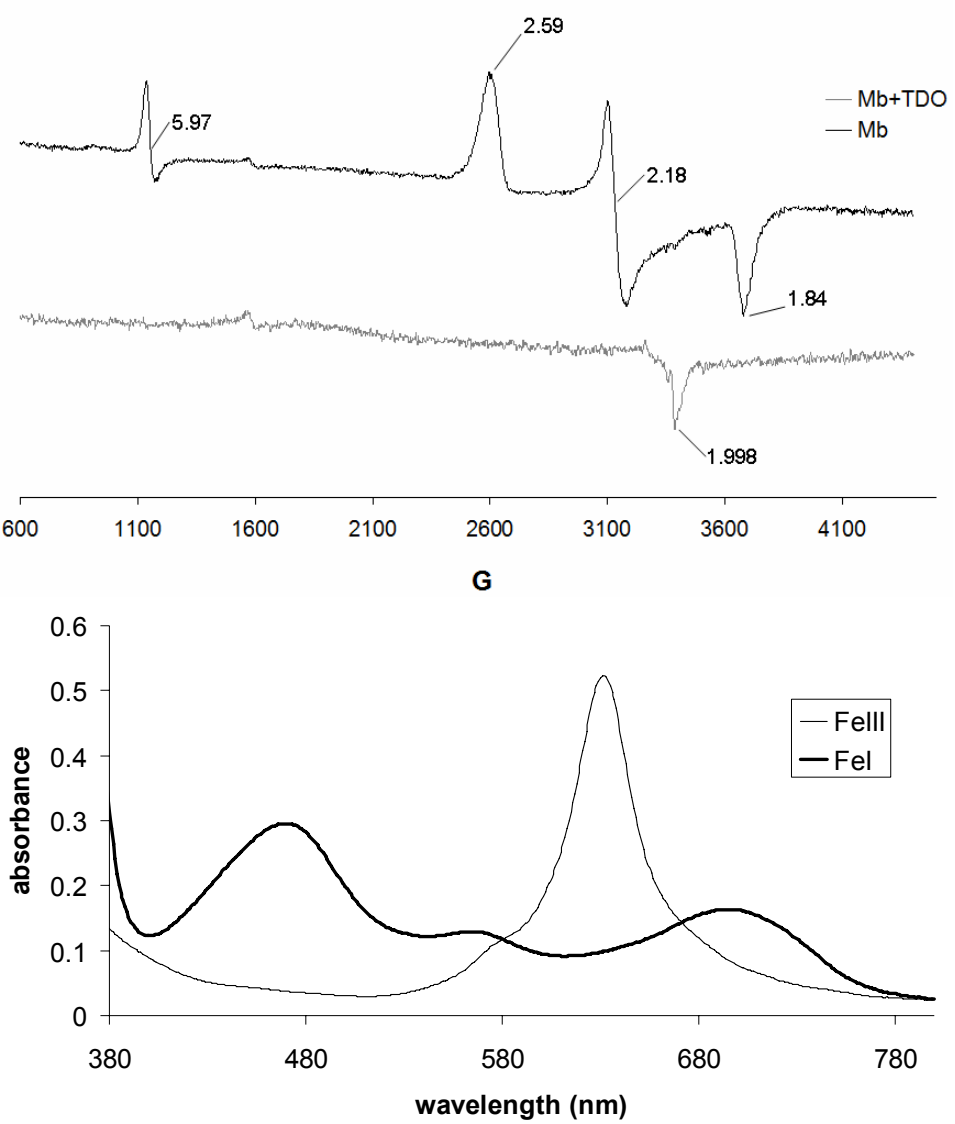


Figure 1. Top panel: 10K EPR spectra of met myoglobin before and after treatment with sulfoxylate-releasing agent thiourea dioxide (TDO). The g-values of the signals are indicated. [13] Bottom panel: UV-vis spectra of a phthalocyanine at neutral pH in aqueous medium and room temperature, in ferric and Fe(I) states, where Fe(I) is also generated by TDO treatment. The drastic change in intensity at the Soret band in Fe(I) compared to Fe(III) may indicate a change in phthalocyanine orbital occupation, and hence a reduction centered on the macrocycle, implying a macrocyle anion radical coupled to an Fe(II) state. The EPR spectrum of this state (not shown) resembles that of myoglobin shown in the top panel.

Makarov and co-workers likewise found, using UV-vis spectroscopy, that Fe(0) states can be generated in iron phthalocyanines at room temperature with chemical reducing agents.[14] Such states can also be produced, albeit in lower yields and for shorter times, electrochemically[15] or by pulse radiolysis.[16] We have recently analysed the electronic structures of a series of heme Fe(0) models, based on results implying Fe(0) macrocycles in carbon dioxide reduction.[13,16,17] Electronic structures were assigned to various models, ranging from partial Fe(0)

character to Fe(I) + porphyrin anion radical and to clean Fe(II) descriptions, with the porphyrin ligand often delocalizing the excess electrons. A heme iron adduct with carbon dioxide was predicted, which to our knowledge was unprecedented both in terms of its unique Fe-C bonding and in terms of its distinct Fe(0) character when compared to all other models examined so far. Figure 2 shows frontier orbitals for two models of these series – the six-coordinate CO_2 adduct and a pentacoordinate model. While in the carbon dioxide model 4 molecular orbitals are found featuring occupancies at the iron in the range 0.7-0.9 on each manifold, implying 8 d electrons and hence Fe(0), the pentacoordinate mode clearly has two empty d orbitals and is therefore Fe(II) with a two-electron-reduced porphyrin ligand.

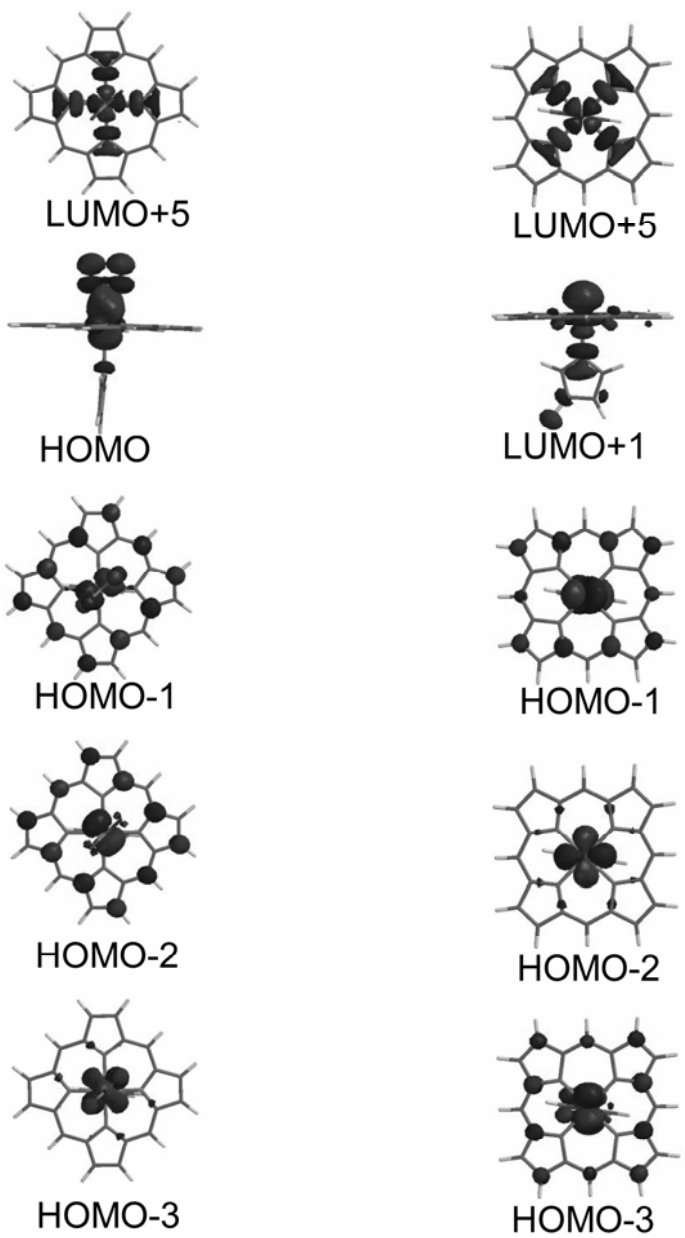


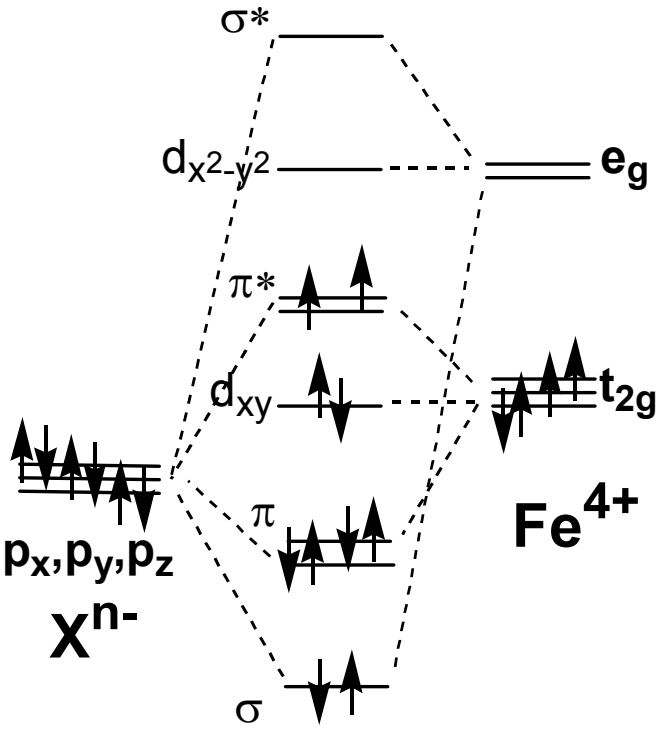
Figure 2. Metal-localized frontier orbitals for models of a putative Fe(0) state in hemoglobin, either in a pentacoordinated state (right) or six-coordinated state with carbon dioxide as a ligand (left).

The data shown in Figure 2, indicating a tendency for the reduction of Fe(II) to be ligand-centered, is verified by a range of unpublished results from our group

with macrocycles other than heme, metals other than iron, and ligation systems other than those shown in Figure 2 (data not shown).

Fe(IV), Fe(V)

High-valent iron complexes at the formal Fe(IV) and Fe(V) oxidation levels are well established as intermediates in heme and non-heme proteins as well as in other catalytic processes. An oxo ligand often accompanies and presumably stabilizes the high-valent iron; nitrido ligands have also been reported with Fe(IV) and Fe(V) systems.[7] A salient feature of high-valent Fe=O and Fe≡N adducts is the strong covalence between the metal and the oxo/nitride moiety, as evidenced by experimental and theoretical studies;[7] a schematic MO diagram for a general S=1 Fe(IV)-O/N/S system in an octahedral ligand field is shown in Scheme 1. For Fe(IV)=O systems, previous theoretical studies employing density functional (DFT) methods have indicated that the electrons in the σ and π bonding system, and in particular the two electrons in the π* orbitals, are essentially equally shared between Fe and O, in a bonding picture reminiscent of the dioxygen molecule; similar pictures were obtained from earlier semiempirical and CASSCF calculations.[7] On the other hand, more recent post-Hartre-Fock (HF) calculations have indicated that the highly-covalent Fe(IV)-O^{2-} picture drawn from DFT may be artefactual, and that the electronic structure of these systems may well involve Fe(III) bound to an oxyl radical.[7,18] This difference between DFT and non-DFT results is illustrated in Figure 3. Furthermore, detailed analysis of the DFT orbitals eventually indicates that they are also consistent with a non-Fe(IV) description for the iron in such formally Fe(IV)=X systems.[7]



Scheme 1

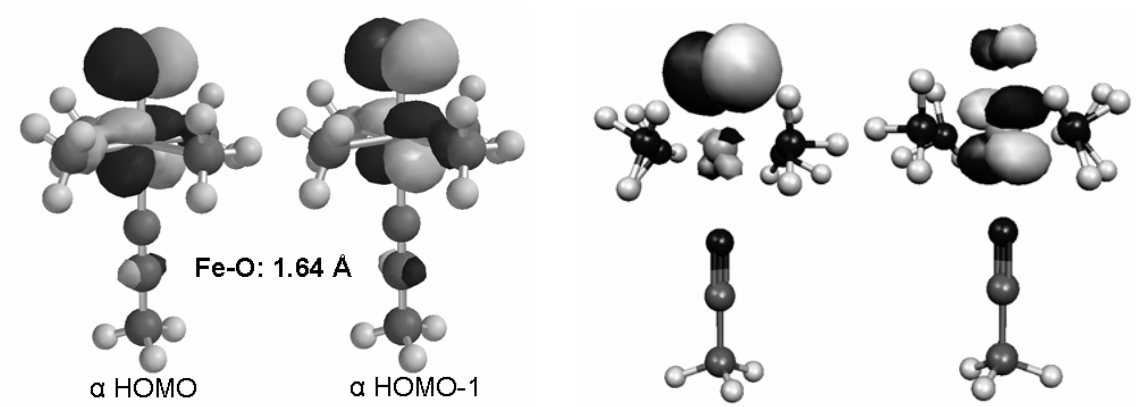


Figure 3. Side-by side comparison of DFT (left) and Hartree-Fock (right) results on the two iron-oxygen π* orbitals (cf. Scheme 1) for an octahedral ferryl model, with four equatorial amine ligands and an axial acetonitrile, illustrating a high degree of covalence.[19,20]

High-valent centers such as illustrated in Figure 3 are found in model compounds, but a strong reason for their study has been the idea, proven for some systems and awaiting proof for others, that Fe(IV) and Fe(V) systems with oxo ligands are implicated in protein catalyzed oxidations, via mechanisms illustrated in Figures 4 and 6.

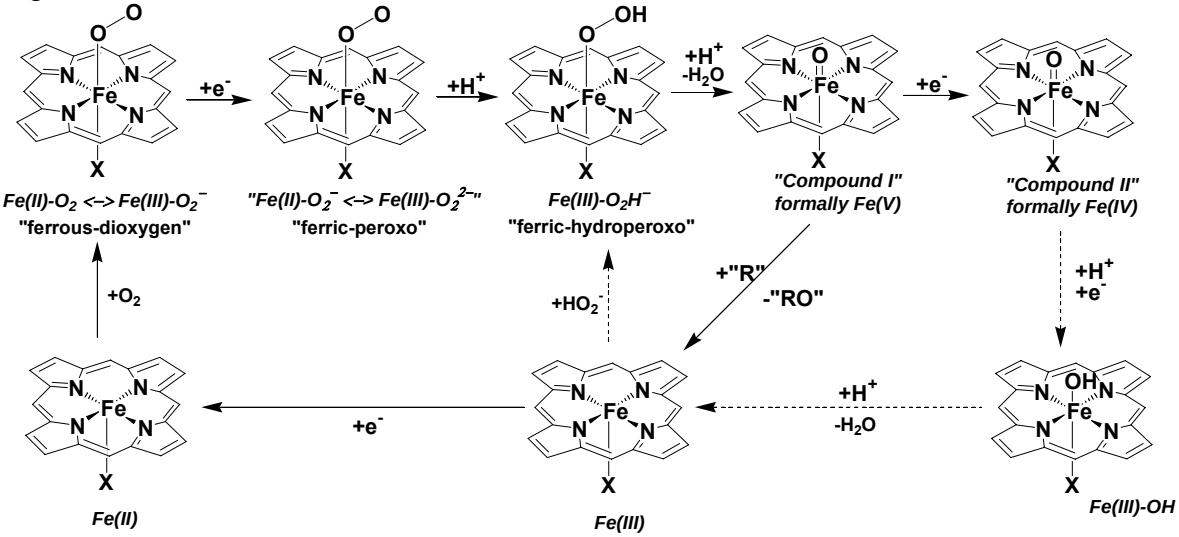


Figure 4. Physiologically-relevant reactions of active site hemes with O_2 and/or H_2O_2. "R" denotes an organic substrate molecule. "X" may be a protein-derived cysteinate, tyrosinate or histidine ligand. Various pathways may be preferred by various enzymes/proteins (e.g., peroxidases, catalases, cytochromes P450, globins), [4-11] depending on axial ligation ("X")) as well as on protein environment.

The high-valent states labeled Compound I and Compound II in Figure 4 were long believed to be stabilized by the heme ligand, which in fact delocalizes one of the oxidizing equivalents in Compound I, so that this species is typically described as Fe(IV)+porphyrin cation radical, as opposed to Fe(V). We and others have proposed that at least some of the ferryl species are in fact protonated under physiological conditions. Figure 5 illustrates stopped-flow UV-vis data coupled with kinetic measurements, indicating that a protonation event controls the reactivity of the ferryl species in globins, where this phenomenon has physiological relevance; DFT results as well as MCD (magnetic circular dichroism) measurements indicate that this protonation occurs at the oxo ligand of the species labeled Compound II in

Figure 4.[4] Others have also indicated, with more direct data (EXAFS, resonance Raman, Mössbauer,[21-24] X-ray crystallography[25-28]) that such protonation occurs in a range of heme ferryl species. However, a debate remains on whether protonation occurs for all ferryls or not.[4]

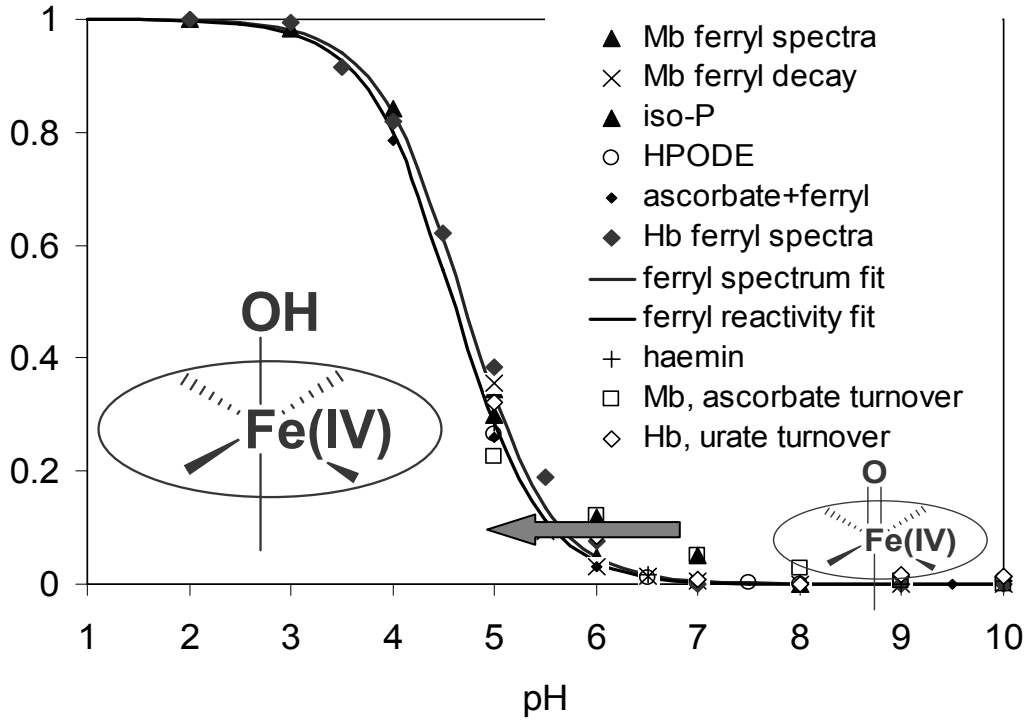


Figure 5. Overlay of UV-vis titration of globin ferryls (from stopped-flow experiments) with turnover ferryl-related reactivity data, indicating a ferryl protonation event dictates reactivity in these systems.

Non-heme iron dioxygenases present an interesting mechanistic problem, illustrated in Figure 6. Thus, these systems are expected to reach an oxidation state similar to that of Compound I form Figure 4, which would be formally Fe(V). However, while in the heme version of Figure 4 one oxidizing equivalent resides on the macrocycle, the non-heme systems must either employ a true Fe(V) center, or avoid the high-valent pathway altogether by directly reacting the peroxide intermediates with the substrates. The peroxo and Fe(V) intermediates have not been observed yet, but our theoretical DFT predictions favor the Fe(V) pathway.[29]

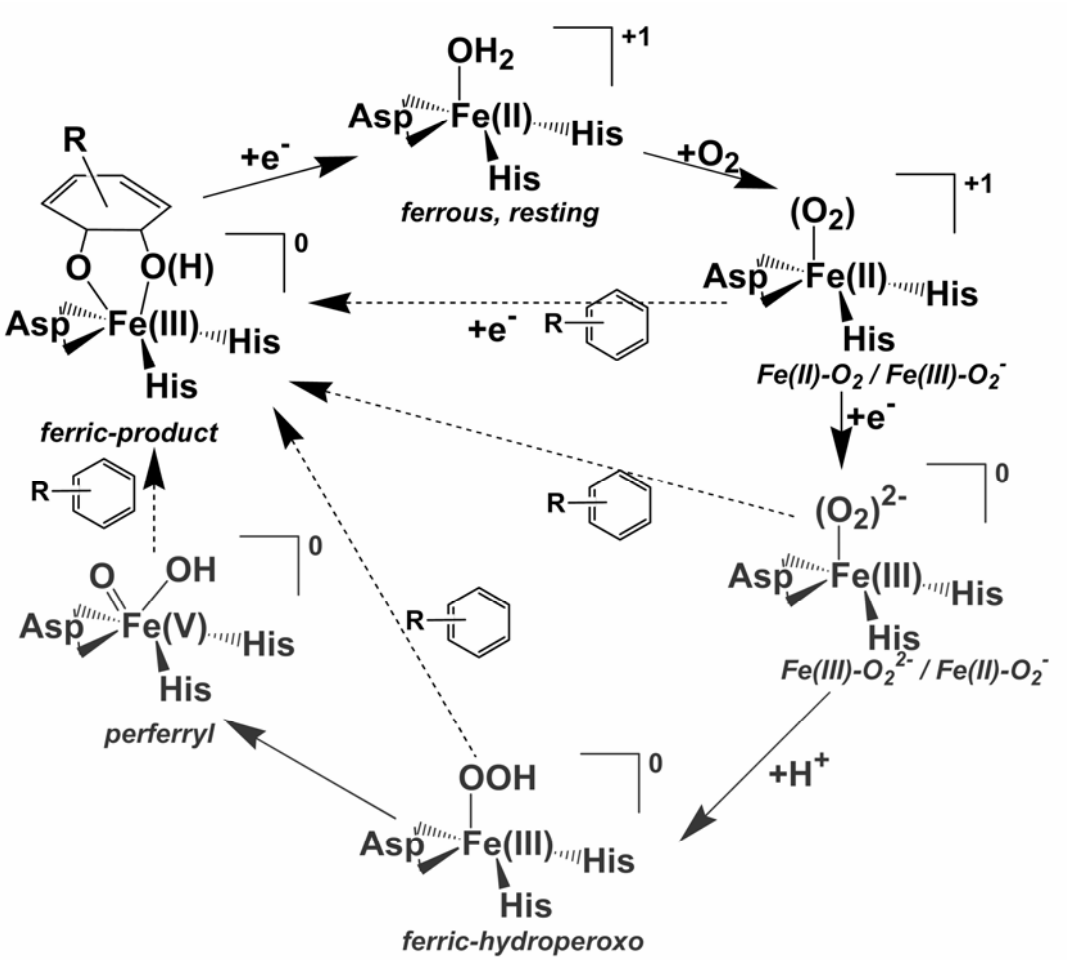


Figure 5. Proposed catalytic mechanisms for a representative class of non-heme dioxygenases (Rieske dioxygenases, RDO). Species shown in grey have never been observed in RDO. The identities of the protein-derived iron ligands are indicated. The aromatic substrate may be benzene (R = hydrogen), substituted benzenes, or other aromatics (including heterocyclic compounds).

Study of the peroxide reactivity of hemoglobin has led to the perhaps unexpected result, that this protein appears to exhibit the highest affinity for ascorbate of those known to date;[30,31] thus, a low-micromolar K_m was inferred from Michaelis-Metne analysis of the ascorbate peroxidase activity of hemoglobin. This has prompted us to look for direct evidence for the existence of such a complex, as the implication would be that red blood cell ascorbate is always bound to hemoglobin rather than remaining free in the cytosol. Proof of an ascorbate-hemoglobin adduct has come from NMR measurements, which clearly indicate ascorbate signals shifting in the presence of hemoglobin (Figure 6). Such shifts, although with different signs and magnitudes, are also seen with myoglobin and serum albumin, with further physiological implications.[31]

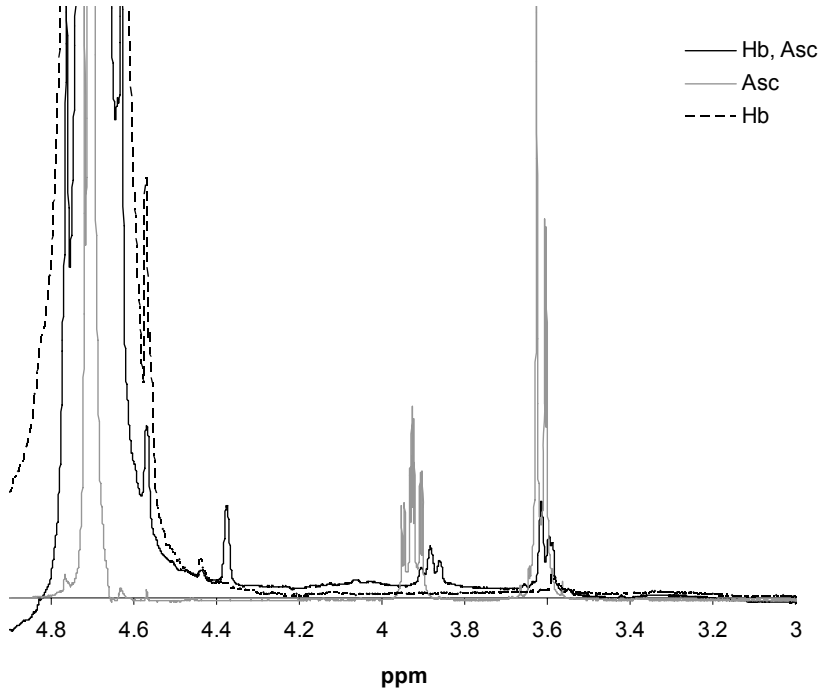


Figure. 6. NMR spectra of ascorbate, met hemoglobin and an equimolar ascorbate-met hemoglobin mixture. [31]

As shown above, high-valent states such as Compounds I and II are not only parts of catalytic cycles delineating the physiological function of various enzymes; in cases such as globins, they can also constitute products of side-reactions with physiological relevance. One other case where a growing interest for putative peroxide-derived high-valent species is cytochrome c. The reactivity of this mitochondrial protein towards peroxides has been linked to apoptosis, and various indirect evidence for its reaction with peroxides have been provided, such as turnover with exogenous sacrificial reductants, or free radical trapping. Our current efforts are aimed at direct detection of reactive intermediates upon treatment of cytochrome c with peroxide employing fast kinetic techniques aided by indirect measurements such as enzyme assay techniques, all of which appear to provide evidence for a ferryl species in cytochrome c.

Acknowledgements: the work shown here has been supported by the Romanian Ministry for Education and Research (ID565/2007).

References:

1. George, P.,Irvine, D. H. The higher oxidation state of metmyoglobin. *Biochem. J.*, **1953**, *53*, xxv
2. Keilin, D.,Hartree, E. F. The combination between methæmoglobin and peroxides: hydrogen peroxide and ethyl hydroperoxide. *Proc. Roy. Soc. B*, **1934**, *117*, 1-15
3. Silaghi-Dumitrescu, R. Horseradish peroxidase - a versatile catalyst. *Horseradish peroxidase - a versatile catalyst. Research Signpost, India*, **2006**,
4. Silaghi-Dumitrescu, R.;Reeder, B. J.;Nicholls, P.;Cooper, C. E.,Wilson, M. T. Ferryl haem protonation gates peroxidatic reactivity in globins. *Biochem J*, **2007**, *403*, 391-5
5. Silaghi-Dumitrescu, R.,Cooper, C. E. Transient species involved in catalytic dioxygen/peroxide activation by hemoproteins: possible involvement of protonated Compound I species. *Dalton Trans.*, **2005**, 3477-3482

6. Silaghi-Dumitrescu, R. Halide activation by heme peroxidases: theoretical predictions on putative adducts of halides with Compound I. *Eur. J. Inorg. Chem.*, **2008**, 5404-5407
7. Silaghi-Dumitrescu, R. Electronic structures of Fe(IV) and Fe(V) systems with oxo, sulphido and nitrido ligands in octahedral environments. *Rev. Chim.*, **2007**, *58*, 461-464
8. Silaghi-Dumitrescu, R. The nature of the high-valent complexes in the catalytic cycles of hemoproteins. *J. Biol. Inorg. Chem.*, **2004**, *9*, 471-476
9. Sono, M.;Roach, M. P.;Coulter, E. D.,Dawson, J. H. Heme-containing oxygenases. *Chem. Rev.*, **1996**, *96*, 2841-2888
10. Meunier, B.;de Visser, S. P.,Shaik, S. Mechanism of oxidation reactions catalyzed by cytochrome p450 enzymes. *Chem Rev*, **2004**, *104*, 3947-3980
11. Que, L., Jr.,Ho, R. Y. N. Dioxygen activation by enzymes with mononuclear non-heme iron active sites. *Chem. Rev.*, **1996**, *96*, 2607-2624
12. Lu, C. C.;Saouma, C. T.;Day, M. W.,Peters, J. C. Fe(I)-Mediated Reductive Cleavage and Coupling of CO2: An FeII(í-O,í-CO)FeII Core. *J. Am. Chem. Soc.*, **2007**, *129*, 4-5
13. Makarov, S. V.;Salnikov, D. S.;Pogorelova, A. S.;Kis, Z.,Silaghi-Dumitrescu, R. A new route to carbon monoxide adducts of heme proteins. *J. Porph. Phthalocyan.*, **2008**, *12*, 1096-1099
14. Kudrik, E. V.;Makarov, S. V.;Zahl, A.,van Eldik, R. Kinetics and mechanism of the iron phthalocyanine catalyzed reduction of nitrite by dithionite and sulfoxylate in aqueous solution. *Inorg Chem*, **2005**, *44*, 6470-5
15. Nevin, W. A.;Liu, W.;Melnik, M.,Lever, A. B. P. Spectroelectrochemistry of cobalt and iron tetrasulphonated phthalocyanines. *J. Electroanal. Chem.*, **1986**, *213*, 217-234
16. Grodkowski, J.;Dhanasekaran, T.;Neta, P.;Hambright, P.;Brunschwig, B. S.;Shinozaki, K.,Fujita, E. Reduction of Cobalt and Iron Phthalocyanines and the Role of the Reduced Species in Catalyzed Photoreduction of CO2. *J. Phys. Chem. A*, **2000**, *104*, 11332-11339
17. Kis, Z.,Silaghi-Dumitrescu, R. The electronic structure of biologically-relevant Fe(0) centers. *Int. J. Quant. Chem.*, **2009**, in press
18. Balland, V.;Charlot, M. F.;Banse, F.;Girerd, J.;Mattioli, T. A.;Bill, E.;Bartoli, J. F.;Battioni, P.,Mansuy, D. *Eur. J. Inorg. Chem.*, **2004**, 301-308
19. Silaghi-Dumitrescu, R. Bonding in Biologically-relevant high-valent iron centers. *Int. J. Chem. Model.*, **2009**, *2*, 1-17
20. Silaghi-Dumitrescu, R. "High-valent" ferryl-oxo complexes: how "high" are they really? *Studia Univ. Babes-Bolyai, Chemia*, **2005**, *50*, 17-21
21. Green, M. T. Application of Badger's rule to heme and non-heme iron-oxygen bonds: an examination of ferryl protonation states. *J Am Chem Soc*, **2006**, *128*, 1902-6
22. Green, M. T.;Dawson, J. H.,Gray, H. B. Oxoiron(IV) in chloroperoxidase compound II is basic: implications for P450 chemistry. *Science*, **2004**, *304*, 1653-6
23. Behan, R. K.,Green, M. T. On the status of ferryl protonation. *J. Inorg. Biochem.*, **2006**, *100*, 448-459
24. Behan, R. K.;Hoffart, L. M.;Stone, K. L.;Krebs, C.,Green, M. T. Evidence for basic ferryls in cytochromes P450. *J Am Chem Soc*, **2006**, *128*, 11471-4
25. Berglund, G. I.;Carlsson, G. H.;Smith, A. T.;Szoke, H.;Henriksen, A.,Hajdu, J. The catalytic pathway of HRP at atomic resolution. *Nature*, **2002**, *417*, 463-468
26. Bonagura, C. A.;Bhaskar, B.;Shimizu, H.;Sundaramoorthy, M.;McRee, D. E.;Goodin, D. B.,Poulos, T. L. High-resolution crystal structures and spectroscopy of native and compound I cytochrome c peroxidase. *Biochemistry*, **2003**, *42*, 5600-5608
27. Hersleth, H. P.;Dalhus, B.;Gorbitz, C. H.,Andersson, K. K. An iron hydroxide moiety in the 1.35 A resolution structure of hydrogen peroxide derived myoglobin compound II at pH 5.2. *J. Biol. Inorg. Chem.*, **2002**, *7*, 299-304
28. Hersleth, H. P.;Ryde, U.;Rydberg, P.;Gorbitz, C. H.,Andersson, K. K. Structures of the high-valent metal-ion haem-oxygen intermediates in peroxidases, oxygenases and catalases. *J Inorg Biochem*, **2006**, *100*, 460-76
29. Silaghi-Dumitrescu, R.,Silaghi-Dumitrescu, I. Computational Inorganic Chemistry - a useful tool, and more. *Chemtracts Inorg. Chem.*, **2005**, *18*, 595-619
30. Cooper, C. E.;Silaghi-Dumitrescu, R.;Rukengwa, M.;Alayash, A. I.,Buehler, P. W. Peroxidase activity of hemoglobin towards ascorbate and urate: a synergistic protective strategy against toxicity of Hemoglobin-Based Oxygen Carriers (HBOC). *Biochim Biophys Acta*, **2008**, *1784*, 1415-20
31. Deac, F. V.;Todea, A.;Bolfa, A. M.;Podea, P.;Petrar, P.,Silaghi-Dumitrescu, R. Ascorbate Binding To Globins. *Rom. J. Biochem.*, **2009**, in press

AUTHOR INDEX