CONTENTS

Metal Elements in Environment, Medicine and Biology

Tome X

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EDITORS OF SYMPOSIA SERIES

METAL ELEMENTS IN ENVIRONMENT, MEDICINE AND BIOLOGY
Tome X

Edited in collaboration with
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Having in view that in the last days before the meeting M.E.E.M.B. 2010 November 11-12, Timişoara, the organizers received by e-mail some scientific papers which were not included in this volume, if there will be requested by participants a second edition will be printed and will include all the received papers.

Editors of
Tome X of M.E.E.M.B.

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FOREWORD

The publication of Tome X of the Proceedings of the 10th Symposium „Metal Elements in Environment, Medicine and Biology" (M.E.E.M.B.) proves the contribution of participants to a large thematic area concerning metallomics and represents a continuity by the integrative and interrelational character of the topics from basic investigations to applicative research.

The series of Symposia „Metal Elements in Environment, Medicine and Biology" has as starting point the activities of the «Working Group for Metal Research in Biological Systems» founded in 1979. Approaching problems of inter- and multidisciplinarity the members of this group started to publish their papers in 1980.

At the beginning, the M.E.E.M.B. Symposia Series were organized under the auspices of Roumanian Academy (with the approval of Acad. Nicolae Cajal, 1993) and of the Biochemical Commission of the Roumanian Academy (with the support of Acad Mihai Serban). The *in extenso* papers of the symposia were published in Tomes: I (1994); II (1996); III (1998); IV (2000); V (2002); VI (2004); VII (2006); VIII (2008) – in Timişoara; Tome IX (2009) - in Cluj-Napoca and Tome X (2010) - in Timişoara.

From 2008 the series of symposia takes place under the auspices of Cluj-Napoca Branch of the Roumanian Academy and of Timişoara Branch of the Roumanian Academy with the perspective to extent the internal and foreign collaborations and the thematic area. Starting with 2009 the meeting is organized annually, alternatively in Cluj-Napoca and Timişoara.

In the context of contemporary development focused on environmental, nutritional, pathobiochemical, pharmacological and public health problems, preoccupations in the domain of metallomics present a special interest.

We hope that the readers of this volume will begin to feel the challenge of these research areas and thereby be encouraged to explore them further, and even participate in future research.

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EFFECT OF SELENIUM ON METALLIC AND NON-METALLIC TRACE ELEMENTS

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ABSTRACT

The effects of Se application on the trace elements levels in plants, animals and humans in Se deficient area were investigated. Investigations were conducted during over two decades on crops foliary treated with different Se levels, experimental and domestic animals, and humans unexposed and exposed to various toxic agents and other stressors. Obtained data showed that each organism has its own beneficial Se ranges. When Se concentration is inside the optimal interval organism have, in comparison with organisms with lower or higher Se concentrations, better regulated machinery for import, excretion, translocation and redistribution of essential (Zn, Cu, Mg, Mn, Fe) and toxic (Al, As, Hg, Cd, Pb) trace elements. Due to the powerful homeostatic mechanisms, the inadequate Se intake often became obvious when organisms are exposed some forms of internal or external stresses. Organisms deficient in Se and exposed to internal and/or external stressors have, as a rule, much stronger correlations between Se and other trace elements. On the basis of all available data it can be assumed that Se play crucial role in formation of common sense in plants, animals and humans. When Se and other essential elements are inside the optimal ranges communication system that allows living organisms to integrate their own systemic physiological processes, respond better to stress-related phenomena.

Key words: selenium, trace elements, plants, animals, human.

INTRODUCTION

Selenium (Se) is one of naturally occurring, for life essential, elements. Its distribution in soil is uneven. From the point of human and animal nutrition low Se areas are spread on the Earth more often than adequate or Se-toxic areas (Oldfield, 1999). It has been proved that extremely low, as well as high Se concentrations in soils and plants can be a crucial risk factor for human and animal health (Tan and Huan, 1991; Franke, 1934; Nelson et al.,1943).

Our understanding of the mechanisms that link Se to its specific vital functions is still fragmentary. We know that Se concentration affects the concentrations of other trace elements in the biological tissues but the interrelationships have not been studied in detail. The interest in the relationship between Se and other trace elements arises in connection with the dietary insufficiency or excess consequences which may contribute to abnormalities of other elements metabolism and utilization.

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Published data indicates that most of Se deficiency signs appear when vitamin E or antioxidant metabolism is suboptimal. Human and animal diseases involving selenium apparently are not simple selenium deficiencies (Prasad, 1978, Neve, 1999). Suggested possibilities include various toxins, hypoxia, or infectious agents, particularly viruses (Djujić et al., 1995, Bulat et al., 2000, Popović et al., 1995, Beck et al., 2003).

Studies in which selenium deficiencies or excesses have been examined in humans and animals organisms exposed to some form of nutritional, metabolic, hormonal or physiologic stress indicates that the inadequate Se intake becomes obvious only when the body is stressed in some way. Otherwise, due to the powerful homeostatic mechanisms of the body a search for a simple deficiency or excess of trace elements was unlikely to be found (Djujić et al., 1991a, Djujić et al., 2000c).

One of important Se characteristics is its interaction with other elements that may be present in water, foods, workplace and environment (As, Pb, Cd, Hg, Cu, Zn, Mn, Ni, Co, Cr, Mo, Al, Sn, Bi, Ag, Au, Tl, F, U etc.) The sequestration of elements by Se represents an efficient natural detoxification mechanism for some of these elements but also results in the physiological inactivation of Se (Gunther, 1974, House and Welch, 1989, Schrauzer,2009).

In the present study the focus of Se research has been on impact that its deficiency, beneficial range and excess will have in the relationships with other trace elements important for plants, animals and humans tissue protection from harm.

MATERIALS AND METHODS

Plant assays were conducted on wheat, soybean, maize, ray, barley, oats, beans, livestock peas, adzuki and alfalfa planted in low Se fields (Se content in top-soils below 200 μg/kg). Crops were at defined growth stage sprayed with combinations that contained different Se concentrations in SeO_3^{2-}, form (from beneficial to toxic) (Djujić et al., 2002c, Djujić et al., 2003a, Djujić et al., 2003b). Supplementation effects on the oxidative changes, antioxidant capacity and seed composition were analyzed and compared with data obtained for untreated control plants. In order to assure proper Se interval in treated plants and to monitor the influence of factors that induce external stresses (high or low temperature and light conditions, increased environmental contamination) on plants the experiments were repeated during 4 - 12 consecutive years. In plant tissues were after mineralization, by use of atomic absorption spectrometry (AAS) and inductively coupled plasma emission spectrometry (ICP-ES) determined the concentrations of Ca, Mg, Fe, Mn, Zn, Cu, Co, Se, Al, As, Pb, Cd and Hg. Certified reference materials IAEA H-9, NIST RM 8431 were used for checking the applied analytical methodology and assuring the quality of analytical work during determining.

Animal studies were conducted on Wistar rats and Japanese quail that, due to Se deficiency in fed contained suboptimal Se levels in tissues and animals that obtained Se adequate diet.

Throughout the experiments Wistar rats that received 0,7 mcg Se/day through standard fed and 1,2 mcg Se/day through water enriched with Se-yeast (SeY) were exposed to ionizing radiation, Adriblastina RD, Cytosar, physical stresses and aging process. In defined time intervals animals were sacrificed. Blood samples were obtained by heart puncture under narcosis, while liver, kidney, urinary bladder, spleen, pancreas, heart, lung, skin, femoral muscle, bone, tongue, eyes, front brain, hind brain, hypothalamus, pituitary gland, pineal gland, thyroid glands, lingual tonsils,

thymus, adrenal glands and testes were from sacrificed animals excised, cleaned of connective tissue and stored at -20^{0}C for trace elements determination.

Japanese quail were raised at a local farm under standard conditions required for the commercial production of these birds. In each cage were 8 laying quail and 4 male quail. Intake of Se by 1st group that received a mix feed diet, composed of Se rich ingredients (bio-fortified crops with Se), were 5,5 mcg Se/day while in 2nd group that received regular diet, originating from Se deficient region, were 2 mcg Se/day . The experiment lasted 12 months. For quail feed manufacturing, the following ingredients biofortified with Se were used: maize, wheat, soybean, oat, barley, sunflower and dehydrated alfalfa. Nutritional value of mix feed for quails made from crops biofortified with Se was due to optimized content of many other nutrients (proteins, amino acids, fats, fatty acids, vitamins A, C, E, essential elements - C, Mg, Zn, Cu, Fe, Mn) much better (Djujić et al., 2000b, Djujić et al., 2004a, Djujić et al., 2002b). Birds were controlled at 1st, 3rd, 6th and 12th month of the experiment. Eggs were separately collected during 3 successive days and stored in a chilly place. After 3 consecutive days of egg collecting, eggs obtained from one cage were mixed and saved frozen at -25^{0}C for trace elements determinations. At the same time intervals (1st, 3rd, 6th and 12th month of the experiment), 6 females and one 3 quail from each cage were sacrificed, their tissues separated and saved frozen at for trace elements analysis.

In tissues of Wistar rats, quail eggs and meat (breast, thigh and wing muscles) were after mineralization analyzed the concentrations of Ca, Mg, Fe, Mn, Zn, Cu, Co, Se, Al, As, Pb, Cd and Hg. The accuracy of the measuring process was checked by the certified reference material for animal tissues (IAEA H-4, NBS 1777a - Bovine liver, CM 184 - Bovine muscle and CRM 186 - Pig kidney).

Human Studies were realized on Se deficient conditionally healthy volunteers, non-exposed and exposed at their working places to: ionizing radiation, Pb, Cd, Al, As, phenols, cigarettes smoke, as well as on adult volunteers with disrupted health by cancer (lung, colorectal, breast, uterus, ovary), cardiovascular diseases, diabetes 2, asthma, hyperthyroidism, multiplex sclerosis. They consumed 6 - 48 weeks 100 -300 mcg/Se daily in as Se-yeast; diet composed of Se enrich wheat products, soybean products, quail eggs, and quail meat in which Se intake ranged from 31 to 91,5 mcg (Djujić et al., 1991b, Djujić et al., 2000a, Djujić et al., 2003e, Djujić et al., 2005).

For assessment Se and trace elements status of volunteers during the test were used blood plasma, erythrocytes, hair and daily urine occasionally. In blood plasma and erythrocytes were beside Se analyzed, Zn, Cu, Mn and Fe while in hair were beside them analyzed concentrations of: I, Co, Mo,Cr, Ni, Si, Sn, Al, As, Pb, Cd, Hg, Ca and. Mg. For determination of all elements except iodine, which was determined by ion-selective electrodes, was used atomic emission spectrometry with inductively coupled argon plasma (ICP-AES). The accuracy of the method was confirmed by simultaneous analysis of the certified reference material (Chinese human hair GBW09101 powder).

RESULTS AND DISCUSSION

Effects of Se on other trace elements in plants - Our earlier investigations indicated that Se foliary applied as selenite converts in leafs easily and rapidly to organic forms (Djujić et al., 2000c, Djujić et al., 2001a, Djujić et al., 2001b). On the basis of aquired knowledge we developed own procedure and combination for foliar Se application to crops growing on Se deficient soils (Djujić et al., 2003e, Djujić et al.,

2003f). The dominant Se form in such enriched crops, over 80% of total Se, is L-(+) selenomethionine (SeMet), the favorable Se form for humans and animals.

The conducted experiments during many consecutive years on crops, planted in low Se fields, foliary treated with different Se concentrations showed that: In general, plants are much more resistant to increased Se concentration than to insufficent (Djujić et al., 2002c, Djujić et al., 2003a, Djujić et al., 2003b, Djujić et al., 1998); When is added in proper range, Se exerts beneficial effects trough improved chemical composition, increased tolerance to oxidative stress (Djujić et al., 2002b, Djujić, 2008a, Djujić, 2008b, Djujić, 2006a) and other unpleasant events.

Proper Se concentrations for investigated crops were, on the basis of our researches, in average 10-20 times higher than are usual. Thus, proper interval for Se in wheat grains was from 0,120-0,300 mg/kg (5-11 times higher than in corresponding control samples) and in soybean seeds was from 0,170 – 0,900 mg/kg (10-45 times higher than in corresponding control samples). Inside this ranges Se exerts its beneficial effects to: oxidative stress levels (TBARS), antoxidative defense (α- and γ- tocopherol concentrations, GSHPx activity), amino and fatty acid composition, other chemical elements of importance for living organisms (Djujić et al., 2000c, Djujić et al., 2001a, Djujić et al., 2001b, Djujić et al., 2000c, Djujić et al., 2001a, Djujić et al., 2004c).

Monitoring of relationships between Se and other elements of importance in crops (Ca, Mg, Fe, Mn, Zn, Cu, Co, Se, Al, As, Pb, Cd and Hg) showed that Se may influence levels of other elements, in this case that were Zn, Cu and Mg. Values for Zn, Cu and Mg obtained in wheat grains and soybean seeds, produced in uncontaminated mild deficient in Cu and Mg fields, which were foliary treated with different Se concentration and its relationships with Se are presented in Figure 1 and Figure 2.

These results and other experimental data also revealed the dual effect of Se on plants:

- **Detrimental** - when plants suffer due to insufficiency or excess of Se. In both cases plants have not the needed antioxidative capacity to counteract the oxidative stress that causes oxygen radicals produced by internal metabolic or external factors (high oxidative stress, insufficient antioxidative capacity, reduced content of many essential nutrients, disrupted amino acids composition, increased content of toxic substances).
- **Beneficial** – when Se amount is inside the proper range. In this case Se through multiple mechanisms exert beneficial effects in plants (increased antioxidative capacity, optimized nutrient content and amino acid composition, reduced oxidative stress, promoted plant growth, delayed senescence).

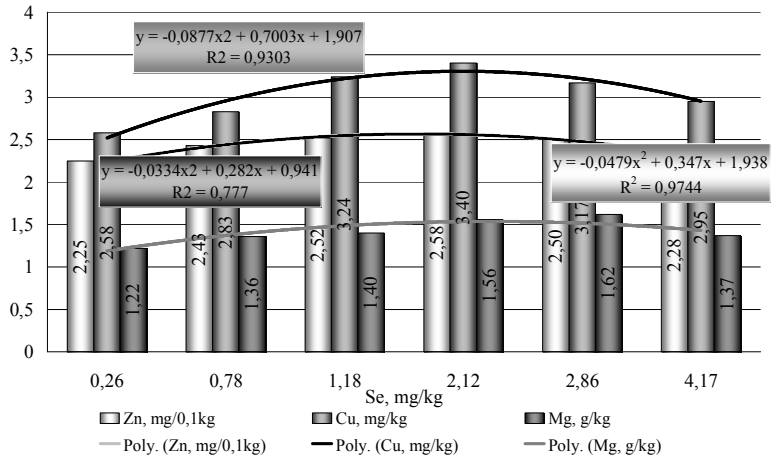

Fig. 1. Effects of foliar Se supplementation on Se, Zn, Cu and Mg in wheat grains

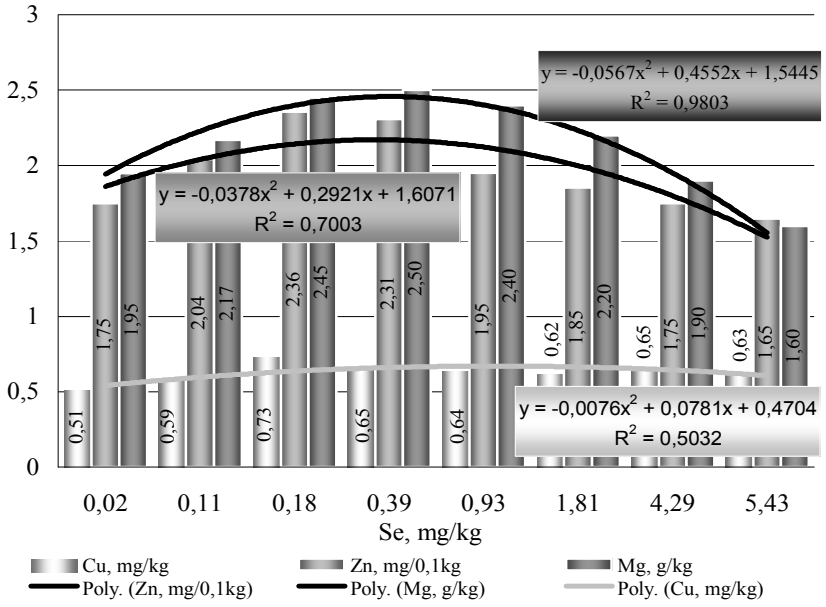

Fig. 2. Effects of foliar Se supplementation on Se, Zn, Cu and Mg in soybean seeds

Resent findings indicates that in most mineral stresses Se, when is present in proper levels, play important, complex, poorly understood regulatory role in the interactions and multiple processes that influences uptake, growth and allocation between plant parts. Obviously without proper Se level plants have not good "common sence" and can not respond on right way to stressors. - improving plant adaptation to stresses.

Research results of Yu et al. (2003), Fang et a.l (2008), Hartikainen (2002), Hu et al. (2003) showed that by application of low Se concentrations or proper combination of Se, Zn and Fe improved plant adaptation to stresses, as well as discoverings thet relates to some of Se form functions in plants made by Hua-Fen et al. (2008) that determined Se aptake, translocation and speciation in wheat, Shao-Fen et al (1994) that determined GSH-Px as one of essential Se forms in higher plants, Mullineaux et al (Mullineaux et al., 1998) that isolated from pea leaf RNA PHGPX and determined its localisation in the chloroplast and root, toogether with our findings that plants with proper Se concentration may optimize circadian regulation (Djujić, 2008b) support this hypothesis.

Effects of Se on other trace elements in animals

The effects of 4 weeks supplementation with SeY and Y on Se, Cu, Zn, Mn and Fe contents in the tissues of male **Wistar rats** are presented in Figures 3 - 7. Obtained results showed that supplementation with SeY induce: increased tissue Se retention, changes in tissue Se Cu, Zn, Mn and Fe retention and distribution (Djujić et al., 1995b).

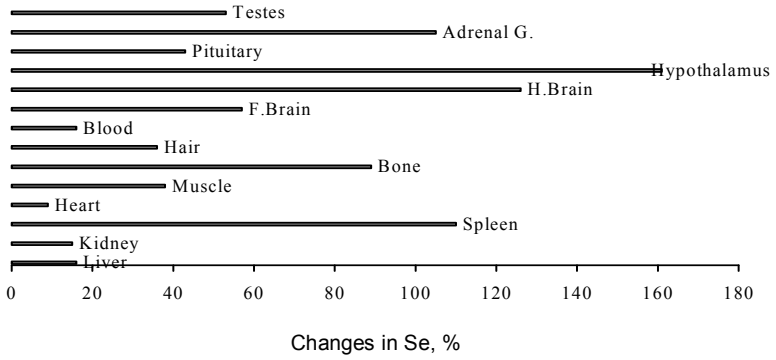

Fig. 3. Changes in Se content after supplementation with SeY

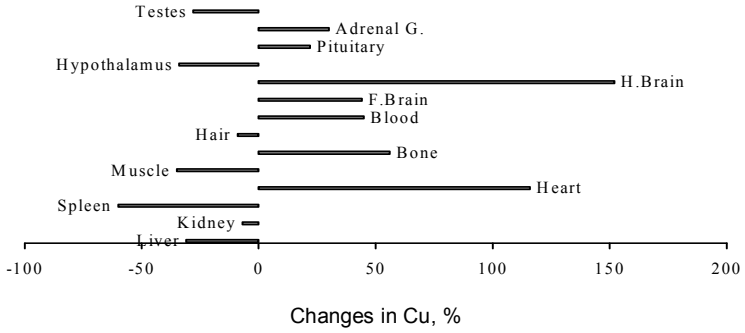

Fig.4. Changes in Cu content after supplementation with SeY

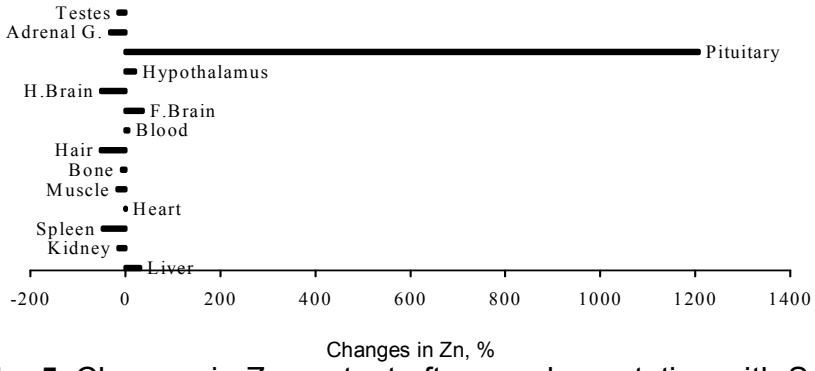

Fig. 5. Changes in Zn content after supplementation with SeY

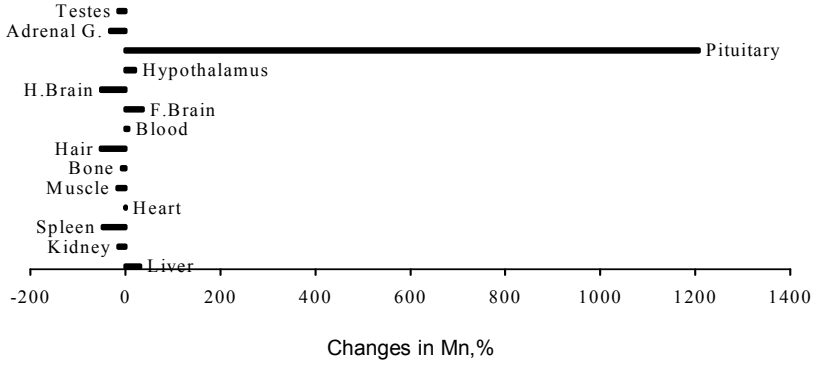

Fig. 6. Changes in Mn content after supplementation with SeY

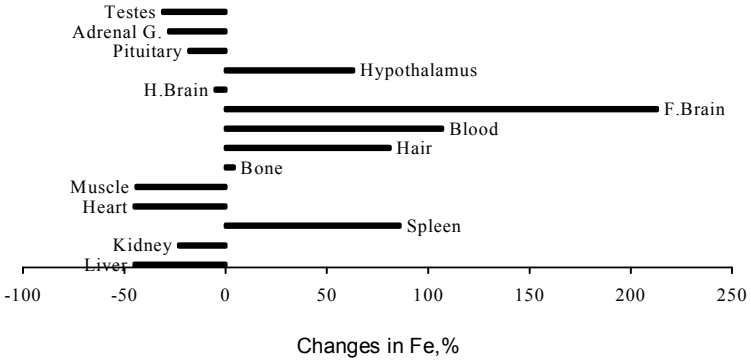

Fig.7. Changes in Fe content after supplementation with SeY

Data for the concentrations of Se, Cu, Zn, Mn and Fe in animal tissues supplemented with SeY and Y on the 7th day after irradiation with a single dose of 4.2 Gy oḍ γ-rays indicates that ionizing radiation induces in animal significant changes in microelement content and distribution (Fig. 8-12) (Djujić et al., 1991a, Djujić et al., 1992, Djujić et al., 1995a). Changes in tissue distribution and content of Se, Cu, Zn and Mn were reduced in most of the studied tissues when Se concentration is higher. Exception was Fe where changes are both sided. Conclusion is that adequate nutrition with Se offer better protection from the toxic action of ionizing radiation and changes in red-ox balance caused by microelement disturbances.

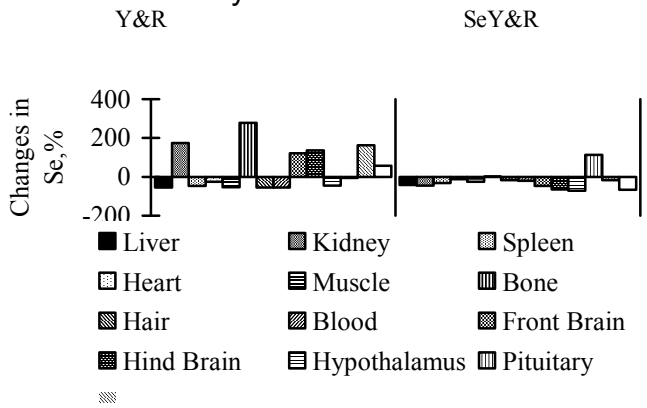

Fig. 8. Changes in Se content on the 7th day after irradiation with 4.2 Gy

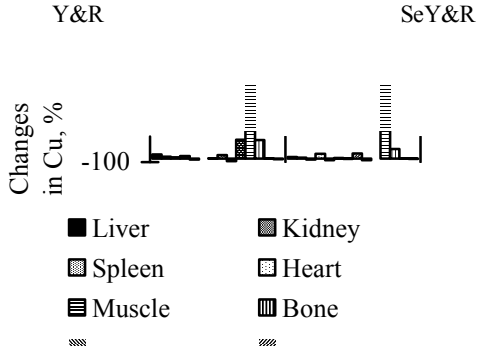

Fig. 9. Changes in Cu content on the 7th day after irradiation with 4.2 Gy

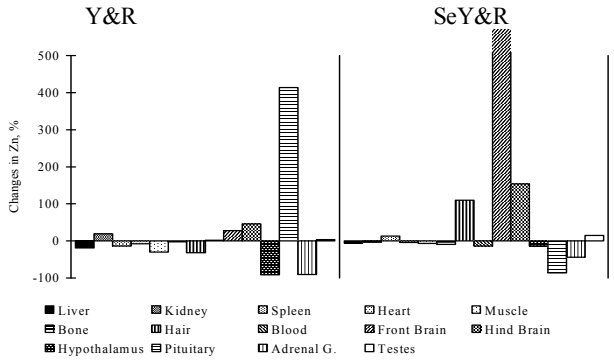

Fig. 10. Changes in tissue Zn content on the 7th day after irradiation with 4.2 Gy

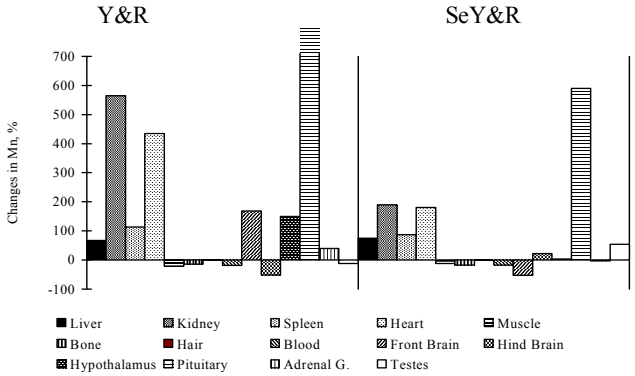

Fig. 11. Changes in Mn content on the 7th day after irradiation with 4.2 Gy

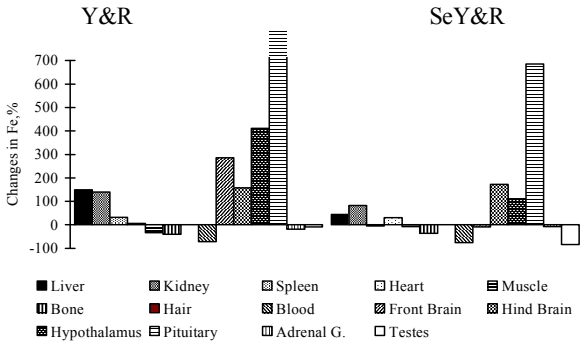

Fig. 12. Changes in Fe content on the 7th day after irradiation with 4.2 Gy

Researches the effect of long term supplementation with moderate amounts of Se in a form of SeY on age related changes trace elements content showed that it delay appearance of unwonted changes in rat tissues, enabling increased tolerance to stresses (Jozanov-Stankov et al., 2000, Jozanov-Stankov et al., 2004, Demajo et al., 2006). In Figures 13 - 17 are presented changes in trace elements in selected rat tissues.

Tests with animal that received Adriblastina RD and Cytozar confirms conclusion, that animals supplemented with SeY have reduced, changes in trace elements content and lower oxidative stress than control, Se deficient, animals (Jozaanov-Stankov et al., 1995, Jozanov-Stankov et al., 2002, Jozanov-Stankov et al., 2003a, Jozanov-Stankov et al., 2003b).

The results confirm the existence of very specific relationships between the levels of elements relevant to AODS depended on the tissue specificity and the time of observation. Generally, correlation between Se and other trace elements are higher in Se deficient animals exposed to stresses.

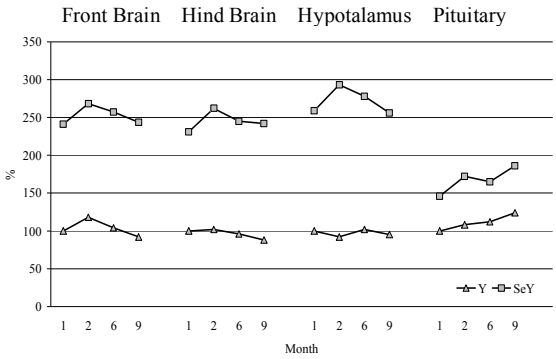

Fig. 13. Changes of Se during aging

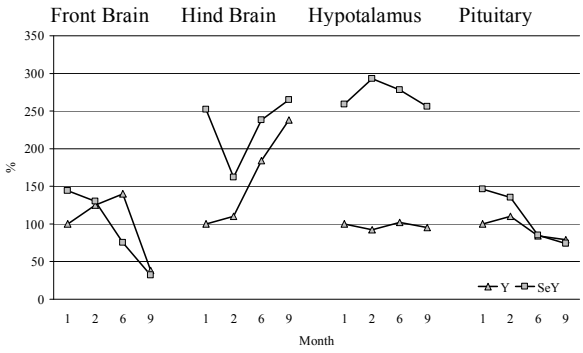

Fig 14. Changes of Cu during aging

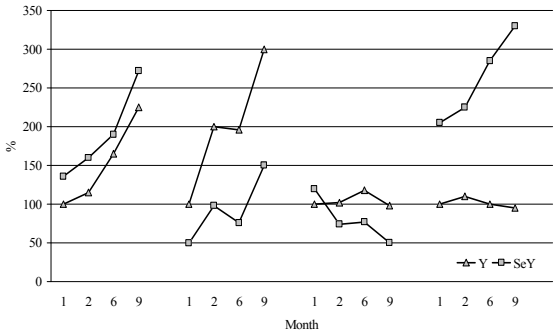

Fig. 15. Changes of Zn during aging

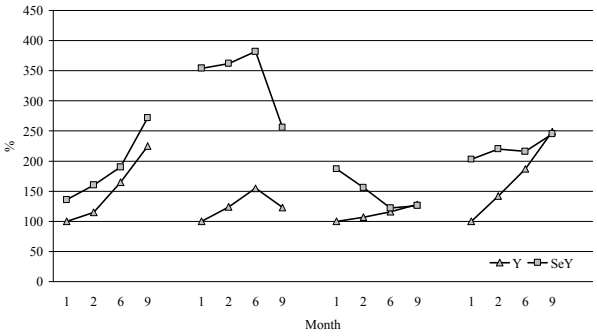

Fig. 16. Changes of Mn during aging

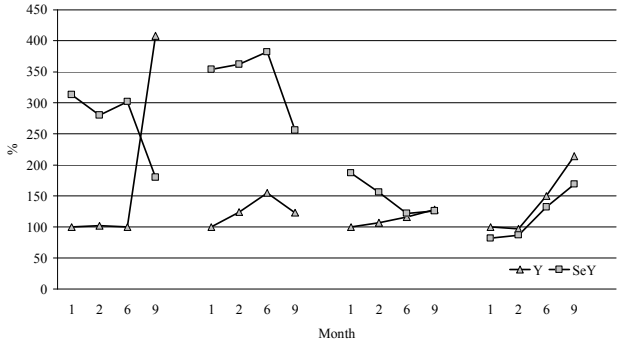

Fig. 17. Changes of Fe during ages

Our studies suggests that proper balance of Se, Cu, Zn, Mn and Fe is needed for a normal functioning of the immune system and as such could have an important role in viral suppression and might be implicated in delaying the aging process. Our

data suggest that in Se supplemented animals, Se could restore the pool for Zn in tissue as is pineal gland when Zn required for regulation of key homeostatic mechanisms of the body, including immune response.

Investigations of influences different supplementation strategies for the enrichment animal diet with Se conducted on **Japanese quails**, showed that mix feed diet fortified with inorganic Se and SeY) are less beneficial than mix feed diet composed of Se rich ingredients (crops biofortified with Se foliar). Investigations showed that eggs quails feed with mix feed diet composed of crops biofortified with Se are an extremely rich source of Se and other commonly deficient nutrients. Furthermore such produced quail eggs have over 50% lower cholesterol content, much less toxic elements in eggshell and inside part of egg and are richer in many in nutrition often deficient nutrient as are choline, vitamin A, riboflavin, vitamin B12, pantothenic acid, minerals - J, Cu, Cr, Si, Ca, Fe, Zn and essential amino acids than eggs obtained by ordinary farming procedure (Figure 18). Muscle analysis data confirms that meat of such nourished birds represent also extremely rich source of well balanced nutrients (Djujić et al., 2004b, Djujić et al., 2006a, Djujić, 2008d, Djujić, 2008b).

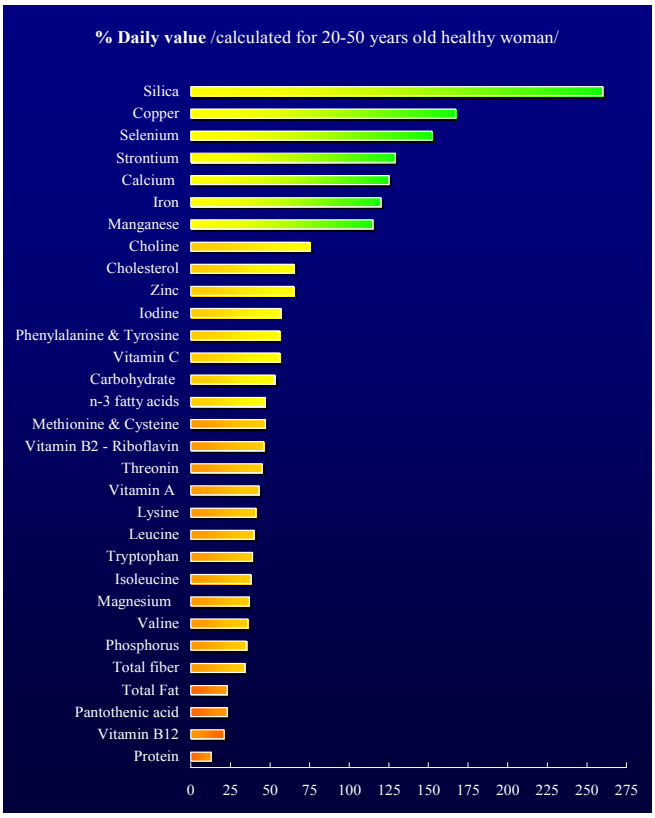

Fig. 18. Nutritive value of eggs obtained from quail nuriched with mix feed diet composed of crops biofortified with Se

World's healthiest foods rating	
-excellent	DV≥75%
-very good	DV≥50%
-good	DV≥25%

Effects of Se on other trace elements in humans

Investigations conducted on population that has marginally low Se intake (95% takes in about 40 % of the RDA) showed that intake of other essential trace elements by sizable percentage of population is not adequate (40% males take in more Fe

while 60% females take in less than is the RDA; 50% take in more Mn than is the upper RDA value for Mn; 15% take in less Cu and chromium Cr than is the RDA; 85% take in 15 /25% less Zn than is the RDA). At the same time, intake of toxic elements (Hg, As, Cd, Pb and Al) has increasing trends (Djujić, 1996, Djujić et al., 2000b, Djujić et al., 2001a, Jeremić et al., 2004).

Many individuals particularly under psychological stress have impaired nutritional status, of the trace elements important in establishing cellular defense against oxidative stress. In the low Se region, Se deficiencies are often confounded by concurrent trace-element inadequacies. Our researches of essential trace elements important for overall oxidative stress status in different groups of volunteers exposed more or less to various toxic agents confirm these findings (Bulat et al., 1995, Djujić et al., 2002a, Djujić et al., 2003f, Djujić et al., 2006b).

Table 1. Trace elements important for redox processes in erythrocytes of volunteers from Serbia

Element	Mean ± SD
Se **(μmol/l)**	0,40 ± 0,07
Zn **(μmol/l)**	102,00 ± 10,07
Cu **(μmol/l)**	9,70 ± 0,08
Mn **(μmol/l)**	16,48 ± 2,41
Fe **(μmol/l)**	153,12 ± 52,22

n = 790; SD - standard deviation

Presented data showed that in all studied groups of volunteers Se concentrations in erythrocytes was significantly lower (optimal range 2,53 – 5,07 μmol/l), while concentrations of Zn, Cu an Fe are in the range found for other European populations. Mn concentrations were in upper permitted level (Table 2).

Table 2. Changes in investigated parameters in erythrocytes of volunteers exposed to harmful agents (%)

Group from	Se	Cu	Zn	Fe
Nuclear institute[a]	-23[1]	-10[1]	-4[3]	+12[2]
Nuclear medicine[a]	-14[1]2	-11[1]	-1[3]	+8[2]
Thermal power plant[b]	-8[3]	-9[2]	+14[1]	-8[2]
Al electrolysis plant[b]	-5[3]	-11[1]	-2[3]	-21[1]
Pb battery plant[b]	-2[3]	-15[1]	+20[1]	-17[1]
Cd battery plant[b]	-14[2]	-11[1]	-5[3]	-20[1]
Chloralcaly plant[b]	-4[3]	-7[2]	+9[1]	-5[2]
Rubber plant[b]	-8[3]	+14[1]	-4[3]	-16[1]
Farms[b]	-7[3]	-2[3]	-1[3]	+22[1]
Schools[a]	+3[3]	+16[1]	-2[3]	+7[2]
Hospitals[a]	-1[3]	-10[2]	+1[3]	-25[1]
Pensioners[a]	+5[3]	-4[3]	-7[2]	-9[2]

[a] as control used volunteers from the university; [b] as control used volunteers from the limekiln plant; [1]p<0,001; [2]p<0,01; [3] NS

The effects of supplementation with Se enriched brewery yeast (SeY), with over 86% Se in the form of Se-methionine (SeMet) on redox element status investigations were conducted few time on the conditionally healthy subjects (CHS) and patients with cancers (colorectal - CCa and lung - LCa) and cardiovascular diseases, and exposed to toxic agents at their working places (Djujić et al., 2004c, Djujić et al., 2003e, Djujić et al., 2003c, Djujić et al., 2003d, Djujić et al., 2000c). Here we will present results of studies in which we fallow up effects of supplementation with 100 and 300 μg Se/day during 2 - 6 months (Table 3 and Table 4).

Table 3. Essential microelements in plasma and erythrocytes of investigated subjects before supplementation with SeMet

	Control		CCa		LCa	
Element	**Plasma**	**Erytrocytes**	**Plasma**	**Erytrocytes**	**Plasma**	**Erytrocytes**
Se (µmol/l)	0,36±0,05	0,48±0,06	0,25±0,05**	0,29±0,05**	0,26±0,05**	0,46 ±0,05
Zn (µmol/l)	11,60±1,02	96,72±9,01	10,04±0,73*	90,25±3,01*	9,85±1,03 * *	88,45±6,43 *
Cu (µmol/l)	12,28±1,08	9,92±0,79	11,30±1,05*	9,70±0,87	13,07±1,10 *	10,92±1,06 *
Fe (µmol/l)	18,30±1,90	153,92±15,17	20,93±2,61*	149,40±27,97	21,67±2,58 *	169,22±19,36 *
Mn (µmol/l)	62,80±2,96	1286,84±308,87	61,86±3,50	1171,57±167,61	64,58±3,80	1076,50±145,28

**p<0,001; *p<0,05

Relationship estimation showed that in patients with:

- **CCa** exist correlations between the fallowing elements: Cu/Fe in plasma ($r = 0.888$, $p<0.001$); Se/Fe in plasma ($r = -0.418$, $p<0.05$); Se/Cu in erythrocytes ($r = -0.323$, $p<0.05$); Se/Mn in erythrocytes ($r = -0.336$, $p<0.05$).
- **LCa** exist correlations between the fallowing elements: Se/Fe in plasma ($r = -0.462$, $p<0.05$); Cu/Zn in plasma ($r = -0.348$, $p<0.05$); Fe/Mn in erythrocytes ($r = 0.356$, $p<0.05$).
- **In control group** did not observed such relationships.

Presented data showed that to reach recommended Se levels in plasma and erythrocytes control group need from 100 - 300 µg Se/day. In cancer patients, supplementation with 100 µg Se/day was even insufficient to prevent its decreasing trend during the 6 months period. Daily intake of 300 µg Se expressed its beneficial effects on Se status in CCa patients after surgery and LCa patients during standard radiotherapy (RT) and concurrent administration of chemotherapy. In less than 20% of the cancer patients supplemented with 300 µg Se/day, its levels in plasma and erythrocytes reaches average Se concentrations for non-supplemented CHS. Longitudinal study of Zn in subjects supplemented with SeY showed that it did not reflect significantly to Zn status in plasma and erythrocytes of mild Zn deficient control group, but acted beneficial to Zn levels in patients with CCa and LCa that have stronger expressed Zn deficiency. Monitoring of Se supplementation effects on Cu status, showed that supplementation with SeY induces in plasma and erythrocytes of control group increase of Cu content , while in patients with CCa and LCA it had no effects. The supplementation with SeY caused in the investigated subjects a significant decline of Mn concentration in plasma and erythrocytes (~40% in control group and ~20% in CCa and LCa). The observed Fe status showed that supplementation with SeY causes a rise of Fe in erythrocytes of control group and CCa patients, but not in LCa patients. In plasma, the effect of Se supplementation was opposite, reduced Fe levels in control group and CCa patients and increased in LCa patients.

Bearing in mind that average intakes of Se and Zn in the investigated population are under the RDA (Recommended dietary allowance), Mn intake exceeds upper RDA level of 5mg/day, as well as that Cu and Fe intakes significantly varied, supplementation with 100 -300 µg Se/day as SeY, generally acted beneficially to trace elements important for maintaining redox balance status. The benefits depended on existing disturbances in the metabolism of trace elements in control group and patients with CCa and LCa.

Table 4. Effects of Se supplementation investigated in:

A - Healthy Se deficient subjects

Changes, %	100, µg Se - 2 month		300, µg Se - 2 month	
	Plasma	Erythrocytes	Plasma	Erythrocytes
Se	+56	+35	+112	+47
Zn	+8	-3	+5	+7
Cu	+4	-2	+6	+4
Fe	+11	+7	+6	+10
Mn	-5	-12	-15	-11

B - Patients that after surgical removing CCa received 6 months

Changes, %	100, µg Se - 2 month		300, µg Se - 2 month	
	Plasma	Erythrocytes	Plasma	Erythrocytes
Se	+8	-3	+74	+84
Zn	-4	-2	+8	+6
Cu	-2	-2	+6	+1
Fe	-5	+3	+11	+13
Mn	-1	+2	-6	-11

C - Patients with inoperable primary non-small cell LCa that 2 weeks before, during and 3 months after treatment (RT or chemotherapy with concurrent RT) received

Changes, %	100, µg Se 2 month		300, µg Se 2 month	
	Plasma	Erythrocytes	Plasma	Erythrocytes
Se	-32	-18	+8	+12
Zn	-14	-8	-6	+2
Cu	+11	+5	+6	-3
Fe	-14	-24	-8	-14
Mn	-12	-14	-6	+4

Examinations of the health benefits that offer consumption of products prepared with crops biofortified foliar with Se and eggs obtained from quails that consumed feed mix composed of biofortified crops showed that its contribution to daily Se intake was high enough that Se deficient population of Serbia can assure even more Se in natural form than is RDA (Fig. 19), as well as that, due to much better nutritional characteristics of such food, its use assures optimized intake of many essential elements (Si, Cu, Se, Cr, Ca, Fe, Mn, I, Zn, Mg, P) and nutrients for quail eggs presented in Figure 18 (Djujić et al., 2005, Djujić, 2007,).

Fig.19. Influence of food rich in natural Se consumption by Se deficient volunteers on its average daily intake, concentration in plasma and lipid peroxidation

Data obtained for Se status in plasma of investigated groups of volunteers after 6 weeks of consuming Se rich products (Figure 19) showed that on average groups that consumed two or more Se rich products assured plasma Se concentration that is required for optimal plasma GSHPx activity (around 95 µg/l) and optimize its mineral and antioxidant status (Djujić, 2006a, Djujić et al., 2001b, Djujić et al., 2005, Djujić, 2007). Investigations of antioxidative enzymes and oxidative stress level in blood plasma and erythrocytes, as well as elements determinations in hair confirmed that consumption highly valuable food products in which all nutrients are in natural form and inter-balanced offer immeasurable benefits to its consumers.

CONCLUSIONS

1. When present in proper level or added in proper concentration and form to Se deficient organisms, Se through various regulatory processes express its beneficial role.
2. Monitoring of relationships between Se and other essential and toxic elements in plants, animals and humans showed that Se, when is present in proper range, induce desired changes in other element status and distribution.
3. Division of elements to synergists and antagonists can not be applied to organisms with Se concentration inside proper level.

Taking into account the various important functions of the organs with highest Se concentrations and order of changes in Se concentrations and/or Se-enzymes activities during exposure to stressors it is evident that Se is involved through Se-amino acids, Se-proteins and Se-enzymes in processes that regulates behavior of tissues, organs and whole organism. In extreme situation reaction of plant and animals with proper Se levels always was directed to provide order of physiological processes that allows assuring needed quality and survival.

Therefore we hypothesized that Se in the form of Se-amino acids or proteins participate in regulatory processes that influence functioning of common sense in the living organisms.

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SUPEROXIDE REDUCTASE: A DEBATED MECHANISM, COMPARISON WITH SUPEROXIDE DISMUTASES

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ABSTRACT

The superoxide detoxification in certain anaerobes involves the superoxide reductase (SOR) enzyme. SORs have an important role as a novel oxygen detoxification system utilized by all anaerobic microorganisms that cannot permit the presence of dioxygen which was produced from superoxide by the action of SOD. During the computational analysis of how these enzymes work, detailed investigations were performed by researchers only for the SOD enzymes. Our current aim is to evaluate the transformation of superoxide to hydrogen peroxide within the iron active-site center of SOR by QM/MM and molecular dynamics computations.

Key words: superoxide reductase, superoxide dismutase, detoxification,non-heme iron, computation

INTRODUCTION

The only existing superoxide detoxification enzyme was though to be the superoxide dismutase (SOD), but recently (Jenney et al., 1999; Abreu et al., 2000; Coulter et al., 2000; Lombard et al., 2000; Kurtz, 2004; Niviere et al., 2004) it has been discovered that for the detoxification of the superoxide some anaerobic or microaerophilic bacteria use a different enzyme system. The one-electron reduction of superoxide, in which peroxide is formed, is catalyzed by the superoxide reductases (SORs). SOR enzymes in comparison with the SODs, that catalyze the dismutation of superoxide radicals forming molecular oxygen and hydrogen peroxide, are non-heme iron proteins classified into one-iron SORs (Jenney et al., 1999; Abreu et al., 2000; Jovanovic et al., 2000; Lombard et al., 2000) which contain only one iron active-center and two-iron SORs (Coulter et al., 2000; Lombard et al., 2000; Rodrigues et al., 2007) possessors of an additional rubredoxin-like $[Fe^{3+}\text{-}(SCys)_4]$ center.

$$SOR{\cdot}Fe^{2+} + O_2^- + 2H^+ \rightarrow SOR{\cdot}Fe^{3+} + H_2O_2 \quad (a)$$

$$SOD{\cdot}M^{(n+1)+} + O_2^- \rightarrow SOD\ M^{n+} + O_2 \quad (b_1)$$

$$SOD{\cdot}M^{n+} + O_2^- + 2H^+ \rightarrow SOD\ M^{n+} + H_2O_2 \quad (b_2)$$

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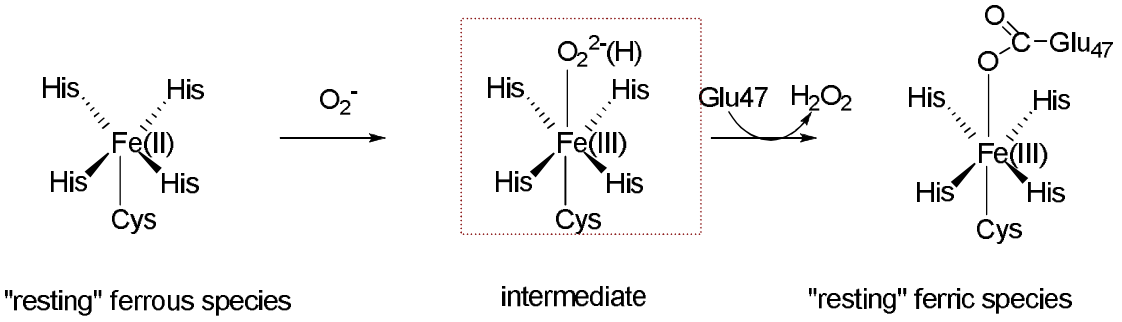
The SOR enzymes have an important loop region (Ile2-Ile20 amino acid sequence) that contains two amino acid residues (Glu14 and Lys15) with role in the superoxide transformation in hydrogen peroxide.

An intermediate of the reaction of ferrous site from SOR enzyme with superoxide was identified by pulse radiolysis (Coulter et al., 2000; Emerson et al., 2001) exhibiting an absorption at ~600 nm. The intermediate showed in Scheme 1 was thought to be a ferric-(hydro)peroxo species and the nature of this has not been established. The pulse radiolysis studies have described one (Abreu et al., 2001) or two (Lombard et al., 2001; Niviere et al., 2001) intermediates at other SOR enzymes, some of them described as ferrous-superoxo, ferric-peroxo, or ferric-hydroperoxo species. Density functional and ZINDO/S semiempirical calculations were employed to show that only one intermediate should be observable in SOR, and that this intermediate should be a ferric-hydroperoxo species, whose UV-vis maximum at 600 nm arises fro thiolate-=to-iron charge transfer transitions, which some contribution from iron as well (Silaghi-Dumitrescu et al., 2003). Subsequent elaborate calculations from several groups have thoroughly confirmed this conclusion, although, unlike the initial study, they have all concluded that preferred the spin state of the hydroperoxo intermediate is S=5/1 not S=1/2. This debate over the nature of the catalytic intermediate in SORs is in sharp contrast with what is known for the equivalent reaction in SODs: superoxide reduction to peroxide in superoxide dismutases has, to date, not allowed for detection of any reaction intermediate in the several SOD classes known to date – nickel-SOD, iron-SOD, manganese-SOD, or copper, zinc-SOD (Surarawatanawong et al., 2010).



Recently DFT and QM/MM calculations of the mechanism of the reaction catalyzed by SOD were performed (Srnec et al., 2009). Although investigations of the reaction catalyzed by SOR at DFT level were effectuated for a small part of the iron center, detailed evaluation of the role of surrounding amino acid residues using QM/MM method was not effectuated until now.

MATERIALS AND METHODS

Selection of the SOR crystal structure for analyzing of the active centers was necessary.

There are twelve crystal structures determined for superoxide reductase enzyme (Brookhaven Protein Data Bank codes: 1DO6, 1DQI, 1DQK, 1VZG, 1VZH, 1VZI, 2JI1, 2JI2, 2JI3, 1YO7, 2AMU, 2HVB). Among the available structures the 1DQI structure was selected for computational investigation of SOR. This is the only

structure which contains a monomer (B chain) with Glu14 and Lys15 out from the square-pyramidal center and revealed an opened conformation of the Glu14- and Lys15-containing loop region. We took into account only the SOR structures with one iron active center.

Investigation were performed using the GAUSSIAN 09 (rev. *A.1*) - 2009 and GaussView (version 3.09) – 2003 as front-end. Optimizations were carried out by the DFT method using Becke's three parameter hybrid functional combined with the Lee-Yang-Parr correlation functional (B3LYP) (Lee et al., 1988; Becke 1993) with the 6-31G or 6-31G(d,p) basis sets.

RESULTS AND DISCUSSION

The active site of SOR is concerning on a non-heme Fe^{2+} center in an square-pyramidal $[Fe^{2+}\text{-NHis})_4$ $(SCys)_1]$ pentacoordination, with a vacant position. The rubredoxin-like center apparently is not involved in catalysis (Emerson et al., 2003) According to an inner-sphere superoxide reduction mechanism, H_2O_2 product is formed leading to a hexacoodinated ferric active-site in which the carboxylic group of the Glu14 (Yeh et al., 2000; Berthomieu et al., 2002) glutamic acid residue is bonded.

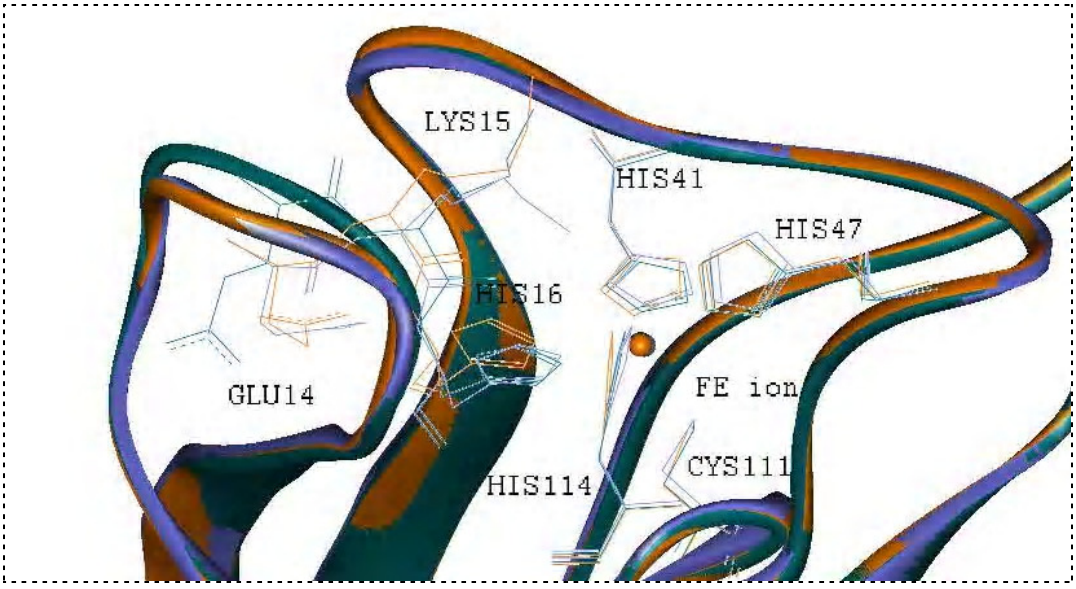


Fig 1. The mobile Glu14-, Lys15-containing loop region and the iron site within superoxide reductase determined from the hyperthermophilic archaeon *Pyrococcus furiosus*. Comparison of three mobile-loops of three SOR structures: 1DO6 (in violet), 1DQI (in dark green) and 1DQK (in orange).

In the Figure 1 representation of the iron active centers from the 1DO6 (in violet), 1DQI (in dark green) and 1DQK (in orange) SOR structures is showing. In the case of 1DQI crystal structure the ferrous center could be observed.

In the first step including of seven water molecules (calculations in which more than seven water molecules were included were aborted) at the top of the iron center was deemed necessary. The possible orientation of the water cluster could be the one presented in the figure 2 – as suggested by an initial molecular mechanics

optimization of this cluster only, leaving the rest of the model frozen. These calculations were followed by DFT geometry optimization. The water molecules are expected to be essential in delivering protons for formation of the ferric-hydroperoxide intermediate, but also for subsequent release of hydrogen peroxide from the active site. As previously discussed (Lombard et al., 2001), incorrect delivery of protons may allow the hydroperide bound to iron to decay via a different route, leading to toxic free radicals and high-valent iron species.

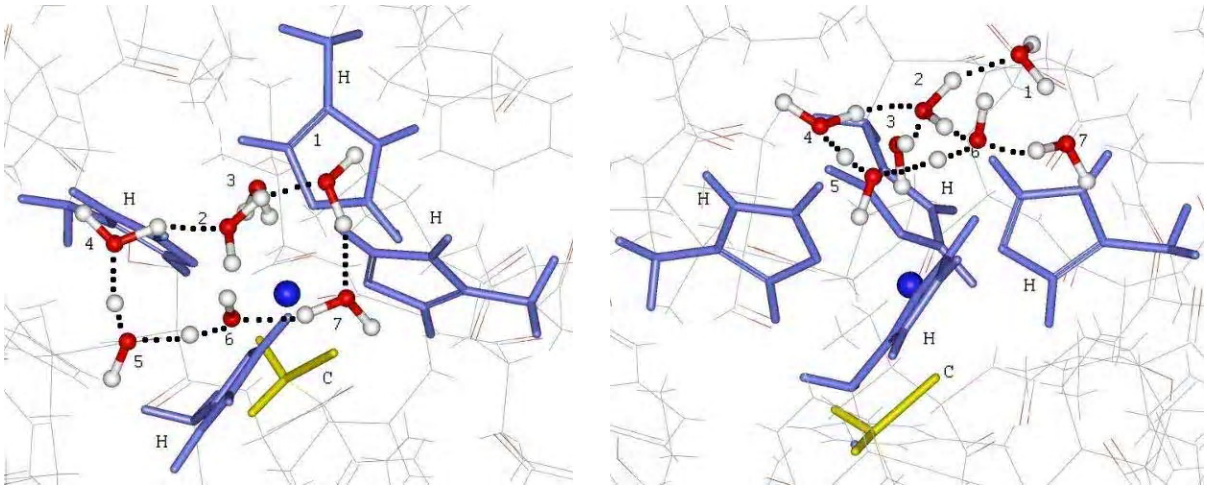


Fig. 2. Arrangement of the seven water molecules beyond the opened non-heme iron center of the B chain of 1DQI crystal structure of superoxide reductase. The left (A) and right (B) panels show two views of the model.

We intend to define the way in which the superoxide radical is bonded and transformed within the iron center of SOR by using QM/MM calculations. For this we propose the QM treatment of the iron center (iron ion, Cys111, His41, His47, His114, His16, Glu14, Lys15, superoxide and water molecules) and MM treatment of the surrounding enzyme environment taking into account a 13Å region of the enzyme.

Figure 3 shows the electrostatic potential surface of the two-iron superoxide reductase, illustrating that although there are two iron centers, only the catalytic one diplays a patch of positive charge fit to attract superoxide, whereas the electron-transport iron-sulfur center will actually repel superoxide due to its negative charge. This justifies a strategy for investigating superoxide interaction with the surface of the protein only in the close proximity of the 'SOR site' (the catalytic center), as done in Figures 1 and 2, and not at further distances or at the other metal center – even though the distance between the two iron centers is too large and hence may raise doubts as to why the iron-sulfur center, typically seen in other proteins as electron-transporter, would have to sit so close to its presumed partner (the catalytic site) in SOR.

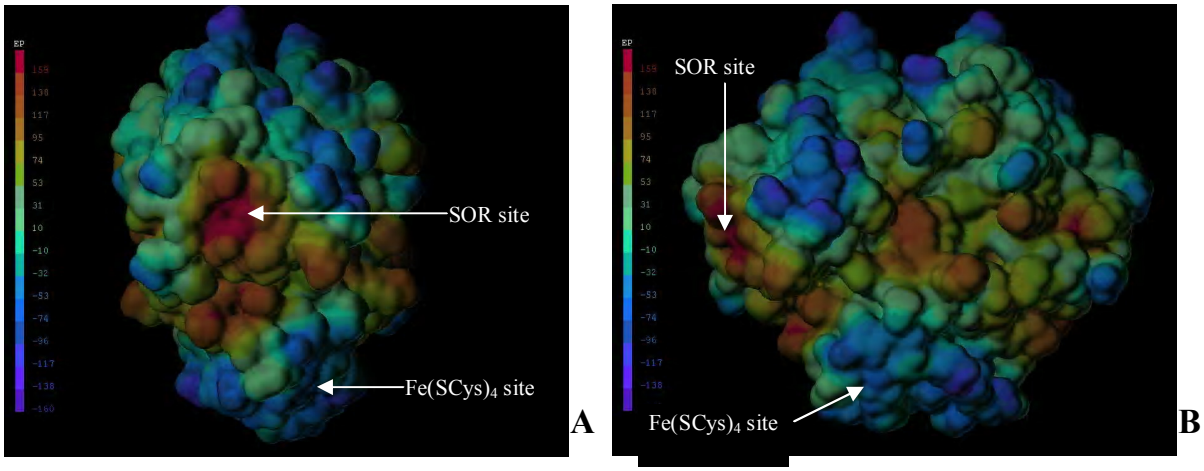


Fig.3. Electrostatic potential surface of two-iron superoxide reductase; panels A and B show two views (differing by a 90º-rotation) of the same molecule.

CONCLUSIONS

The crystal structure of superoxide reductase with the IDQI PDB code determined from *Pyrococcus furiosus* has the B chain with the Glu14-, Lys15-containing loop region in opened conformation and hereby we could perform DFT calculations in order to check the possible arrangement of some water molecules and superoxide above the square-pyramidal ferrous-center of the SOR. During the calculations we concluded that seven water molecules form a water cluster next to the active center. To our knowledge this is the first report where the SOR structure is examined computationally at this level, taking into account the entire protein.

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COMPARATIVE STUDY OF SPA WATERS IN SOME OF SOUTH-EAST EUROPEAN COUNTRIES

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ABSTRACT

This paper is coping with thermal mineral waters which has a good cemical and physical characteristics for use to spa waters in South-East European Region including Serbia, BH and Romania. The detailed analysis was done for Spa Junakovic (Backa) compared with others: Novi Sad, Temerin, Becej, Kanjiza (in Backa), Lazarevac, Torda, Rusanda, Jermenovci (in Banat), Olovo (in Bosnai and Hercegovina) and Spa waters in Lakes Sovata (Bucharest, Romania). In the work emphasised the content of metalic cations in balance with characteristic anions. The content limits the stay in water and influences the terapheutic values of spa water, also helps to determine the treatment regime for rehabilitation, recreation purposes especially in preparation of athletes (lakes in Sovata) for recovery of reconvalescents with bone fractures and rheumatism (Junakovic, Rusanda, Olovo). The analysis of Junakovic spa water where done by means of standards and contemporary methods in Institute of Hygiene (Sombor).

Key words: Spa Water, Cations, Anions, South-East Europe.

INTRODUCTION

In this paper are presented spa waters originated in Serbia in Region of Vojvodina, in Backa and Banat in comaprison with Olovo in BH and water in lakes in Sovata. Among of the investigated waters there were hypermineralized, oligomineralized and slightly mineralized waters. In Backa are important thermal mineral water sources which used for exploatation for installing spas in Junakovic, Novi Sad, Kanjiza, Becej and Temerin. The mineralization of thermal mineral waters in Backa varies between 3-20g/dm^3, with maximum value arround 40 g/dm^3. On that base they differ to four hydro-geological systems as: $NaHCO_3$ type (3-9g/dm^3), $NaHCO_3$-NaCl type (4-20g/dm^3), NaCl type (15-25g/dm^3) and the type low mineralization. The temperature varies in 75-90^{O}C, 50-75^{O}C and bellow 50^{O}C Bogdanovic, 2000).

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Spa Junakovic

The Junakovic spa is located 10km from Hungarian border nearby Prigrevica. The first well was opened 1912. and a deeper (590m) in 1977. In 1981. were made hydrothermal wells trough Miocene limestone layers in depth of 700-800m. The water is alkaline with pH 7.5 with Na^+, K^+, Ca^{2+}, Mg^{2+}, NH_4^+ cations and balanced chloride, hydrogen-carbonate and sulphate anions. The water from Prigravica-1 was characterised as alkaline muriatic iodine hypo-thermal water (Table1). Junakovic spa is an important point for medical tourism. Spa water of Junakovic is successful in the treatment of degenerative rheumatism, asthma and the rehabilitation of bone fracture.

Thermal mineralwater of Novi Sad

The thermal mineralwater of Novi Sad was discowered in Futoski park 1897. The water contained hydrgen-carbonates, chlorides, hydrogen-sulfide and rich with Na^+. The temperature in NS1/H sources is 42□C and in NS2/H sources is 35□C (Milosavljevic, 1997). The use of Novi Sad thermal mineral water started with medical treatment 1910. of rheumatic, vascular and nerve illness.

Temerin spa

This thermal mineral water is hydrgen-carbonate type of water with Na^+ content. The spa in use about 100 years. The spa is known for treatment of degenerative disease and skin and bone disease. Chemical content of Temerin‘s termomineral water; cations (mg/dm^3): Na^+(995.00), Mg^{2+}(21.75), Fe total (1.00), K^+(5.6); anions: HCO_3^-(1910.50), Cl^-(514.17), J^-(012) (Bogdanovic,1999).

Kanjiza spa

Kanjiza spa is on the Nord Backa, about 10km to Hungarian border. The termal mineral water found 1908, started 1913. The metal cations Ca^{2+}, Mg^{2+}, Fe^{3+}, Al^{3+}, are on in the balance with Cl^-, SO_4^{2-}, CO_2 and SiO_2. The spa water is hydrogen-carbonate-Na type with fluoride and/or nitrite and chloride. Kanjiza spa is good for treating rheumatic illnesses, arthritis and hardening or inflamtion of spine.

Rusanda spa

Rusanda lake. The pH value in lake water is 6.8. The mineralization 16.54dm^3, contains Na^+, Ca^{2+}, in balance with HCO_3^-, Cl^- and SO_4^{2-}. The everage salinity is about 4% (Tomic, 2000). The mud contains K^+, Ca^{2+}, Fe^+, Al^{3+}, in balance with HCO_3^-, CO_3^{2-}, SO_4^{2-} and H_2S. Rusanda spa is a center for physical medicine and rehabilitation. The spas Olovo and Sovata lakes were studied with aim to compare the qualities of sapas abroad near Serbia.

MATERIALS AND METHODS

For the preparation of samples was used is the microwave oven Aurora, 680 MW to 350 psi, with 6 Teflon cuvette 60ccm, according to standards EPA 3015, 3051, 3052… Elemental analysis was done using the device, Aurora, AAS AI1200 with 5 lamps that are powered at the same time, the burner is titanium, teflon chamber and the diffuser glass and security provided a software monitoring. Characteristic of device: optics with high bandwidth and automatic correction of deviation, 0.3m Czerny-Turner monochromator installed, automatically moving the slit grating with 1200/mm, a wide range of high-sensitivity PMT 185-900 nm, spectral

width of the openings of 0.2, 0.6, 1.2 nm and the reduction of the slit height of 0.6 nm, 1000Hz sampling rate data HCl self reversal (Smith-Hieftje) background correction for accurate correction of the D2 with automatic adjustment of intensity. Reference substances are brand Accustandarad NO HAZ, and samples were withdrawn automatically switches the device for sampling along with all the precautions and recommendations of the manufacturer of the instrument and chemical standards.

RESSULTS AND DISCUSSIONS

One of the important indicators of the spa water is its mineral composition, where the cations play an important role. Therapeutic values are determined in relation to the concentration of certain metal ions. Usually emphasize the ion concentrations of $Fe^{2+/3+}$, Mg^{2+}, Ca^{2+}, K^{+}, Li^{+}. In some of cases trace elements have significance for the treatment of certain skin diseases and for reconvalescents, especially with fractures and other injuries and for patients with inflammation and rheumatism. The water in the spa Junakovic contains 0.0035 g/dm^3 (Table 2). Unlike spa Junakovic, water of Spa Olovo containes nearly 10 times more iodine (0.02 mg/dm^3). Iodine is the most valuable component on the medical meaning. Depending on the cations are in balance certain anions. Iodine is important for the balance of sodium and potassium, which influences the balance between carbonates, hydrogen-carbonates and sulfates. Cations Mg^{2+}, Mn^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+} and Ca^{2+} have special therapeutical values. Pb content preferably should be reduced because it is considered to be toxic and not recommended for patients with open injuries. Chemical characteristics of spa water Junaković show that there are certain turbidity, but because of the low content of toxic substances can not be considered to have negative effects in therapy.

Analysis of thermal waters AQUATERM ”Lead” (”Olovo”) did Geoinstitute IMTH Belgrade in 1985. year. Unlike spa Junakovic, which has a hyper-thermal spa water, spa Olovo has only thermal water, between 30 and 40°C, but there are many more minerals. In particular it should be noted that in the spa Olovo iron ions are not detected, whereas, in small quantities, in the spa Junakovic there. H_2S content is significantly higher in the water of spa Junakovic. Waters of Olovo and spa Junakovic not contain lead, arsenic and other toxic elements (Tables 1, 2, 3, 4, 5).

Table 1. Chemical content of spa water (Spa ”Junakovic”)

Content of cations	mmol/dm^3	mg/dm^3	Content of anions	mg/dm^3	mmol/dm^3
Sodim, (Na^+)	92,6086	2,1300	Hydrocarbon (HCO_3)$^-$	1,5614	25,5967
Potasium, (K^+)	0,9256	0,0361	Cloride (Cl^-)	2,5600	72,1126
Lithium, (Li^+)	0,1685	0,00118	Bromide (Br^-)	0,0060	0,0750
Ammonium, (NH_4^+)	1,1111	0,0200	Iodide (I^-)	0,0035	0,0275
Calcium, (Ca^{2+})	0,8233	0,0330	Fluoride (F^-)	0,0033	0,1736
Magnesium, (Mg^{2+})	0,7401	0,0180	Nitrate (NO_3^-)	-	-
Strontium, (Sr^{2+})	0,0642	0,00563	Phosphate (PHO_4^{3-})	0,00003	0,00003
Manganese,	0,10001	0,00001	Sulphate	0,0040	0,0416

(Mn^{2+})			(SO_4^{2-})		

Table 2. Characteristics of spa water (Spa ”Junakovic”)

Other components and parameters	Mass fraction, g/dm^3
Siliicon (IV) oxide, (SiO_2)	0,0950
Aluminum (III) oxide, (Al_2O_3)	0,0120
Iron (III) oxide, (Fe_2O_3)	0,0015
Metaboric acid, (HBO_2)	0,0690
Free hydrogen sulfide, (H_2S)	0,0012
Dry residue	5,4720
Specific weigth, kg/m^3	1,00348
Spa water temperature, °C	50,8

Table 3. Chemical content of spa water, mikroelements(Aquaterm, Spa ”Olovo”)

Microelements	Li	Rb	Sr	P	Br	I	Zn	Cu	Pb	Mn	Cr	Al
mg/l	0,1	0,01	0,34	0,04	0,06	0,02	0,05	0	0	0	0	0,14

Table 4. Chemical content of spa water (Aquaterm, Spa ”Olovo”, BH)

Content of elements	mg/l	mg-ekvl	%ekvl
NH_4^+	0	0	0
Fe^{3+}	0	0	0
Fe^{2+}	0	0	0
Na^+	5,1	0,22	3,9
K^+	1,3	0,03	0,5
Mg^{2+}	17,8	1,48	26,2
Ca^{2+}	78,3	3,91	69,4
NO_2^-	0	0	0
NO_3^-	4,15	0,07	1,2
CO_3^{2-}	0	0	0
HCO_3^-	323,3	5,3	93,5
SO_4^{2-}	5	0,1	1,8
Cl^-	7,1	0,2	3,5
F^-	0,14		
C	0,05		
SiO_2	16		
Mineralization	459		
Dry residue	297		

Table 5. Characteristic of spa water (Aquaterm, Spa ”Olovo”)

Solybility of free gases	mg/l	ml/l	%ml
CO_2	79,2	39,6	N 86,5
H_2S	0,14	0,1	He (+Ne) 0,031
O_2	7,49	5,24	Ar (+Kr +xe) 1,1876
Total hardness	2,69	15,06 d	
Carbonate hardness	2,65	14,84 d	
E-condivity	270 µs/cm		
Color (Color scale)	3 jedinice		
turbidity	2 stepena		
Eh	+ 85 mV		
pH	8,1		
Spa water temperature	34-36°C		

Spa water Rusanda slightly thermal water, with relatively low mineral content and low temperature of 28°C. According to its chemical composition is hydrogen-carbonate water, with minimal presence of free hydrogen sulfide and ammonia in trace amounts. Nitrates are present in somewhat larger amounts.

Table 6. Chemical content of spa water (Spa ”Rusanda”)

Content of cations	g/dm^3	Content of anions	g/dm^3
Sodim, (Na^+)	5,8	Hydrogen-carbonate (HCO_3)$^-$	1.464
Calcium, (Ca^{2+})	0.24	Chloride (Cl^-)	8.52

Table 7. Characteristic of spa water (Spa ”Rusanda”)

Other components and parameters	Mass fraction,
Salinity	4%, variable
Free hydrogen sulfide, (H_2S), g/dm^3	0.0005
Dry residue, g/dm^3	180°C 15,58
Water temperature, °C	28

Analysis of thermal waters some of the lakes in Sovata did The Institute of Physical Medicine, lake-climatology and medical recovery, Bucharest.

In the waters of the Sovata lakes the ions of sodium, magnesium and calcium are stratified. (Table 8).

Table 8. Chemical content of spa water some of lakes in Sovata (Bucharest)

lake / Characteristics mg/dm^3	Ursu (bottom)	Ursu (surface)	Verde (bottom)	Alunis (bottom)	Alunis (surface)	*Rosu* (bottom)
Cl^-	60 276	179 909	39 606	24 820	171 966	155 124
Br^-	5	17	8,5	6	10	13
SO_4^{2-}	720,1	2 656	6 580	266	1 346	1 218
HCO_3^-	256,3	470	348	244	367	183
Na^+ (mg/l)	39 224	116 105	60 811	16 171	111 324	100 214
Ca^{2+}	244	715	212	76	701	298
Mg^{2+}	22	72	32	10	71	73
Mineralization	100 809	299 944	257 207	41 613	285 879	257 137
Year	1980	1980	1980	1980	1982	1980

The difference of concentrations are very significant in lakes Ursu and Alunis. This difference is seen in Rusanda spa, where the salinity varies. The higher value is in the place there the salt water springs appear from the bottom of the lake. The higher values of concentration of iones in Ursu and Alunis are on the surface. It is important for the time of stay in water, to terapy and for recreative activities.

CONCLUSIONS

1. Spa waters in South-East Europe are generally thermal or hypothermal and have a high mineral content.

2. The chemical composition of the spa waters are carbonated waters with hydrogen- carbonate, with a high content of sodium, potassium, magnesium and calcium, and have a low content of heavy metals, don't contain lead and mercury.

3. Of particular importance is the balance in the concentration of anions and cations at a given temperature, especially in thermal and hypothermal spa water, which reflects the high quality of the spa water with health effect.

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MAGNESIUM OROTATE IN CARDIOLOGY - A FORTY YEAR OLD STRUGGLE

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ABSTRACT

Magnesium is on place four of all the cations in the body and it is present in more than 300 enzymatic systems, its presence being crucial for ATP metabolism. The role of magnesium is of enzyme activator in regulation of cellular energy metabolism, vascular tone and cell membrane ion transport.

Low serum magnesium and potassium levels were shown to be directly related to prevalence of premature ventricular contractions in healthy subjects in the Atherosclerosis Risk in Communities Study - ARIC (Simpson et al., 2002). Intravenous magnesium sulfate has been used therapeutically in critical situations such as torsade de pointes and ventricular arrythmias caused by digitalis (Fox et al., 2001, Chang et al., 2002) and proven to be safe and effective at the onset of myocardial infarction, leading to a 24% reduction in mortality (Yusuf et al., 1993). In patients with chronic heart failure, even on chronic cardiac glycoside, beta-blockers and associated antiarrythmic regimens, a worsening of their clinical condition is frequently due to supraventricular or ventricular arrythmias. In many cases these arrythmias prove to be life-threatening and are difficult to manage. Precipitating factors for arrythmia include associated potassium deficiency secondary to chronic diuretic therapy regimens, digitalis toxicity and ischemia.

Keywords: magnesium orotate in therapy

INTRODUCTION

Magnesium therapy has proven to be beneficial in counteracting all phases and processes of ischemic heart disease, from low-risk arrhythmias to sudden death.

Concomitant magnesium deficiency in K-depleted patients was reported to range from 38% to 42% (Whang et al., 1992). Uncorrected magnesium deficiency impairs repletion of cellular potassium, a condition referred to as refractory potassium depletion. According to Whang et al, refractory K depletion as a consequence of Mg deficiency may be operative in patients with congestive heart failure, digitalis toxicity, cisplatin therapy and in patients receiving potent loop diuretics. Therefore, they recommend that serum Mg be assessed routinely and hypokaliemic patients be treated with both Mg as well as K to avoid the problem of refractory K depletion due to coexisting Mg deficiency (Whang et al., 1992).

In his excellent review on digitalis toxicity, delivered in e-format (eMedicine) on Pub Med, Patel (2002) points out that long-term digoxin users often have hypomagnesaemia secondary to diuretic usage. Patients with hypomagnesaemia,

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hypokalemia or both become cardiotoxic even at therapeutic digitalis levels, because hypomagnesaemia increases myocardial digoxin uptake and decreases cellular Na+/K+-ATPase activity.

Sueta et al. (1994) have found that the risk of developing potentially fatal ventricular arrhythmias was reduced by more than half in patients with ischemic heart failure who received large intravenous doses of magnesium, 0.3 mEq/kg injection followed by continuous infusion of 0.08 mEq/kg/h over 24 h. They conclude that more studies are needed for establishing to what extent oral supplements of magnesium can be as efficient as intravenous doses (Sueta et al., 1992).

Investigators in Baltimore, Maryland report that an intravenous infusion of 2 grams of magnesium chloride in patients having undergone coronary bypass surgery significantly lowers the incidence of severe ventricular arrhythmias.

Although all these reports have documented the effectiveness of magnesium in correcting lethal arrythmias, the rank of magnesium administration has not been well established in standard algorithms for arrythmia therapy.

Few controlled studies exist regarding the therapeutic uses of oral magnesium supplementation in chronic cardiovascular disease, although in the US daily allowances are recommended to prevent the risk of dying from heart disease (Ford, 1999).

1. MAGNESIUM SERUM LEVELS AND TISSUE STORES

Many investigators agree that normal serum levels of magnesium could still be associated with low tissue stores responsible for clinical effects (Patel et al., 2002). A recent study of Klevay et al. (2002) tested the hypothesis that an intake of magnesium considerably below the recommended allowance could produce evidence of depletion. The study was carried out on 22 postmenopausal women who ate a diet of conventional foods containing less than half the recommended dietary allowance of magnesium. Holter monitors showed a significant increase in both supraventricular and ventricular ectopy, although serum Mg, K, and Ca concentrations remained normal (Klevay et al., 2002). Despite normal values of blood-ionised magnesium, Chang et al (2002) report drastically reduced incidence of ventricular arrhythmia after administration of 2 grams of magnesium sulphate intravenously.

Intracellular magnesium depletion has been shown to occur despite normal serum magnesium level in the study of Patel. Iv magnesium sulphate 2 g over 5 min has been shown to terminate cardiac arrhythmias and aside from successful replacement of intracellular Mg, it also may act as an indirect antagonist of digoxin because hypomagnesaemia decreases cellular Na+/K+-ATPase activity (Patel et al., 2002).

Gottlieb et al. (1990) have studied the prognostic significance of an abnormal serum magnesium concentration in 199 patients with heart failure. The serum magnesium concentration was less than 1.6 mEq/l in 19% of patients, within normal range in 67% of patients and greater than 2.1 mEq/l in 14% of patients. Patients with low serum magnesium concentration had more frequent ventricular premature complexes and episodes of ventricular tachycardia than did patients with a normal serum concentration. Patients with hypomagnesaemia had more severe symptoms, worse renal function, but fewer ventricular arrhythmias. The authors recommend routine measurement of serum magnesium concentration in patients with chronic heart failure.

2. MAGNESIUM AND INSULIN RESISTANCE

Available research suggests an association between magnesium deficiency and insulin resistance. In two patient populations normally associated with insulin resistance, overweight and type 2 diabetic individuals, magnesium deficiency is a relatively common occurrence. Depletion of intracellular free magnesium has also been found to be a characteristic feature of insulin resistance among subjects with essential hypertension (Dominguez et al., 1998).

Nadler et al (1993) reported a decrease in insulin sensitivity with magnesium deficiency in all subjects studied Humphries et al (1999) reported a clear association between the lowest consumption of dietary magnesium and the highest degree of insulin resistance among non-diabetic subjects. Dominguez et al (1998) confirmed this observation, finding that among both normotensive and hypertensive subjects, a higher magnesium level corresponded to a greater degree of sensitivity to insulin. Looking at this association from another perspective, research indicated an infusion of insulin lowered the ability to accumulate intracellular magnesium, and this response to insulin might be even more exaggerated among individuals with higher degrees of insulin resistance (Paolisso and Ravussin, 1995). Lefebvre et al (1994), in their evaluation of magnesium's role in glucose metabolism, concluded, "...magnesium deficiency results in impaired insulin secretion while magnesium replacement restores insulin secretion. Furthermore, experimental magnesium deficiency reduces tissue sensitivity to insulin.

In efforts to clarify the relationship between insulin resistance and magnesium, several research groups have examined the effects of magnesium supplementation and glucose handling. Paolisso et Ravussin (1995) conducted a double-blind, randomized, crossover study to test the impact of magnesium supplementation on, among other factors, insulin resistance in elderly individuals. They provided subjects with 4.5 grams magnesium daily for four weeks, which resulted in a significant increase in erythrocyte magnesium concentrations. This intervention also resulted in an improvement in insulin sensitivity, and this improvement correlated with the improved magnesium status (Paolisso et Ravussin, 1995).Unfortunately, similar improvements in glucose control were not found in a study of magnesium supplementation in people with type 2 diabetes. While oral magnesium supplementation (30 mmol/day) for three months resulted in a significant improvement in plasma magnesium levels, this improvement was not sustained following discontinuation of magnesium, and no significant changes in the metabolic control of blood sugar were observed.

2. MECHANISM OF ARRHYTHMIAS RELATED TO MAGNESIUM DEPLETION

Lack of magnesium was shown to determine a decrease in the concentration of intracellular potassium and an increase in calcium levels (Reinhart, 1991).

In the ischemic myocardium, cellular calcium overloading is a major factor in the pathogenesis of arrhythmias and cell death. Magnesium ion is accepted as a natural calcium antagonist (Ziskoven, 1989). Mg deficiency reverses the optimal intracellular Ca:Mg ratio, the excess calcium becoming toxic to the cell. In response to high calcium levels in the ischemic myocardium, calmodulin, a calcium-sensing

protein, binds to the tail end of the sodium channel protein causing a malfunction of these channels and consequently irregular cardiac activity (Balser, 1999).

Transsarcoplasmatic ionic movement is one of the major cellular functions of myocardial and skeletal muscle cells. The structural components of the sarcolemma are glycoproteins, disposed in oligosaccharide chains. The usual terminal monosaccharide in these chains is sialic acid, a relatively strong acid, with a pKa=2,6. Cations such as sodium, pottasium and magnesium bind to sialic acid, but calcium is bound preferentially in a 1:1 ratio with an affinity constant KA=121 mole-1. By binding calcium, sialic acid retards the influx of calcium into the cell, being thus a regulator of membrane permeability to calcium ions. This was first evidenced by in 1977 by pretreatment with neuraminidase to remove sialic acid in order to prevent binding of calcium, which resulted in a marked increase in calcium uptake by myocardial cells.

3. OROTATE

Orotic acid is a naturally occuring substance and a key intermediate in the biosynthesis of pyrimidines. Previous investigations suggest that orotate can protect recently infarcted hearts against a further ischemic stress and may be beneficial in certain types of cardiomyopathy.

In the seventies many Russian and Bulgarian clinical investigators reported beneficial effects of potassium orotate in the treatment of angina, myocardial infarction and chronic heart failure. These investigators observed improved contractility, as assessed by the ventricular ejection period on nuclear angiography, lower incidence of complications and lower mortality rates in the patients on potassium orotate treatment compared with patients on cardiac glycosides, oxygen and anticoagulants (Lukomski et al., 1967; ; Ignatev, 1969; Zharov, 1972).

In the Western world, Hans Nieper was the first to use orotate clinically prior to 1980 (Nieper, 1974). His argument was that being neutrally charged, they pass easily through cell membranes and ferry mineral atoms into cells, producing higher intracellular concentrations. Nieper combined potassium and magnesium orotates to treat cardiovascular disease.

3.1. Role of magnesium orotate

The first International Symposium on Orotic Acid and Magnesium Orotate was held in november 1991 in Rudesheim, Germany. According to the studies of Williams (1992) and Munsch et al. (1991) performed on a rat model, the mechanism for the cardioprotective effect of orotic acid is consistent with the increase of the activity of all enzymes of the de novo pyrimidine pathway. Orotic acid stimulates the synthesis of pyrimidine bases in the heart, kidney and liver, by increasing the activities of uridine kinase and uridine phosphorylase, neither requiring phosphoribosyl-pyrophosphate (PRPP). These bases are transported by “salvage pathway” mechanisms from the kidney and liver to the heart for pyrimidine base and nucleoside synthesis, thus sparing the ischemic myocardium PRPP for the more needed ATP synthesis. In the failing heart, this mechanism seems to be crucial for maintaining the energy charge of the “high energy” adenylate compounds at an elevated level in the cytoplasm. This optimization of the phosphorylation state of the adenine nucleotides secures the energy metabolism of the stressed myocardium by “metabolic supplementation” .

The conclusions of the Hamburg symposium on magnesium orotate held in 1998 were more reluctant and stated that a number of studies indicate that orotic acid and its magnesium salt have a modest beneficial effect on the myocardium under conditions of stress and that further clinical testing is indicated to determine if the effects described could be of significant clinical benefit in the treatment of heart disease (Rosenfeldt, 1998).

3.2. Oral supplementation of magnesium orotate

Reluctancy in the use of oral administration of other magnesium salts (oxide, carbonate) is linked to the laxative properties of these salts in higher doses, an effect completely absent in orotate.

Although most of the studies regarding the benefit of magnesium therapy were conducted with intravenous supplementation of magnesium sulphate, clinical evidence is growing that oral supplementations may be as efficient as intravenous use in chronic patients. Most clinicians seem reluctant to administer higher than daily-recommended doses because of the undesirable laxative side effect of Mg oxide and carbonate. However, this is not the case of Mg orotate, a very well tolerated preparation, which offers the advantage of orotic acid supplementation with all the benefits derived from the key function it holds in regulation of energetic metabolism.

Shechter et al performed a randomised, placebo-controlled trial on 50 CAD patients to test the efficacy of oral magnesium supplementation in reducing endothelial dysfunction and improving exercise tolerance. They measured tissue magnesium levels in scraps of sublingual endothelial cells, found to correlate well with levels found in heart tissue. 72% of patients had lower than normal Mg tissue levels, and were randomised to receive either placebo or 365 mg of Mg oxide or carbonate daily. After 6 months, endothelial function and exercise duration were significantly better in the magnesium group, compared to the placebo group. It was also highly significant that none of the patients in the magnesium group experienced any arrhythmia during exercise. The authors conclude that magnesium may protect the heart against the detrimental effects of calcium overload and improve intracellular ATP production and glucose use (Shechter et al., 2000).

Favourable effects of oral magnesium orotate on exercise tolerance and left ventricular function have also been reported by Geiss et al (1998) in a pilot study on 14 CAD patients, active participants in an ambulatory rehabilitation program. Magnesium orotate decreased significantly LVESV, increased significantly EF and exercise duration.

4. ANIMAL STUDIES

The favourable effects of oral potassium and magnesium orotate supplements in heart failure and arrythmia treatment and prevention were also confirmed by animal studies. These studies also contributed to a better understanding of the mechanism of action of orotates, at sarcoplasmatic and intracellular level.

Wrogemann et al studied the effect of orotate delivered as dietary supplement on an animal model of chronic heart disease, the hamster hereditary cardiomyopathy (Wrogemann et al., 1978). Marked reductions in mitochondrial oxygen consumption and increases in mithocondrial calcium concentration have been shown to

accompany the spontaneous necrotic lesions in heart and skeletal muscle. All these effects were attributed to an inability of the sarcolemma to restrict the entry of calcium ion into the cell, because of reduction of sarcolemmal sialic acid, a potent regulator of membrane permeability. In the same animals, sialic acid was significantly less in myocites from myopathic animals fed a normal diet, than in myocites of animals fed sodium or potassium orotate. Thus, orotate in the diet prevented both the reduction in sialic acid content and calcium binding capacity (Bailey et al., 1980).

Jasmin et al (1998) repeated the experiment 20 years later, on the same model of hamster hereditary cardiomyopathy, this time together with ECG recordings. ECG recordings revealed that magnesium orotate diet significantly reduces myocardial damage, especially the severity of calcific changes. ECG recordings clearly demonstrated a significant shortening of QTc and PR intervals, resulting in partial electrical stabilization of failing hearts, with a significant delay in systemic congestive changes.

Recents studies on animal models have shown that magnesium administration lowers the incidence of arrythmias and has an infarct size limiting effect attributable to augmentation of adenosine mechanism (Matsusaka et al., 2002).

QUESTIONS TO BE ANSWERED

Since orotate was proven to be efficient as transsarcoplasmatic carrier for Mg and also contributes to “metabolic supplementation” of the stressed myocardium, magnesium orotate seems to be the ideal oral administration form of magnesium, in order to obtain maximal efficiency with minimal side effects in treatment of ventricular arrythmias occuring in patients with heart failure.

1. Is orotate only efficient as transsarcoplasmatic carrier for Mg, while the increase of intracellular levels of Mg and consequent decrease of Ca is responsible for the antiarrhythmic effect?
2. Since orotic acid has been shown in several studies to have a protective effect on recently infarcted myocardium and is a key intermediate in the biosynthetic pathway of pyrimidines via respiratory-chain coupled DHODH (mitocondrially-bound dihydroorotate dehydrogenase), is the antiarrhythmic effect only due to metabolic supplementation?
3. Are both magnesium and orotate responsible for the effect, taking into consideration all their mentioned possibilities of action?

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STUDY OF NONCOVALENT INTERACTIONS IN TRANSITION METALS SYSTEM

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ABSTRACT

Noncovalent interactions are present in all molecular systems. They play very important role in biological systems and in environment. If transition metals and π -systems are present quite specific noncovalent interactions form. In transition metal systems noncovalent interactions with π -systems can be formed in two ways; (a) ligands coordinated to the metal can interact with π -systems or (b) π-systems of ligands can form noncovalent interactions. Stacking interactions of terpyridyl square-planar complexes in crystal structures were studied analyzing the data from Cambridge Structural Database. In most of crystal structures two terpyridyl complexes were oriented "head-to-tail" or "head-to-head", with "head-to-tail orientation" prevalent. The number of structures with other orientations was very small. Based on the analysis of interacting geometries, we classified overlaps of terpyridyl complexes in six types. The types were defined by values of several geometrical parameters and all interactions of the same type had very similar overlap pattern.

Key words: Noncovalent interactions, aromatic molecules, transition metals, chelate rings

INTRODUCTION

Noncovalent interactions are present in all molecular systems. They play very important role in biological systems and in environment. If transition metals and π -systems are present quite specific noncovalent interactions form. In last decade several noncovalent interactions involving π -systems and transition metals were recognized (Zaric, 2003; Milcic et al., 2006; Suezawa et al., 2002; Bogdanovic et al., 2002;Medakovic et al., 2004;Jiang et al., 2005; Stojanovic et al., 2007; Tomic et al.; 2006; Sredojevic et al., 2007; 2010).

In transition metal systems noncovalent interactions with π -systems can be formed in two ways; (a) ligands coordinated to the metal can interact with π-systems or (b) π -systems of ligands can form noncovalent interactions. Ligands coordinated to a metal interact with the π systems in cation- π interactions and in metal-ligand X-H/ π interactions (Zaric, 2003).

Ligands in transition metal complexes can form specific π -systems; chelate rings with a metal atom as a member of the ring can be planar and can have

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delocalized π -bonds. These chelate rings can be involved in the noncovalent interactions similar to aromatic organic molecules. Chelate rings can form X-H/π and stacking interactions. Several studies of chelate rings with delocalized π-bonds involved in noncovalent interactions (Bogdanovic et al.,2002; Castineiras, 2002; Suezawa et al., 2002; Zaric, 2003; Medakovic et al., 2004;Jiang et al., 2005; Milcic et al., 2006; Stojanovic et al., 2007; Tomic et al.; 2006, 2007, 2010). In ways similar to aromatic organic molecules (Pitonak et al., 2008), were published. Chelate rings can be involved in CH/ π interactions as hydrogen acceptors with organic moieties and in stacking interactions with aryl rings and other chelate rings. The delocalized π -system of chelate rings can be considered as a soft base, similar to double, triple bonds or aromatic rings. These observations could be connected with an assumption that planar chelate rings with delocalized π -bonds bonds can have aromatic character (Masui, 2001; Milcic et al., 2007). Several studies about interactions where the π-systems of chelate rings interact with C–H groups, belonging to an organic moiety, were published (Bogdanovic et al.,2002; Medakovic et al., 2004;Jiang et al., 2005; Stojanovic et al., 2007), including C-H/ π interactions with chelate rings of coordinated porphyrin in transition metal porphyrinato complexes and in porphyrin containing proteins (Medakovic et al., 2004;Jiang et al., 2005; Stojanovic et al., 2007). The results showed that these interactions contribute to the stability of porphyrin containing proteins and may play some role in the function of these proteins (Stojanovic et al., 2007).

Our previous results show that there are stacking interactions between chelate rings with delocalized π -bonds, and aryl rings containing six carbon atoms (C_6-aryl), in crystal structures of square-planar transition-metal complexes (Tomic et al.; 2006; Sredojevic et al., 2007; 2010). Studies show that interactions between chelate and phenyl rings exist in square-planar complexes of different transition metals. In these crystal structures the geometry of the stacking interaction between C_6-aryl rings and chelate rings is similar to the geometry of the stacking interaction of two benzene rings (Pitonak, 2008).

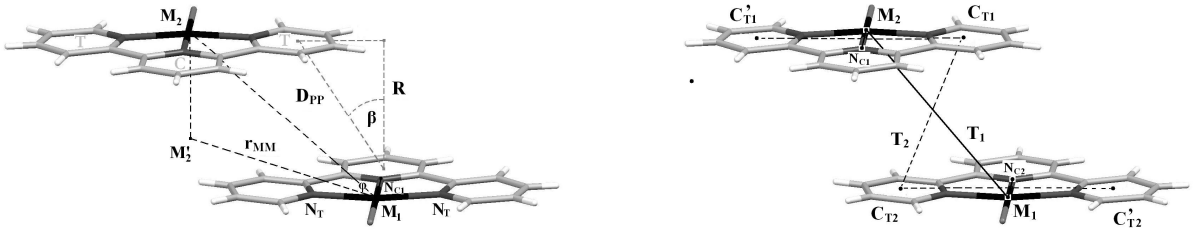
Terpiridine (2,2';6',2"-terpyridine) molecule coordinating to a metal ion forms large planar system of five rings, three pyridine fragments and two chelate rings. This planar system has propensity to form stacking interactions (Bugarcic et al., 2004; Li et al., 2004). Propensity for stacking interactions is important for using these complexes in biochemistry, supramolecular and medicinal chemistry. For example, it is known that terpyridyl complexes interact with DNA by intercalating between base pairs of DNA (Lippard, 1978; Messori et al., 2005).

In order to gain better inside in stacking of terpyridyl complexes in this work we analyze the geometries of stacking interactions between the terpyridyl square planar transition metal complexes in crystal structures from the CSD.

MATERIALS AND METHODS

The study is based on the crystal structures archived in the Cambridge Structural Database (Allen, 2002). The crystal structures involving terpyridyl complexes with coordination number 4 were screened for intermolecular contacts. The CSD search program ConQuest 1.10 (Allen et al., 1991) was used to retrieve structures satisfying the following criteria: a) the crystallographic R factor < 10% b) the error-free coordinates according to the criteria used in the CSD c) the H-atom positions were normalized using the CSD default X-H bond lengths

(O-H = 0.983 Å; C-H = 1.083 Å and N-H = 1.009 Å), d) no polymer structures. In order to find intermolecular stacking interactions between terpyridyl (terpy) complexes, we searched for the structures with the distance between centroids of any pyridine fragments (Dpp distance, Figure 1) below 4.6Å. The same criterion was used before in study of stacking interactions of aromatic nitrogen-containing ligands [15]. The geometric parameters used for analysis of the stacking interactions of terpy complexes are presented in Figure 1.



(a) (b)

Fig. 1. (a) Geometrical parameters describing interactions. In one of the complexes T and C denotes terminal and central pyridine fragments, respectively. (b) Torsion angles T_1 and T_2 used in analyzes of geometries.

RESULTS AND DISCUSSION

In the CSD 77 crystal structures of terpy square-planar complexes with the distances between centroids of the two pyridine fragments (Dpp, Figure 1) below 4.6 Å were found. In these structures there are 131 interactions of terpy ligands. The interactions were studied analysing geometrical parameters. The most important geometrical parameters are normal distances R (Figure 1a) that indicate stacking, and torsion angles (T_1 and T_2, Figure 1b) that show mutual orientation of two complexes. The distribution of the normal distances of the interacting terpy complexes shows pick at 3.4-3.5 Å, while in large number of interactions the normal distance is 3.3-3.4 Å (Figure 2). These normal distances are typical for stacking interactions (Janiak,2000)

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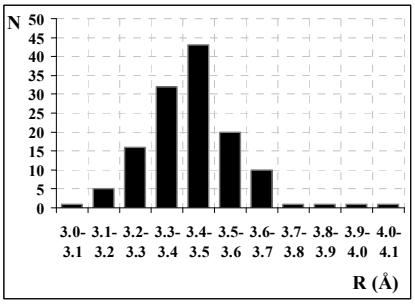

Fig. 2. The histogram of the distributions of the normal distance R for interactions of terpy complexes in square-planar complexes.

The distribution of the values of T_1 torsion angle (Figure 1) shows two

preferred orientations; the first one, with the angle from 0° to 10° ("head-to-head" orientation), and the other one with the angle from 170° to 180° ("head-to-tail" orientation) (Figure 3). The majority of interactions occur with the T_1 torsion angle close to 180°. The distribution of T_2 torsion angle (Figure 1) also shows two preferred orientations; the first orientation with T_2 values of 0° to 10° and the second with 170° to 180° (Figure 3). The values of T_2 torsion angle of 0° to 10° correspond to the interactions with overlap of large part of the terpy ligand, while the values of 170° to 180° correspond to only partial overlap of one terminal pyridine fragment. The interactions with the values of T_2 in the range of 0° to 10° are encountered more often. Hence, four possible orientations can describe most of the intermolecular stacking interactions of the terpy ligands in square-planar complexes. Based on the values of torsion angles T_1 and T_2 we defined four types of overlap (Figure 4, Table 1).

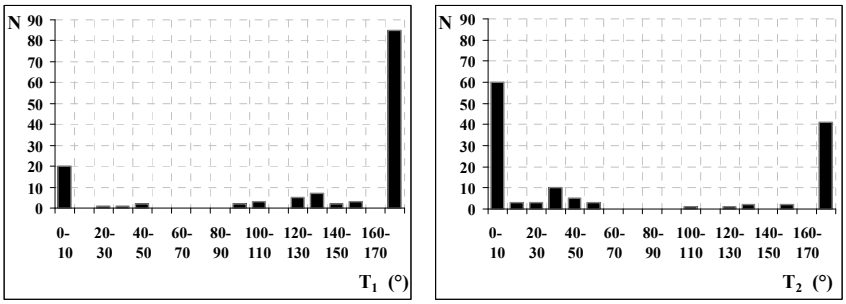

Fig. 3. Histograms showing the distribution of torsion angles T_1 and T_2, for interactions of terpy ligands in square-planar complexes.

The other two geometric parameters, angle φ and offset r_{MM} (Figure 1) were found to be also very important for the description of the mutual orientation of terpy complexes. The diagrams of the angle φ versus the offset r_{MM} are shown in Figure 4 for every type separately. In the diagrams the interactions of the same type are clustered in the same region of the diagram. For types III and IV the points in the diagrams are separated in two subgroups.

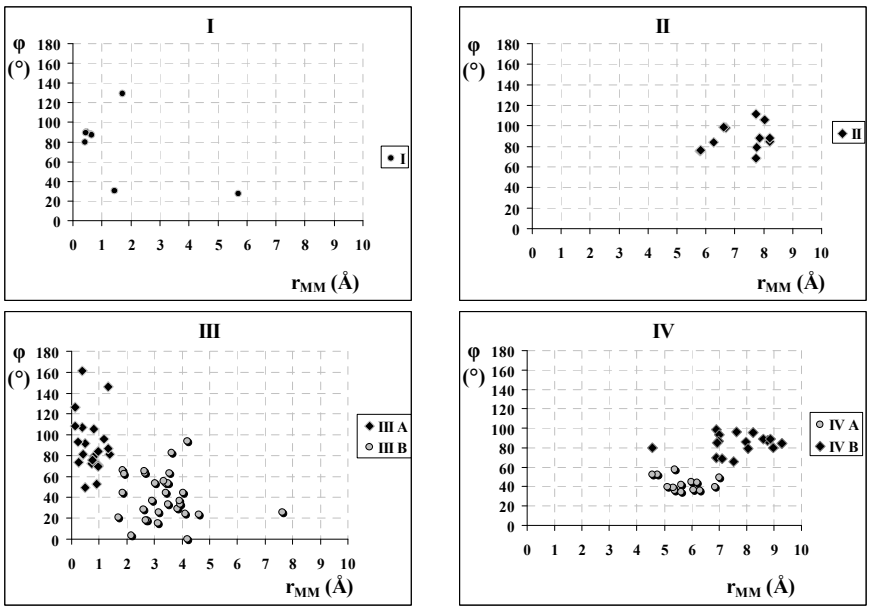

Fig. 4. The plots of the angle φ versus the offset r_{MM}, for overlap types I, II, III, and IV.

The group of structures with overlap type I is the smallest group, it includes only five structures with eight interactions. Because of the small number of the structures and the interactions, the conclusions about clustering and the properties of the interactions in this group could be perceived as questionable. In this group, interacting ligands are oriented "head-to-head". In almost all observed interactions, except one, ligands overlap with whole surface, and normal distances between planes are quite small, 3.2 to 3.4 Å. All rings participate in overlap, and besides that, rather short metal-metal distances are noticed, hence the values of r_{MM} offset are also small (Figure 5).

The group of structures with overlap type II is larger than group I, and includes 11 structures with 11 interactions. In this overlap type, terpy ligands are oriented "head to head", and only small part of the ligand is involved in the overlap. In most of the structures the overlap involves terminal pyridine rings, while in some structures the overlap involves also one chelate ring. Metal ion does not overlap with terpy ligand, as suggested by the values of the angle φ, larger than 70° and values of r_{MM} displacement, above 5.5 Å (Figure 4). The second ligands are usually bulky. The interaction of two complexes is additionally stabilized by the interaction of hydrogen atom of terpy ligand with second ligand of the other interacting complex. This stabilization occurs very rarely in the structures of the other types.

Group of structures with overlap type III, with torsion angle T_1 close to 180°, and T_2 close to 0°, is the most numerous one and includes 51 interactions, found in 35 crystal structures. The overlap manner in this group is not a unique one, as indicated by the plot of the angle φ versus r_{MM} value (Figure 4). Namely, one can notice two clusters of points in this plot. One group are interactions with very short offset values (r_{MM}), up to 1.5 Å, and angle φ in the range from 70° to 110° (overlap type III_A). This group covers 19 structures (21 interactions). In these interactions two complexes are in „head-to-tail“ orientation with small overlap of terminal pyridine fragments (Figure 5). A metal ion lies only in a small number of structures above the ligand ring, i.e. has the value of angle φ smaller than 80°. In some structures terminal pyridine fragments overlap a little with chelate rings. The crystal structures in IIIA are structures of Pt(II) (17 structures) and Pd(II) complexes (2 structures), with very short metal-metal distances, in the range from 3.2 to 3.6Å. In half of these structures metal-metal distance is below sum of VDW radii, indicating metal-metal interactions. Because of metal-metal interactions, the interaction of two complexes is stronger than in other types, and it is supported by shorter normal distances in IIIA.

Structures with overlap type IV are the second largest group; we found 23 crystal structures with 31 interactions. The diagram of the angle φ versus the offset r_{MM} shows that structures are clustered into two subgroups. In Figure 4 structures of type IV_A are the points with angle φ less than 60° and r_{MM} values in the range from 4 to 7 Å. These are interactions where the central pyridine ring overlaps with terminal ring (Figure 5). The chelate ring that is between them is with small area involved in overlap, while the metal ion is not involved in overlap. The type IV_A overlap was found in 12 structures (14 interactions). In this overlap type terpy ligand either interacts with the ion from the external sphere of the complex, which is located above metal ion or terpy ligand interacts with the second ligand. The structures with IV_B overlap type have angle φ larger than 60o, and offset r_{MM} values between 7 and 9 Å. This group counts 15 structures (17 interactions). In these interactions one terminal pyridine fragment overlaps, while the chelate ring is involved in overlapping in a small

number of structures (Figure 5). In these structures, ions from the external sphere of the complex pack above terpy ligand.

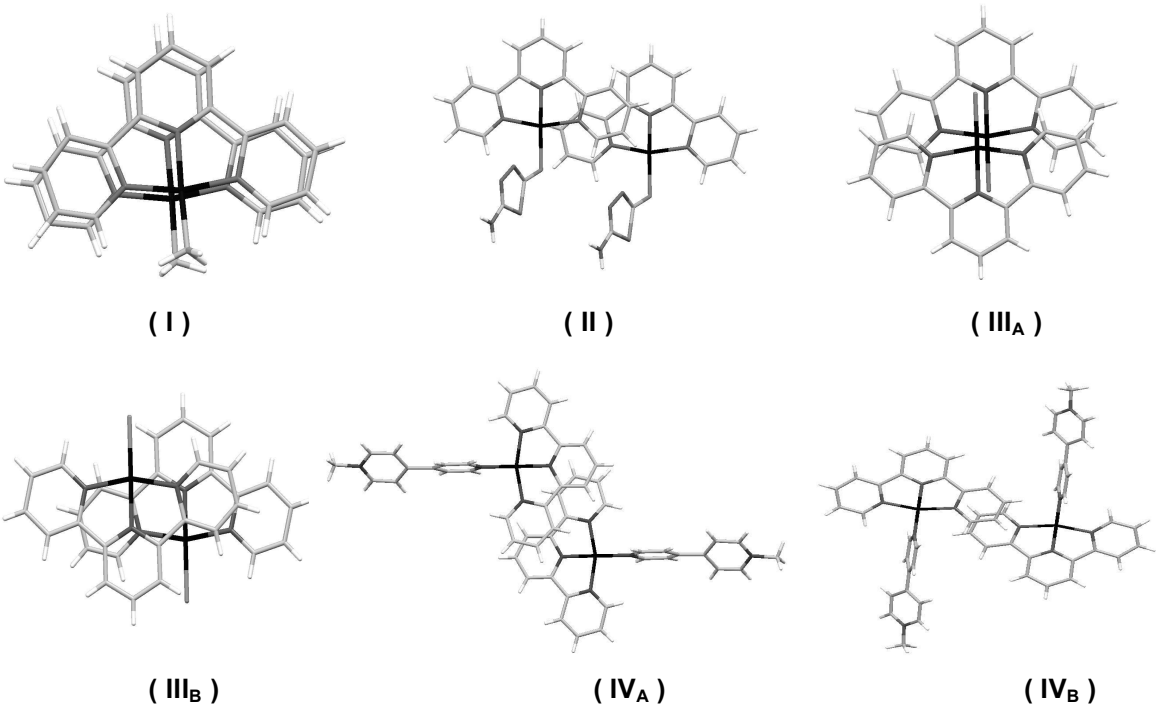

Fig. 5. Illustrations of overlap types in crystal structure of terpy complexes.

Our results showed that in 77 structures square-planar complexes 131 stacking interactions between terpy ligands occur. The 101 interactions with torsion angles T_1 and T_2 close to 0^o and 180^o are classified in six overlap types, while in 30 interactions torsion angles are not close to 0^o or 180^o (Figure 3). Analysis of packing in crystal structures showed that stacking interactions form stacking chains and dimers. In 53 structures (99 interactions) stacking chains form, while in 23 structures (31 interactions) dimers form. Only in one structure a tetramer was found. In some chains the same overlap is permanently repeated, while in some two types of overlap alternately appear in the chains.

CONCLUSIONS

In the Cambridge Structural Database (CSD) 77 crystal structures of terpy square-planar complexes with 131 interactions of terpy ligands were found. The number of the interactions is not very large, however, we showed that it is possible to classify the geometry of the stacking interactions.

Based on the analysis of stacking interactions between terpyridyl square-planar complexes we classified terpyridyl complex overlaps in six types, I, II, III_A, III_B, IV_A, and IV_B. Types of the overlap are defined by geometric parameters; torsion angles T_1 and T_2, angle φ, and offset r_{MM}. The distribution of both torsion angles, T_1 and T_2, show preferred orientations; both angles have values close to 0° or close to 180°. Structures of the same type are clustered in the plot of the angle φ versus the offset r_{MM}, and have very similar pattern of overlap. The most numerous are structures with overlap types III_A, III_B with „head-to-tail“ orientation of the two terpy complexes, and quite large area of overlap.

The shortest normal distances between planes of interacting complexes are noticed in the structures of type I and III_A with short metal-metal distance, indicating that metal-metal interaction contribute significantly to the interaction of two complexes in those overlap types.

The second ligand at the forth coordination site has influence on the overlap type. Complexes with small second ligand can form all types of overlap, however, they prefer types with large overlap area where overlap involve second ligand. Complexes with bulky ligands cannot form all types of stacking interactions; they can form stacking interactions where second ligand is not involved in the overlap. Large ligands with conformational flexibility allow overlap with involvement of forth coordination site.

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METAL COMPOUNDS WITH ANTIMETASTATIC POTENTIAL

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ABSTRACT

The present paper presents a short overview on recent developments in anticancer metallodrug field, focusing on the novel synthesized antimetastatic compounds. Bioorganometallic chemistry made in the last decade a huge expansion. A large number of medicinal organometallic compounds were prepared and tested, and many of them proven to be active against cancer cell proliferation. They are metals complexes which exhibit not only anticancer activities, but moreover, they are active against the most aggressive cancer cells: the highly metastatic cells and they have the capacity to hold back the cell migration and invasion mechanisms which leads to appearance of secondary tumors. We made an outline of the most recent literature data and present also the authors contribution to the field.

Key words: metal-based drugs, cancer, metastasis.

INTRODUCTION

Despite the clinical success of metal-based drugs, especially platinum-based drugs, the toxicity of these drugs and the intrinsic and acquired drug resistance is a major disadvantage which limits their applicability. The mechanisms involved in drug resistance can obstruct the compounds efficiency: reduced cellular uptake, increased efflux of the drug from the cells, inactivation through binding to proteins (cytosolic and nuclear), glutathione and for drugs that damage DNA, such as cisplatin, increased ability of cancer cells to repair the DNA damage can also occur.

Bioorganometallic chemistry made in the last decade a huge expansion (Hartinger, 2009). A large number of medicinal organometallic compounds were prepared and tested, and many of them proven to be active against cancer cell proliferation, and more, due to ligand design complexes led to new improved therapies against secondary spread of cancer cells, namely metastases. The categories of metal anticancer compounds were established based on their mode of action: (i) the metal has a functional role, (ii) the metal has a structural role, (iii) the

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metal is a carrier for active ligands that are delivered *in vivo*; (iv) the metal compound is a catalyst; and (v) the metal compound is photoactive and behaves as a photo-sensitizer (Gianferrara, 2009). The few metal anticancer drugs that are in clinical use are all believed to be functional compounds.

They are metals which complexes exhibit not only anticancer activities, but moreover, they are active against the most aggressive cancer cells: the highly metastatic cells and they have the capacity to hold back the cell migration and invasion mechanisms which leads to appearance of secondary tumors.

IRON

Among iron compounds, ferrocene is mostly known for his multiple antitumoral effects, and for the antimetastatic mechanisms of action. Very recent papers studied [3]-ferrocenophanyl and ferrocenyl derivates; compounds mean activity was better than cisplatin for breast cancer, leukemia, central nervous system and renal cancer (Görmen, 2010).

Ferrocenyl diphenol butene derivatives have prodrug potential. They are strong antitumor agents against both hormone-dependent and -independent breast cancer cell lines, and two diphenol derivates with 5-membered ring and 6-membered ring were prepared and studied for their estrogen receptor affinity and antiproliferative effects against the hormone-dependent breast cancer cell line MCF-7, and the hormone-independent breast cancer cell line MDA-MB-231 (Plazuk, 2010). Compounds exhibit differentiated effect against the estrogen receptors.

A ferrocene amido acid derivate is active against cancer cells and overcomes different mechanisms of multiple drug resistance (MDR) due to his apoptosis-triggering capacity, while the necrosis induction is minimal (Kater, 2010). Ferrocene derivatives of diethylstilbestrol exhibit cytotoxicity against the hormone-independent MDA-MB-231 breast cancer cell line (Tan, 2009). The compounds are less cytotoxic than their corresponding ferrocenyl phenyl or phenol isomers in which the ferrocene and ethyl moieties are linked to the same carbon atom. A series of ferrocenyl ester complexes cytotoxicity was studied, varying the lipophilic character of the pendant groups (Fig. 1.): Fe(C(5)H(4)CO(2)CH(3))(2), Fe(CpCOOCH(3)) (CpCOO CH(2)CH(3)), and Fe(CpCOOCH(2)CH(3))(2) (Gao, 2009). Their effect against colon cancer HT-29 and breast cancer MCF-7 cell lines were measured and data suggest that as we increase the lipophilic character of the functionalized ferrocene, the cytotoxicity improves.

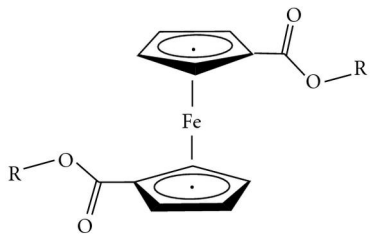

Fig.1. Ferrocenyl ester complexes which exhibit cytotoxic activity against cancer cells (Gao, 2009).

RUTHENIUM

Ruthenium is the metal with the largest number of anti-metastatic compounds. An advantage of ruthenium organometal compounds is the relative inertness of the

metal–ligand complex in the interaction with the deactivating cellular components. It has been shown that additional structural modifications of Ru(II) complexes can suppress certain resistance mechanisms in cancer cells.

The Ru(III) salt: (imH)[trans-RuCl(4)(dmso-S)(im)] (im = imidazole, dmso = dimethylsulfoxide) (Fig. 2.), displays remarkable and specific activity against metastases and simultaneously, a decreased *in vitro* toxicity (Velders, 2004); it was tested in clinical trials Phase I (first ruthenium complex ever to reach clinical testing) and the human body tolerates satisfactory this compound (Bergamo, 2004).

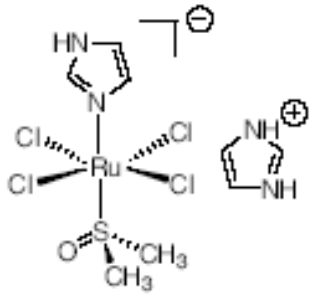

Fig. 2. Chemical structure of NAMI-A.

New classes of ruthenium complexes originated from the NAMI-A were synthesized: NAMI-A-type complexes, changing the nature of the N-ligand, dinuclear NAMI-A-type compounds containing heterocyclic bridging N-N ligands, and new Ru-dmso nitrosyls (Alessio, 2009). Several of these new compounds were found to have antimetastatic activity comparable to, or even better than, NAMI-A. All active NAMI-A-type compounds share the capacity to modify important parameters of metastasis such as tumor invasion, matrix metalloproteinases activity and cell cycle progression.

The complex of formula [Ru(PAn3)(P(An)(phenolate)2)Cl] shows a high cytotoxic activity in ovarian cancer cell lines comparable with cisplatin and defeat the cisplatin-resistance of cancer cells (van Rijn, 2009).

The reaction of metallothionein-2 (MT-2) with the organometallic antitumour compound [Ru(Z6-p-cymene)Cl2(pta)] (Fig.3) : RAPTA-C binds to DNA significantly different as the cisplatin RAPTA-C and it forms monoadducts with MT-2, at variance with cisplatin(Casini, 2009).

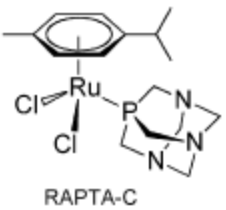

Fig.3 . Organometallic ruthenium complex with antimetastatic properties (Casini, 2009).

The studies have shown that despite the moderate cytotoxicity of these compounds in vitro, they demonstrate a high selectivity toward cancer cells in comparison to non-tumourigenic cells. Moreover, a significant in vivo effect on the growth of lung metastases was established for RAPTA-C9 and for [Ru(Z6-toluene)Cl2(pta)], RAPTA-T. Unlike cisplatin, which exhibits its main chemotherapeutic action through binding to the bases of DNA, the mechanism of action of the Ru(II) compounds may involve interactions with critical intracellular proteins.

Cationic tetranuclear and hexanuclear opened metalla-assemblies incorporating porphyrines and dinuclear arene ruthenium complexes containing cymene-, oxalate- and benzoquinonato moyeties have been assembled and their biologic effect has been established on ovarian A2780 and A2780cisR cancer cell lines (Barry, 2010). The compounds are quite cytotoxic, the most active metalla-assembly being [Ru6(p-cymene)6(dobq)3(tpp)2]6.

Ru(II) complexes that bring together the properties of the dipyrido-phenazine intercalating residue and the properties of metal-coordinating pentaaza macrocycles were found to interact with DNA , both by external binding, both by an intercalation process with lower dppz penetration within DNA slots (Bazzicaluppi, 2010).

Two new flavanone complexes of Ru(II) display highly antiproliferative effect towards the cisplatin resistant, and both complexes are as active as cisplatin in the sensitive cell lines (Ochocki, 2010). They have the ability to overcome cisplatin resistance in the drug resistant sub-lines EJcisR and L1210R. The present evidence suggests that the mechanism of biological activity may be different for these ruthenium compounds compared to cisplatin.

Mononuclear arene ruthenium complexes containing *P*- or *N*-donor ligands or *N*,*N*-, *N*,*O*- or *O*,*O*-chelating ligands, dinuclear arene ruthenium, trinuclear arene ruthenium clusters, tetranuclear arene ruthenium porphyrin derivatives that are photoactive, have been shown to be active against a variety of cancer cells due to their capacity to incorporate both hydrophilic and hydrophobic parts (Suss-Fink, 2010).

OSMIUM

Anticancer capacity of a series of metalla-rectangles of the general formula $[(p\text{-cymene})_4Os_4(OO \cap OO)_2(N \cap N)_2]^{4+}$ has been using ovarian A2780 cancer cell lines (Barry, 2010). The most active metalla-rectangle, $[(p\text{-cymene})_4Os_4(dhbq)_2(4,4'\text{-bipyridine})_2]^{4+}$, shows an IC_{50} value comparable to cisplatin against A2780 cancer cells and against the cisplatin resistant A2780cisR cells.

NIKKEL, ZINC, CADMIUM

The cytotoxic activity of two novel Cd(II) and Zn(II) complexes with the condensation product of 2-formylpyridine and selenosemicarbazide, as well as of five structurally related complexes and the ligand evaluated against eight tumor cell lines (Bjalogrlic, 2010). The new Cd(II) complex showed the highest activity, and cell cycle distribution and apoptosis study showed that Cd(II) complex and cisplatin might have some similarity in anticancer activity. Cd(II) and Zn(II) complexes and cisplatin increased matrix metalloproteinases MMP-2 activity in supernatants of tested cells, while Ni(II) complex with the same ligand decreased the activity, which can confer to this compound the capacity to prevent tumor invasion and metastasis.

PLATINUM

Cisplatin, carboplatin and oxaliplatin are standard anticancer drugs, important antineoplastic agents, but they are known to be toxic, the incidence of cellular resistance and the genotoxicity reduces their efficacy. There is a great interest to synthesize novel platinum agents with a broad spectrum of antitumor activity and reduced toxicity. The therapeutic success of the platinum-based standard drugs has triggered, in the past decades, the development of several metal-based potential chemotherapeutic agents, but few were introduced in clinical trials.

A new Pt(II) complex ([Pt(O,O'-acac)(gamma-acac)(DMS)]) (Fig. 4.) may be a promising therapeutic agent for preventing growth and metastasis of breast cancer MCF-7 cells (Muscella, 2010). Treatment with sublethal concentrations prevented events leading to metastasis via alteration of the anchorage-dependent and -independent growth of the cells, detaching the cancer cells from the surrounding extracellular matrix and alter the migration ability of MCF-7 cells, stromal interactions and MMP activity.

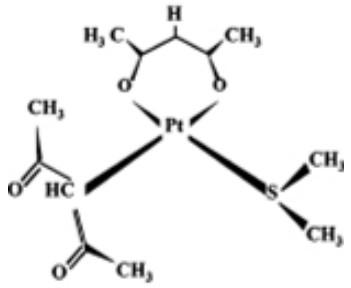

Fig. 4. New Pt complexes with antimetastatic potential (Muscella, 2008)

A platinum(II) coordination complex containing a pyridine nucleus and a dithiocarbamate moiety as ligands, [Pt(ESDT)(Py)Cl was tested for its cytotoxicity, by MTT assay, on various human cancer cell lines also including different cisplatin-resistant cells and in animal models (Marzano, 2004).

The antiproliferative activity of novel complexes derived from N-benzyl-ethylenediamine and oxalate were investigated against human non-small cell lung carcinoma, mouse non-metastatic cell skin melanoma, mouse metastatic cell skin melanoma, human cell breast adenocarcinoma and normal cell lines and compared to cisplatin and carboplatin under the same experimental conditions (Silva, 2010). The presence of oxalate as a leaving group conferred an interesting cytotoxicity profile to the complexes in the tested cell lines.

The square-planar platinum complex $[Pt^{II}(L^1)(L^2)]Cl$ has been found to intercalate DNA. Agarose gel electrophoresis indicates that the complex cleaves supercoiled plasmid DNA *via* singlet oxygen and as determined by MTT assay, exhibits significant cytotoxicity (Mandal, 2010).

Our team synthesized and fully characterized three novel platinum complexes of tertiary arsine ligands: trans-[PtI2(2-iPrOC6H4AsPh2)2] (), trans-[PtCl2(2-MeOC6H4AsPh2)2] () and cis-[PtCl2(2-HOC6H4AsPh2)2] . The three compounds are biologically active against tumor cells and their cytotoxicity is comparable with standard drugs(Fischer-Fodor, 2008). Measurements using the CellScan technology correlate well with the results provided by other bioassay methods.

PALLADIUM

Cytotoxic activity of a new palladium(II) complex with 2-(diphenylphosphino)benzaldehyde (dpba) and ethyl hydrazinoacetate (etha) ligands was tested against a panel of four tumor cell lines, including cisplatin-resistant U2-OS/Pt cells (Malesevic, 2006). The results suggest they have a similar effect to cisplatin, inducing apoptosis followed by arrest of cells cycle.

Biological mechanisms of palladium(II) complexes, especially of palladacycle compounds were studied (Caires, 2007). They are correlations between chemical structures of palladacycle compounds and biological activities: complexes containing ligands derivatives of pyridine and imines in trans position having high antitumoral activities. The intercalation of metallic complexes in the double helix of DNA of cancerous cells causes irreparable lesions in the macromolecule and complexes interact with proteins and peptides and with the thiol group of methionine. The lysosomal cysteine proteinases cathepsins B and L have been implicated in a variety of pathological conditions, especially in diseases involving tissue-remodeling states, such as tumor metastasis.

Palladacycle compounds derived from N,N-dimethyl-1-phenethylamine and the ligand bis(diphenylphosphine)ferrocene were presented as effective antitumoral agents (Spencer, 2009). The palladacycles (Fig. 5.)were evaluated for *in vitro* activity as cytotoxic agents on A2780/S cells and also as cathepsin B inhibitors, an enzyme implicated in a number of cancer related events.

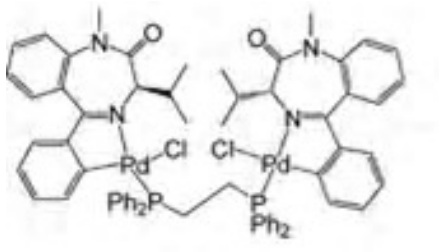

Fig.5. Palladacycles with in vitro anticancer activity (Spencer, 2009)

Biological activity of complex combinations of Cu(II) and Pd(II) with thiosemicarbazone derivatives of 2-hydroxy-8-R-tricyclo[7.3.1.0.^{2,7}]tridecane-13-one (where R = C_3H_7, C_4H_3O) was evaluated in terms of antibacterial or antiproliferative activity; the effect on the proliferation of cervix carcinoma cells was tested (Rosu, 2010).

Following the metallomic trend, we tested new palladium complexes with general formula [PdCl(2)L(2)], where L=heterofunctional organoarsenic ligand: (2-isopropoxyphenyl)diphenylarsine (1), (2-methoxyphenyl)-diphenylarsine (2) and (2-hydroxyphenyl)diphenylarsine (3) (Miklasova, 2009). The lethal doses are comparable with those of standard metal-based chemotherapeutical drugs (carboplatin and oxaliplatin). These palladium complexes exhibit a higher cytotoxicity against tumor cells as against normal cells in vitro. Complex 2 has an important capacity to induce apoptosis in tumor cells. The apoptotic process is triggered due to the interaction of these complexes with secondary structure of DNA in treated cells. The alkaline single-cell gel assay shows that the level of DNA damages induced by compounds 2 and 3 are significantly higher in tumor cells as in normal cells.

GOLD

Gold compounds are a class of metallodrugs with great potential for cancer treatment (Nobili, 2010). Biophysical studies reveal that the interactions of cytotoxic gold compounds with DNA are generally far weaker than those of platinum drugs, implying the occurrence of a substantially different mode of action, involving mitochondrial damage, proteasome inhibition or modulation of specific kinases.

Gold complexes with dithiocarbamat ligands were synthesized (Milacic, 2006) and tested against highly metastatic breast carcinoma cell lines and they found to have antiproliferative effect due to their apoptosis-inducing capacity via proteasome-inhibition mechanisms.

Metal complexes of an *N*,*N*′-disubstituted cyclic thiourea exert significant cytotoxicities to cancer cells and, in particular, the gold(I) thiourea complex exhibits a potent tight-binding inhibition of the anticancer drug target thioredoxin reductases (Yan,2010).

Amino acid and dipeptide complexes of N-heterocyclic carbene- gold halides NHC- Au(I) and NHC-Au(III) showed significant anti-tumor activity (on the HeLa, HepG2 and HT-29 cancer cell lines, and their activity was comparable to the well-known anti-cancer drug cisplatin (Lemke, 2009).

Gold(III) meso-tetraphenylporphyrin , has been shown to be effective in inducing apoptosis and prolonging the survival of hepatocellular carcinoma-bearing rats as well as inhibiting the tumor growth of mice bearing nasopharyngeal carcinoma, neuroblastoma and colon carcinoma(Lum, 2010). The compound prolonged the survival of metastasis-bearing mice and inhibited intrahepatic and lung metastasis, by influencing the neoangiogenesis. Also the complex inhibited the migration and invasion of C666-1 human nasopharyngeal carcinoma cells.

CONCLUSIONS

Based on the success of standard metal-based drugs, many efforts were made in the last years to produce clinically beneficial analogues or completely new compounds. For every new clinically approved compound a large quantity of novel synthesized biologically active compounds has to be evaluated, and the biologic tests should be sensitive, specific, straightforward and insightful as regards the mechanisms of action of the metal and the ligand. It was proven that many other metals except the platinum are able to elicit a complex antiproliferative and anti-apoptotic effect which leads to restrain the metastatic processes. Furthermore, a large number of with tumor-targeting ligands are expected to be created and bind to metals in order to maximizing the impact on cancer cells and minimizing the adverse side effects, and complexes with ligands. As a result of the accumulation and refine of knowledge acquired in these years, medicinal bioorganometallic chemistry is probably ready to make significant steps forward and there are great expectations for future metallodrugs.

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DETERMINATIONS OF SOME NEUTRAL FAT AND HEAVY METALS IN CRYO-DESICCATED FOODS

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ABSTRACT

Drying food by cryo-desiccation process it has many advantages over other methods. This paper, after some experiments, highlights the data that reflect certain changes of neutral lipid and heavy metals concentrations due in the contact of food with different metal alloys during cryo-desiccation process. It confirms once again the risk of contamination of food when the trays are by aluminum alloy, low alloy steel or brass. Also noticed some effects on the environment.

Key words: cryo-dessicated foods, neutral lipids, heavy metals

INTRODUCTION

The need for preservation of foods has led the development of a true industry. Thanks for keeping the food needs of different periods of time, methods and techniques of conservation are diversified. Often adversely affect the operation of environmental conservation and / or subjected to food preservation (Jennings, 1999; Songa et al., 2005). The food cryo-desiccation brings several advantages, the water removal ensuring better preservation, but also a number of disadvantages. The main issue raised by this method of conservation is the fact that food is in constant contact with metal trays which, in specific extreme conditions for this process, may allow the migration of metals in foods subjected to cryo- desiccation process (Băcăoanu et al., 2006; Mnerie et al., 2009).

Lipids are fatty organic substances insoluble in water but soluble in most organic substances which contain hydro-carbon group. They play an important role in the life of living matter. Neutral lipids generally include simple lipid class compounds (Ensminger, 1995).

The category of heavy metals included a series of chemical elements with high toxicity for living organisms. The toxic effect manifests itself in exceeding a certain threshold, below which some metals, such as Co, Cu, Ni, Zn, Fe, may even be essential components of proteins involved in different metabolic pathways (Watt et al., 1975; Garban and Garban, 2003). The toxic metals can be: toxic bio-metals by

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excess of contents (e.g. Co, Cu, Zn, Fe etc.) and potentially toxicogen metals (e.g. Hg, Cd, Pb, etc.). Toxic metals are: Biomet toxic excess of content (e.g. Co, Cu, Zn, Fe etc.) And potentially toxicogen metals (e.g. Hg, Cd, Pb etc.).

Heavy metals are found in different concentrations in soil, water, air, food, vegetable or animal, depending on various factors that determine their pollution (Ensminger et al., 1995; Garban, 2007).. Also, an important source of heavy metal contamination of food can be the contact with machinery, plant or equipment for food processing, the keeping canned of the food.

MATERIALS AND METHODS

In order to investigate the degree of change in the presence of neutral lipids and of heavy metals in cryo-desiccated foods, were analyzed the food samples in cryo-desiccated state, in the specialised Laboratory of the Faculty of Agriculture, University of Ceske Budejovice (Czech Republic) and in the “Laboratory for residue control” of the University of Agricultural Sciences and Veterinary Medicine of Banat Timişoara. On entering in the laboratory, the samples of cryo-desiccated food were identified and marked, for to determine the neutral lipid content, respectively, metallic microelements (Mnerie and Ţucu, 2000; Anghel et al., 2003).

For metals analysis, was required the mineralization of the samples in the microwave, at controlled temperature and pressure, in the presence of concentrated nitric acid. To that end, weighed on analytical balance to an amount of about 0,5000 g of sample, which were introduced in Teflon bottle of mineralizing. Added a volume of 3 ml with concentrated nitric acid 65% p.a. and about 2 ml water, it was been fixed the bottle in the protection shield, then in the mineralizing. The temperature program especially allowed for obtaining clear solutions, without residues, which were diluted with distilled water at a volume of 15 ml. From this solution, were made dilutions as needed, which were analyzed by the spectrophotometer with atomic absorption, type AAnalyst 800 - Perkin Elmer.

The metals Al and Cr were determined from electro-thermal atomization in graphite furnace, equipped with Zeeman background noise correction, using as a matrix modifier mixture of $PdCl_2$ and $Mg(NO_3)_2$, which was injected simultaneously with the sample. Metals Cu, Fe and Zn were determined by atomization in air-acetylene flame, using for the ionization control in flame $LaCl_2$.

At the analysis in flame, that in the case of the analysis in graphite furnace, accuracy was checked by reading the sample in terms of repeatability and the accuracy of reading was examined by analysis of some solutions of control standard.

After the analysis of the solutions with metals content, based on sample mass which was subjected to the mineralization, its where determined the concentrations of metals in the matrices cryo-desiccated in the measurements units dedicated for these findings, [mg/g] and [µg/g].

To the research of this issue was made some food cryo-desication (white onions, potatoes, tomatoes, lemon, cows' milk, yogurt and beef), in the presence of metal alloys (aluminum sheet, brass sheet, black steel sheet, galvanized sheet and stainless steel sheet), which were suspected by the metals releasing that can migrate into food.

The inteprinsed investigations were focused also to the liquid extracted from vapor condensation in the sublimation phase, at more foods cryo-desicated. The

investigation of the presence of metals in residual liquid was done by atomic absorption spectrometry, using the Spectrometer by atomic absorption, type Varian 280 FS SpectrAA.

It was subjected by cryo-desication: fresh milk, vegetables, fruit and beef. The food cryo-desication was made in the presence of the following metal alloys: aluminum sheet, brass sheet, black steel sheet, galvanized sheet and stainless steel sheet.

RESULTS AND DISCUSSIONS

Investigations on neutral lipids have in view - considering biochemical and nutritional aspects - to find out the concentration of compounds belonging to the simple lipids class. It is necessary to mention that in lipidology one can distinguish simple lipids (glycerides, cerides, sterides, etholides) and complex lipids (glycerophospholipids and sphingolipids) – see Pomeranz and Melon, 1971; Gustong, 1983).

From Table 1 it is distinguished important changes of the neutral lipids concentration, in the presence of metals, even in the presence of stainless steel, with significant percentages (eg cow's milk with 4%). The threat of aluminum for the contamination of milk stands also in the table 1 with the influences on the percentage of simple lipids.

Table 1. Changes of the neutral lipids concentration in cryo-dessicated milk

Samples study	**Group samples**	**Nr. samples [n]**	**Cryo-desiccated sample [%]** $\overline{X}$	**Water [%]** $\overline{X}$	**Neutral Lipids [%]** $\overline{X}$
Milk	C	10	10,505	89,495	24,355
Milk / Tal	E1	10	12,135	87,865	21,090
$\Delta E_1 = C - E_1$			- 1,630	3,230	3,265
Milk / T_{am}	E_2	10	12,580	87,420	21,360
$\Delta E_2 = C - E_2$			- 2,080	3,675	2,995
Milk / T_{ol}	E_3	10	11,775	88,225	25,650
$\Delta E_3 = C - E_3$			- 1,270	1,87	- 1,295
Milk / T_{zn}	E_4	10	10,025	89,975	22,210
$\Delta E_4 = C - E_4$			0,480	0,020	2,145
Milk / T_{ix}	E_5	10	12,095	87,905	20,020
$\Delta E_5 = C - E_5$			- 1,590	2,190	4,335

T_{al} – aluminum sheet, T_{am} – brass sheet, T_{ol} – black steel sheet, T_{zn} – galvanized sheet, T_{ix} – stainless stell sheet

It also notes that it has excluded the possibility of using in the black steel trays manufacturing, which can taint the food cryo-desicatred quality, in Table 2, the simple lipids with different percentage values compared with yogurt cryo-dessicated.

Table 2. Changes of the neutral lipids concentration in cryo-dessicated yogurt

Samples study	**Group samples**	**Nr. samples [n]**	**Cryo-desiccated sample [%]** $\overline{X}$	**Water [%]** $\overline{X}$	**Neutral Lipids [%]** $\overline{X}$
Yogurt	C	10	26,110	73,890	57,420
Yogurt / Tal	E1	10	26,450	73,550	57,250
$\Delta E_1 = C - E_1$			- 0,340	0,340	0,170
Yogurt / T_{am}	E_2	10	26,945	73,055	58,550
$\Delta E_2 = C - E_2$			- 0,835	0,835	- 1,130
Yogurt / T_{ol}	E_3	10	27,995	72,005	59,090
$\Delta E_3 = C - E_3$			- 1,885	1,885	- 1,670
Yogurt / T_{zn}	E_4	10	27,980	72,020	58,760
$\Delta E_4 = C - E_4$			- 1,870	1,870	- 1,340
Yogurt / T_{ix}	E_5	10	25,985	74,015	57,660
$\Delta E_5 = C - E_5$			0,125	- 0,125	- 0,240

T_{al} – aluminum sheet, T_{am} – brass sheet, T_{ol} – black steel sheet, T_{zn} – galvanized sheet, T_{ix} – stainless stell sheet

In the investigations following on the crude protein content from the samples subjected on the cryo-desication, there were quite large differences between samples, carried out after repeated using of the base (tray). Thus, through contact of the milk with stainless steel, the crude protein percentage decreased from 23.69% to 13.85%, in the cryo-desication made under the same conditions. As in the case of aluminum, crude protein percentage is much higher (23.68%) for yogurt than milk when that was in contact with aluminum (19.66%), raw material was the same.

The results of the investigations aimed at highlighting the presence of heavy metals in the cryo-desiccated foods are summarized in Table 3.

Due aluminum properties, it is expected to be considered the XXI century, the century of the aluminum. But food industry applications, based, above all, on fairly good machining properties and on the stability of aluminum oxide (Al_3O_2) formed on the surface, its have recently attracted many arguments about the dangers that can cause the aluminum to human health.

Table 3. Changes of the presence of heavy metals in the cryo-desiccated foods

Sample no.	Sample ID	Cu [mg/g]	Fe [mg/g]	Zn [mg/g]	Al [µg/g]	Cr [µg/g]
Tray made from aluminum sheet						
1	Onion / T_{al}	-	-	-	31,49	-
2	Potato / T_{al}	-	-	-	29,73	-
3	Tomato / T_{al}	-	-	-	23,77	-
4	Lemon / T_{al}	-	-	-	21,43	-
5	Milk / T_{al}	-	-	-	15,13	-
6	Yogurt / T_{al}	-	-	-	24,52	-
7	Beef meat / T_{al}	-	-	-	19,49	-
Tray made from brass sheet						
Sample no.	**Sample ID**	**Cu [mg/g]**	**Fe [mg/g]**	**Zn [mg/g]**	**Al [µg/g]**	**Cr [µg/g]**
8	Onion / T_{am}	0,016	-	-	-	-
9	Potato / T_{am}	0,082	-	-	-	-
10	Tomato / T_{am}	0,042	-	-	-	-
11	Lemon / T_{am}	0,059	-	-	-	-
12	Milk / T_{am}	0,069	-	-	-	-
13	Yogurt / T_{am}	0,123	-	-	-	-
14	Meef meat / T_{am}	0,045	-	-	-	-
Tray made from black steel sheet						
15	Onion / T_{ol}	-	0,149	-	-	-
16	Potato / T_{ol}	-	0,917	-	-	-
17	Tomato / T_{ol}	-	0,394	-	-	-
18	Lemon / T_{ol}	-	7,64	-	-	-
19	Milk / T_{ol}	-	0,260	-	-	-
20	Yogurt / T_{ol}	-	1,21	-	-	-
21	Beef meat / T_{ol}	-	0,483	-	-	-
Tray made from galvanized sheet						
22	Onion / T_{zn}	-	-	0,031	-	-
23	Potato / T_{zn}	-	-	0,029	-	-
24	Tomato / T_{zn}	-	-	0,81	-	-
25	Lemon / T_{zn}	-	-	0,76	-	-
26	Milk / T_{zn}	-	-	0,69	-	-
27	Yogurt / T_{zn}	-	-	1,01	-	-
28	Beef meat / T_{zn}	-	-	0,34	-	-
Tray made from stainless steel sheet						
29	Onion / T_{ix}	-	-	-	-	<0,15
30	Potato / T_{ix}	-	-	-	-	< 0,15
31	Tomato / T_{ix}	-	-	-	-	0,17
32	Lemon / T_{ix}	-	-	-	-	0,21
33	Milk / T_{ix}	-	-	-	-	0,24
34	Yogurt / T_{ix}	-	-	-	-	< 0,15
35	Beef meat / T_{ix}	-	-	-	-	0,57

T_{al} – aluminum sheet, T_{am} – brass sheet, T_{ol} – black steel sheet, T_{zn} – galvanized sheet, T_{ix} – stainless stell sheet

From the Table 3 it notes the high particularly impact that the trays construction by aluminum to the food cryo-desiccated, due to the extreme growth in the percentage of the aluminum in food during the cryo-desiccation process: 31,49 µg/g for onion and 3,77 µg/g of tomato is definitive.

The results of the second line of the investigation, the direction which is aimed at the determining of heavy metals from the liquid extracted from the condensing vapor phase sublimation, are given in Table 4.

Table 4. Metal concentration in the residual liquid condensate

Residual liquid	Sample	U.M.	Cu	Zn	Fe	Cr	Ni
Milk	a	[mg/L]	0,013	0,60	0,13	0,033	0,045
	b		0,016	0,30	0,15	0,041	0,036
	c		0,44	0,24	0,18	0,044	0,037
	d		0,011	0,16	0,11	< 0,02	0,057
	e		0,047	1,17	1,25	< 0,02	0,168
Vegetables	f	[mg/L]	0,056	0,25	0,75	0,024	0,026
	g		< 0,02	< 0,02	0,75	< 0,02	0,016
Fruits	h	[mg/L]	< 0,02	0,012	< 0,02	< 0,02	0,016
	i		< 0,02	0,057	0,03	< 0,02	0,010
	j		0,059	0,48	0,05	< 0,02	0,035
Beef meat	k	[mg/L]	0,058	< 0,02	0,11	< 0,02	0,024
	l		0,164	< 0,02	0,14	< 0,02	0,033

The purpose of these measurements was to seize the cryo-desication technology users that this residual liquid is not only water, that has a complex content in relation to food subject on the cryo-desiccation and the metals are in contact.

CONCLUSIONS

During the food cryo-desication, that suffer multiple changes, not just status, but also structural. Some comments may be considered surprising, following the various investigations being undertaken on the effects of metals on food that have been in contact during cryo-desiccation process. Thus, there are changes in the concentrations of nutrients (macro-and micro-nutrients) of water, of some xeno-biological components.

Very important are the results from measurements made on the food cryo-desiccation effects on the environment. The measurements were recorded low levels of hazardous gas concentrations, but do not mean an implicit threat to the environment and human health.

Warnings are drawn from the tests carried out evidence of the risks in the use of metallic materials, determined solely on technological criteria (mechanical), taking into account less chemical reactions accompanying the process, micro-biological effects, which may affect the characteristic fundamental performance of a technical system for the food cryo-desiccation, also for the final cryo-desiccated product quality.

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CHEMICAL AND BIOLOGICAL CHARACTERISTICS OF SALIVA *VS* THE STATUS OF ORO-PHARYNX MICROBIOCENOSIS

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ABSTRACT

For chemical, physical and biological analysis, have become increasingly important the involvement of saliva sampling use, as one of the most noninvasive collection technics in order to perform qualitative and quite sophisticated quantitative tests. Being readily accessible and collectible, saliva may show many advantages over 'classical' biological fluids such as blood and urine, taking into accounts the means of venipuncture and urine collection quality standards identification, as well as patients' discomfort. Modern techniques for saliva patterns are focused on the components affecting oro-pharyngeal microbiocenosis balance, in order to clearly define its role as a diagnostic fluid, for local but also general pathology. Due to incomplete knowledge of saliva as a biological specimen and diagnostic tool, in the present study we propose: saliva enzymes levels, microbes identification, tests indicating the local inflammation degree and metal levels, to be performed, in parallel with features selection and extraction from saliva databases, image processing applied to saliva images and saliva data set information classification, for identification and further use of clinical and paraclinical interrelations, in preventing/controlling local and general pathology.

Key words: saliva, microbiocenosis, quality standars, data set information classification, interrelations, pathology.

INTRODUCTION

Saliva as diagnostic fluid has an ancient history. In some Asian communities, a person judged for a crime, to be declared guilty or non-guilty has to rule the "rice test" regarding the ability to form a spit-and-rice ball, under conditions of stress.

As a body fluid, saliva, is a dilute aqueous fluid containing both electrolytes and protein with an osmolality less than or equal to that of plasma. Contains also

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cells, represented also by debris arising from the epithelial cells of the mouth, food components/residues and metals /external and internal environment sources/. Saliva osmolality depends on the type/secretory activity of the gland, also on: sex, age, diet, emotional state, risk behaviors, season, a variety of diseases (Burford-Mason et al., 1988) and many local agents.

Saliva is a dynamic and complex fluid, produced by specialized glands, being discharged through salivary channels of the glands into the oral cavity Most of the saliva is produced by the major salivary glands (parotid, submandibular, and sublingual), but a small contribution is made by the numerous small labial, buccal, and palatal glands which line the mouth, with a daily synthetized volume, about 1.0-1,5 liters. Saliva should not be considered as a simple ultra-filtrate of plasma, but rather a complex fluid formed by different mechanisms: by a passive diffusion process, by an active process against a concentration gradient, by ultrafiltration through pores in the membrane, or by pinocytosis, being in permanent relation with oro-pharyngeal microbiocenosis and the different chemical substances which reside here /such as prosthetic, orthodontic materials/. The inorganic saliva compounds are represented by the usual electrolytes of the body fluids, such as: sodium, potassium, chloride and bicarbonate. Organic compounds - previously it was noted that saliva supplies enzymes for digestion, including saliva-specific glycoproteins. Saliva is also an adequate source of DNA for analysis and for DNA typing in certain forensic settings.

Some of the important functions of saliva are represented by the ability to - humidify the mucous membranes of the upper aero digestive tract, facilitating the speech and maintaining a certain spectrum of mouth microbiocenosis /in healthy status/; - supply enzymes involved in digestion process; - produce pharmacologically active compounds /such hormones/; - support non-specific and specific self defense mechanisms (Koga-Ito et al*.,* 2003*,* Buciu et al., 2006).

The study is focused on wide spectra of saliva components and features, which will be presented by respecting the 3 main approach ways*: in vivo*, *in vitro* and *in silico*.

MATERIALS AND METHODS

The study, identified further with the acronim ”**SP**” (”**S**alivary **P**roject”) is an on-going one, designed in a complementary approach, with a certain number of variables, each of them selected in order to get the most relevant salivary features – both in physiologic and non-physiologic conditions.

The complementary character, is based on the involvement of different types of specialties involved in this study, such as: stomatology, clinical laboratory, biology, biochemistry, environment protection, informatics, each with well-define materials and methods to be performed and prelucrated.

After several meetings /”brain-storming sessions”/ of the team members, was drafted the study scenario: „scene entry” order, methods to be used, the list of information to be noted, and the final complete recording form for saliva-based data analysis and processing.

Was also completed the calendar of the activities, to have a comprehensive and logical way of ruling the saliva assessment steps.

Below, is a “map” (*figure 1*) of the study, divided into two main domains: the 1st one, targeted on “SALIVA” and the 2nd one, on “LOCAL AND GENERAL PATHOLOGY”. Were also designed 3 steps to be followed: - the interrelation

between saliva features and local/general pathology, - certain salivary patterns identification, - innovative bio adhesive therapeutic products formula, devoted to cure/ameliorate/prevent certain pathological aspects correlated to oro-pharyngeal environment.

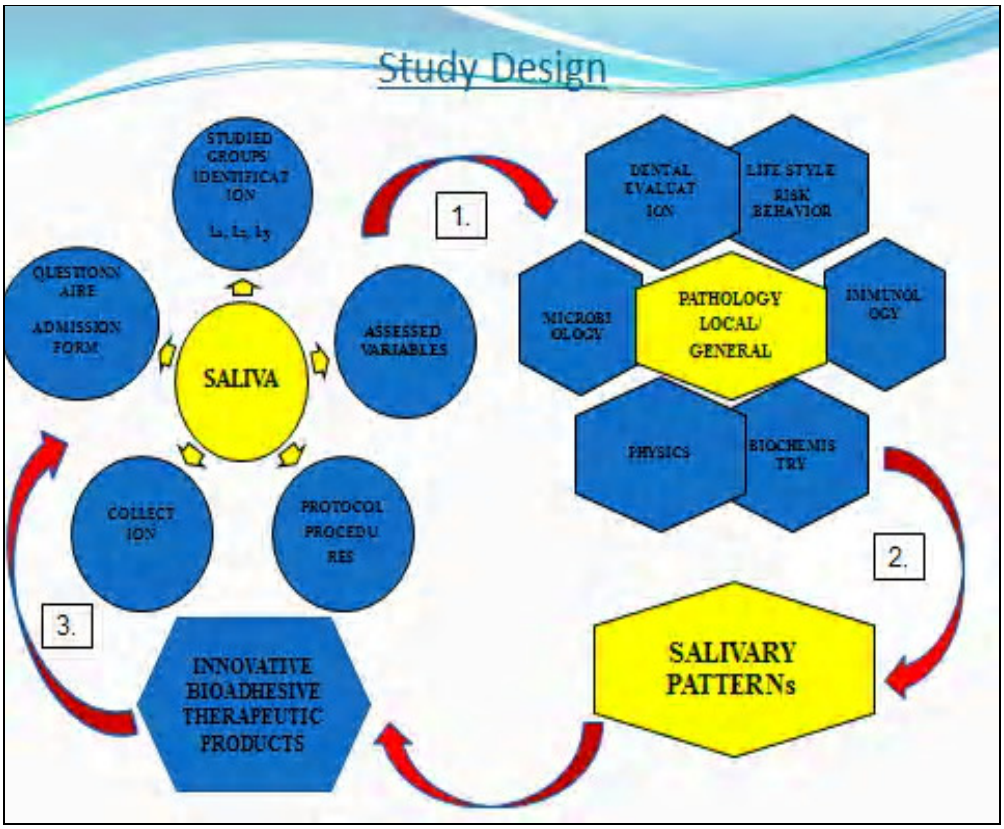

Fig. 1. General design of "Salivary Project"

The first moment of the study, is represented by "studied groups identification" according to the absence or presence of oro-pharyngeal pathology (Pink et al., 2009). During this activity, the role belongs to the stomatologist, who has to follow a complex questionnaire: "**SP- Individual File**" (table 1) in order to get the main information regarding personal, heredo-colateral history, specific dental respects (Jacob et al.,1998), diet, risk behavior, aiming to fulfill the admitting criteria.

For a correct and logical schedule of the procedures, for a maximum degree of both stomatologists' and patients' availability, as well, was timed the "SP – Individual File" completion, which lasts about 40 minutes /noted as "initial visit" in the "SP" questionnaire/.

Table 1."Salivary Project - Individual File"

Study code (1)	**Stomatologist's code (2)**	
General data	ID, Age, Profession, Professional risks, Urban/rural provenience,	
The motive of stomatology visit	Regular, Pain, Physiognomic changes, Functional changes, Infectious processes, Accidents/Acute Complications	
Antecedents 1. Heredo-colateral	General - Chronic pathology	Cardiac pathology, Endocrine pathology, Renal pathology, Respiratory pathology, Others
	General Dental Pathology	Congenital malformations, Macrodontis, Microdontis, Proalveolodontis, Mandibular prognatism
Antecedents	General antecedents	Birth way, Brest fed, Temporary dental eruption,

2. Personal antecedents		Permanent dental eruption	
	Systemic pathology	Cardio-vascular pathology, Bronchial asthma, Neurologic pathology, Endocrine pathology, Phosphor-calcium metabolism disbalances, Allergic pathology, Surgical procedures, Psychiatric pathology	
	Personal history	Pregnancy /n^{o}, Brest feeding /n^{o}	
	General Dental Pathology/ Stomatology treatments	Odontal treatment, Parodontal treatment, Surgical procedures, Prosthetic procedures	
	Dental "mirror" Parodontal examination	Teeth ID, Clinical lesions stage, Cavities topography Enamel lesions/color/topography, Enamel hypoplasia/shape, Cuneiphorm lesions/sites, Occlusions: topography, aspects (physiognomic/ non-physiognomic), metal content Radicular remnants topography, Dental usage: abrasive, dental atritis, Edentation: unidental, Edentation: multi-dental, sub-total, total Prosthesis devices* – fixed, motile, mobilisable, physiognomic, Prosthesis devices*: non-physiognomic, semi-physiognomic Type of metals residing -/specialized procedures/, Gingivitis – localized, and generalized, Marginal parodontopathy, Bone atrophy, Dental mobility, Bacterial plaque, Dental plaque, Halitosis, Buccal mucosal pathology, Parodontal pouch/real, Parodontal punch/false, Buccal hygiene indexes	
	Imagistic records	X-ray, Orthopanthomography	
	Life style	Particularities:	
	Diet	Norm balanced, Hyperglucidic, Hyperlipidic, Hypo protein/fast/, Obesity, Denutrition Fluids up-take: source: tap water, mineral water, artificial flavor cola-like liquids	
	Risk behaviors	Smoke, Alcohol, Psychological stress, Physical stress, Sedendarism	
Admitting criteria fulfilled	Yes	No	
Studied group ID	Group 1: **L1** /healthy study group	Group 2: **L2** /cavities pathology (aged 8-16 years)	Group 3: **L3** /parodontal pathology (aged over 50 years)
Calendar of activities	Stomatology visits	Initial	
		Regular/scheduled	
		Informative – tests result Advisory	
		Saliva collection	Date
			Conditions **Table II*
	Other activities, required by certain organizational aspects		
Patient's signature "I agree to participate to the study"			

When the enrolled patient will sign for "Patient's signature – "*I agree to participate to the study*" will get a flyer (table *2*), about all conditions should be respected, in order to have standardized collection procedure.

Table 2. Saliva Collection Procedure

Time of collection	During morning(at least 12 hours of fast) previously common diet
Condition of collection*	Non-stimulated saliva
Required saliva volume	5 ml
The period of time required for saliva collection volume*	Number of minutes required for collection
Time to get to the Lab	Maximum 60 minutes
Patient's Signature "I was informed about the saliva sample collection procedure, and I will respect it"	

**Saliva collection is performed under medical surveillance, in an intimate environment (to avoid any kind of negative reaction of the patient regarding the procedure). The subject is asked to spit directly into a sterile collection container. This spitting itself is usually a sufficient stimulus to elicit a flow about 0.5 ml/min, but even so, one the admitting criteria – is the record of time required for collection of a 5.0 ml volume.*

Further the collected data are "e" archived by the stomatologist (completing the individual file, devoted to "SP").

After the both forms completion, the information is sent in "e" form to the "core-data -base" devoted to "SP". In this way, the research team's members will be informed about each new enrolled patient, together with all collected data, during this phase.

Another pre-analytical Quality Standard refers to saliva manipulations (Pink et al., 2009). Once the samples have been collected, it is important that they should be properly transported /to get to the laboratory in maximum 1 hour after collection/ and stored /if the testing moment exceeds 2 hours, it should be kept at 4^0C, but not more than 2 hours/. In this way will be assured the specificity and sensitivity of further saliva determinations, which clearly fingerprint the adequate application of analytical procedures.

The chosen laboratory for biochemical and microbiological assessments is possesses accreditation on ISO 15189, having all quality standards implemented, in order to get pertinent information to be used for "Salivary Patterns" identification.

As soon as the saliva samples get to the laboratory, follow the next assessment procedures; their results will be add to the previous data.

The new recorded ***saliva variables*** are represented by: *a. - macroscopic evaluation* / color, consistency, presence of pathologic compounds: blood, pus; *b. - biological and biochemical* parameters /using a strip with 10 parameters/: erythrocytes, leucoytes, glucose, proteins, bilirubine, pH, density, ketonic bodies and nitrits.

Along ***analytical phase***, follows the centrifugation /3000 rpm for 3 minutes/ - saliva samples will be transferred into sterile centrifugation tubes; after centrifugation, will be recorded the ratio between Supernanant/sediment (S/s).

The supernatant will be divided into 1 ml samples: 1 ml for immunologic tests, 1 ml for enzimology, 1 ml for metal detection and 1 ml for inflammation degree activity.

The sediment will be used to get: native saliva smear, stained smear /Blue Methylene and NBT staining procedure/ and cultivation: on Blood –Agar medium, Levin Medium, Drigalski medium and Sabouraud medium.

For the ***native and stained saliva smears***, is used an Olympus microscope, in order to develop the photo-gallery – images which are further computing – assissted, being part of the general data-base in ”SP” / for saliva organic and inorganic components/.

The ***NBT test*** – is based nitrobleutetrazolium (NBT) a salt of tetrazoliu activity; due to its reducing propriety, is used for „*in vitro*” leucocytes oxido-reducing enzymes study. Exists a direct proportion between the intensity of oxido-reducing activity and the degree of inflammation.

Metalic Ions. For the qualitative identification of metal ions, is used a Trace Lab 150 device. The equipment is an electro-chemical one, and allows detection of certain heavy metals, anions and electroactive organic species. Traces of metals can be assessed /up to 0,01 ppb/ using a stripping voltametry analysis /in less than 15 minute; are involved 2 different processes: electrolysis /during this phase, the electroactive species are deposited along the working electrod/ and the second one: stripping process /during tis phase, the species are re-solved in solution/.

In the present study the saliva inorganic ions were assessed by using the absorbtive stripping voltametry method /the buffer was represented by Britton-Robinson /pH 2.8/.

For the quantitative saliva metal assessment, was used the **Atomic Absortion Spectrophotometry ICE 3300** /belonging to the laboratory from the Agency of Environment Protection Bihor County/.

The ***salivary amylase activity*** is measured using a colorimetric method with DNS reagent (3,5-dinitrosalicylic acid) after Hosttettler and co. modified by the authors in order to ensure the appropriate conditions for the starch hydrolyse in human body (Bice and Evans, 1982; Kennedy and Stevenson, 1988).

The amylase activity can be measured following the decrease of the viscosity of a starch solution, the decrease of the turbidity of a starch suspension, the decrease of the intensity of a starch-iodine reaction and the increase of the reducing groups in the reaction medium. The last method is in agreement with the EC-IUB demands (Rouau et al.,1993).

To determine the reducing sugars existing in the reaction medium at initial moment, for all samples were made controls, identically with the tests, except that in the controls there was no enzyme. To transform the optical densities read for the tests and controls in moles maltose it was made a standard curve.

The used Reagents are represented by: soluble starch supplied by Merck, Darmstadt was used in 1% concentration in acetate buffer solution at pH 6, 9 which contain $CaCl_2$ 0,1M. Maltose was used as standard solution (see figure 2) in the concentration as 100 μg/ml. *The biological sample* is represented by an enzymatic extract of human saliva amylase centrifuged 3 minutes at 2000 rpm rpm (Honesey aand Moreau, 1994).

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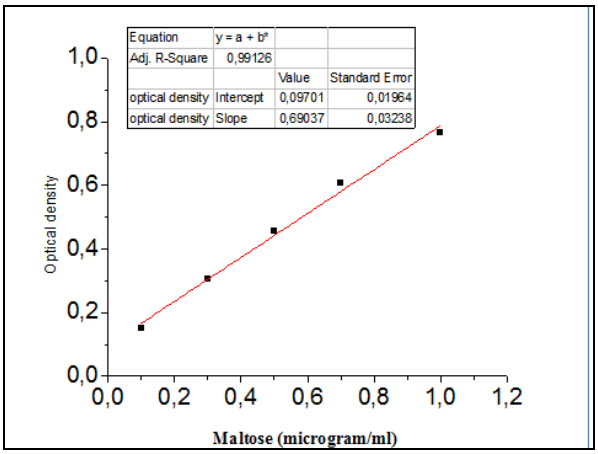

Fig. 2. Standard curve /maltose/

Objectives for saliva-based data analysis and processing 1. Feature selection and feature extraction from saliva databases. Saliva-based data may contain various level measurements of different and important chemical compounds like pH, proteins, glucose, bilirubin, nitrites, leucocytes, and erythrocytes. These levels are provided as numerical values. Based on those levels many medical conditions may be revealed. However, some compounds may not be active for certain medical conditions. Therefore, a preprocessing step named feature selection could be employed to discriminate between active (relevant) and less active (relevant) compounds. On the other hand, when uncertainty about the full data set relevance occurs, another preprocessing step can be carried out, named feature extraction (data transformation). This step mainly refers to capture data with high variance while discarding data with low-level variance, as usually employed by basic statistics, searching for correlation amongst data. Moreover, data dimension is highly reduced down to 3 or 2-dimensional data, allowing 2D or 3D data ***visualization*** (Nixon and Aguado, 2007). As consequence, an automatic clustering can be performed, clusters that share common characteristics and should be easily interpreted by visual inspection, helping doctors or biologists in their decisions. Finally, some other statistics elements such as normalization and standardization may be necessary to de-correlate data prior to clustering. ***2. Image processing applied to saliva images***. Apart from numerical data drawing from chemical compound measurements, valuable information may be extracted from saliva images acquired by a microscope coupled with a CCD camera and computer. Crucial preprocessing steps are here important including: image quality enhancement, automatic (or semi-automatic) segmentation (segmenting important regions of interest from the background) of patterns, edge detection, pattern characteristics measurement (shape, color value, color intensity, density, histogram, etc), so that those measurements may be converted to numerical values (Nixon and Aguado, 2007; Gonzales, 2008). ***3. Saliva data set information classification***. Once features are extracted or clustered, the ultimate purpose of data analysis is provided by the ability of the system to give an accurate and automatic diagnose. This is a complex procedure comprising several issues, such as a relevant database, a reliable feature extraction strategy, a learning algorithm (where the learning is accomplished based on some learning rules together with a training set) and an automatic recognition approach for an unseen test data, where the resulting answer should assist and help the doctor in having an accurate diagnose decision. It must be

noticed that the classification methods are not specific only to medical images. The methods can be employed to any medical information at our disposal (represented by numerical values), for instance, to a diabetic medical conditions set, where the multidimensional data comprises attribute values such as plasma glucose concentration, salivary glucose presence, diastolic, blood pressure, body mass index, etc. (Duda et al., 2000).

The scheme of all variable taken into account in this study, which will be computer-assisted on the „SP" are displayed on Table 3.

Table 3. „Salivary Project" variables

ID patient	Heredo-collateral antecedents	Personal antecedents	Life Style	Dental "mirror"
Parodontal examination	Time required for "S" collection	"S" macroscopic features: color, consistency...	Biologic & chemical "S" markers: 1. glucose	2. "S" pH
3. "S" δ	4. "S" Erythrocytes	5. "S" Leucocytes	6. "S" Proteins	7. "S" Bilirubine
8. "S" ketones	9. "S" Nitrits	"S" S/s (Salivary ratio Supernatant/sediment)	"S" Macroscopic S/s	"S" sediment Native smear
"S" sediment staining procedures: BM, NBT /organic and inorganic elements/	"S" sediment culture *(pathogenic bacteria and C. albicans)*	"S" Supernatant: Ioni metalic ions: Ag, Na, K, Ca, Cu, Fl *TraceLab, SFAA ICE 3300*	"S" Supernatant: enzymes: amilaza, lysosim, ATP-aza *Interferometrie LASER*	"S" Supernatant IgAs *ELISA*

RESULTS AND DISCUSSIONS

So far the total number of assessed saliva samples is 18, identified according to admitting criteria into 3 categories: the control group (**L1**) composed of healthy individuals, the 2nd group (**L2**) composed of patients aged 8 up to 16 years / with multiple dental cavities, the 3rd group (**L3**) composed of pacients aged over 50 years with parodontal pathology.

The total results of the biological, biochemistry and microbiological tests /referring to ID and age patient/ are displayed in the table 4.

Table 4. "Salivary Project" results: biological and biochemistry and microbiological tests

No	Initials	Age	Glu	Pro mg/dl	Bil	Uro	pH	S	Bld mg/dl	Ket	Nit	Leu /ml	microbiology:				
													Strept	Staf	C. alb.	Others	AST
1	CS	42	N	30	neg	N	7.5	1020	0.2	neg	+	500	neg	neg	neg	neg	
2	CI	44	N	30	neg	N	7.0	1020	0,2	neg	neg	500	neg	neg	++	neg	
3	CA	30	N	30	neg	N	7.0	1015	1.0	neg	+	500	neg	neg	neg	neg	
4	CF	13	N	30	neg	N	7.0	1015	0.06	neg	neg	500	neg	neg	+	neg	
5	KL	39	N	30	neg	N	7.0	1015	0.06	neg	neg	500	neg	neg	neg	neg	
6	SD	17	N	30	neg	N	6.5	1020	0.2	neg	neg	500	neg	neg	neg	neg	
7	BM	57	N	100	neg	N	7.5	1020	1.0	neg	neg	500	neg	neg	neg	neg	
8	SI	35	N	30	neg	N	7.0	1020	1.0	neg	neg	500	neg	neg	neg	neg	
9	SD	57	N	30	neg	N	7.0	1015	1.0	neg	neg	500	neg	neg	neg	neg	
10	PI	60	N	100	neg	N	6.5	1025	1.0	neg	++	500	neg	neg	neg	neg	
11	GN	74	N	300	neg	N	7.0	1025	1.0	neg	neg	500	neg	neg	neg	neg	
12	SM	33	N	30	neg	N	7.0	1025	0.2	neg	neg	500	neg	neg	neg	neg	
13	BA	28	N	30	neg	N	6.5	1010	1.0	neg	neg	500	neg	neg	++	neg	
14	IC	42	N	100	neg	N	7.0	1020	1.0	neg	neg	500	neg	neg	+++	neg	
15	AS	56	N	100	neg	N	7.0	1020	1.0	neg	neg	500	neg	neg	neg	neg	
16	BA	68	N	100	neg	N	6.5	1020	1.0	neg	+	500	neg	neg	+++	enter ococ	x
17	TC	53	N	100	neg	N	7.5	1015	0.06	neg	neg	500	neg	neg	+	neg	
18	FV	56	N	100	neg	N	7.0	1020	0.2	neg	neg	500	neg	neg	+++	E. coli	x

Selected images /Blue Methylene Stain/ are presented in figure 3, part of "SP" general data-base.

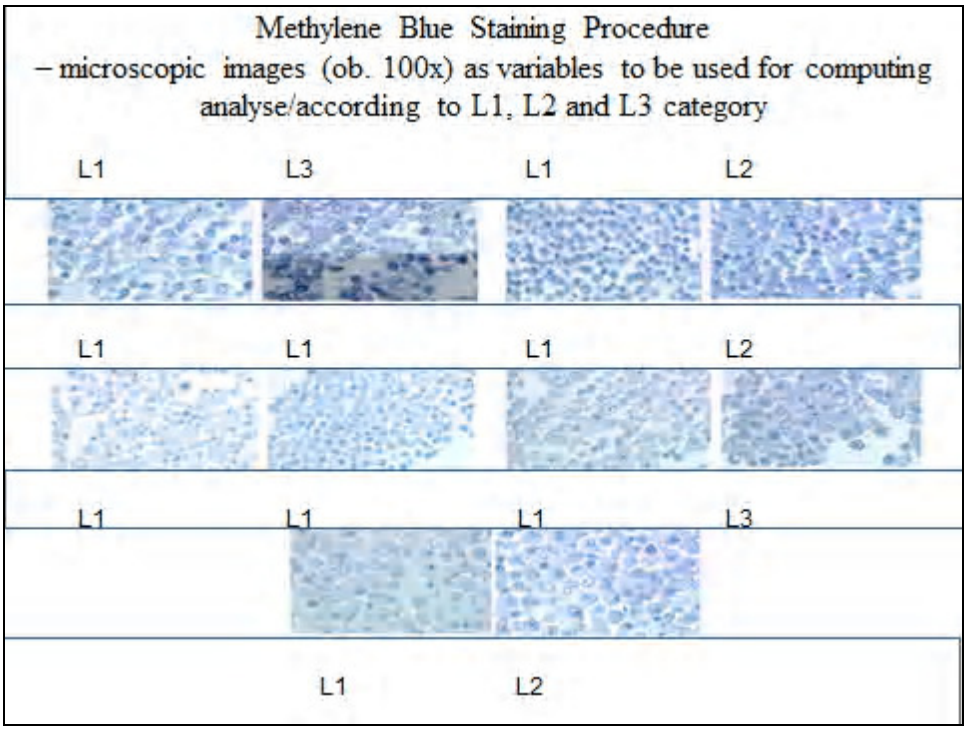

Fig. 3. MB Staining Procedure - Saliva Smears

Selected images /NBT technique/ belonging to each category of patients are represented in figure 4, part of the "SP" general data-base.

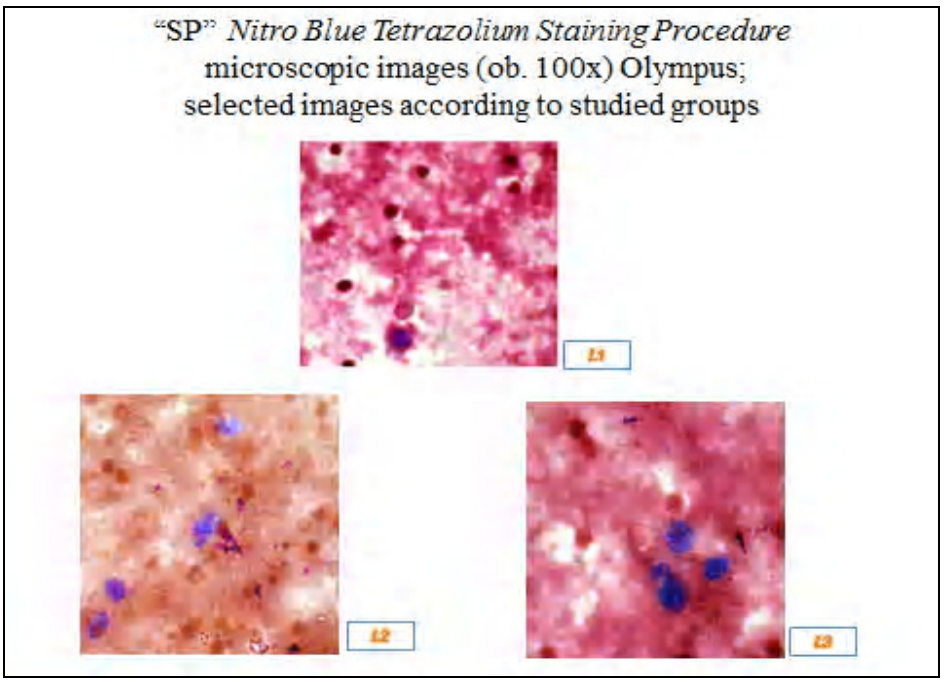

Fig. 4. NBT Staining Procedure - Saliva Smears

Selected diagrams obtained with Trace Lab 150/ belonging to L1, L2, and L3/ are presented in figure 5, part of "SP" general data-base.

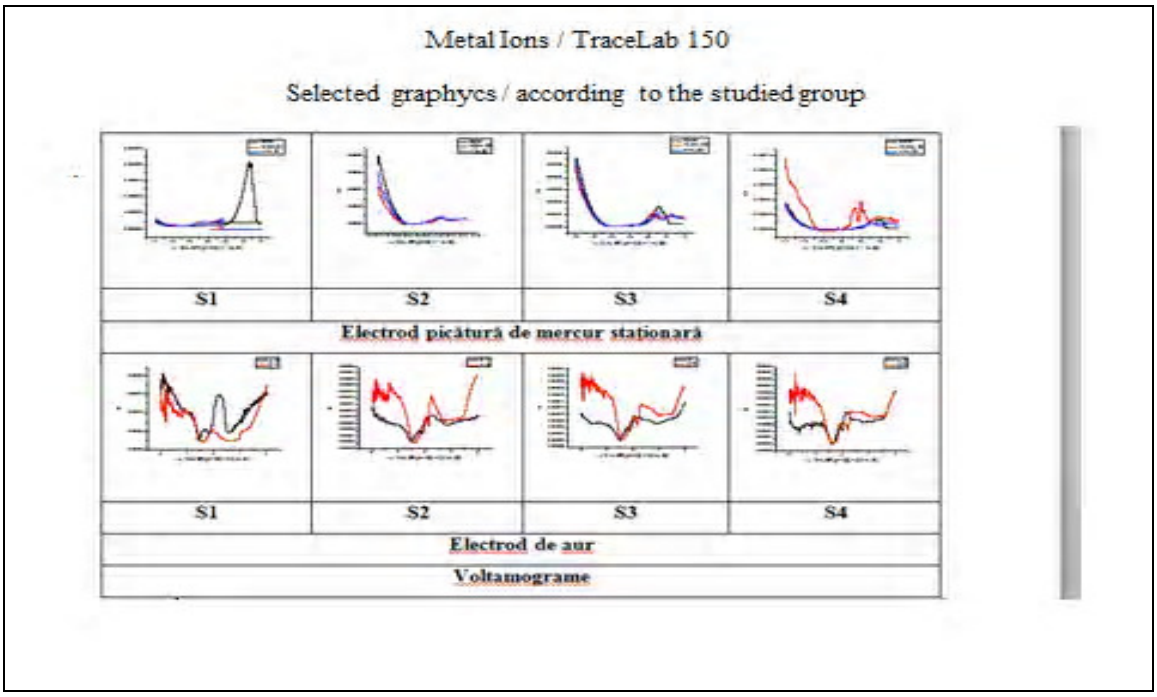

Fig. 5. Qualitative test results – salivary metals

Selected results – obtained with *Thermoscientific AA Spectrometer ICE 3300*) figure 6, part of the „SP" general data-base.

Saliva Sample	Metal (µg/mL)		
	Cd	Ni	Pb
1.	0.0035	0.0051	0.0059
2.	0.0159	0.0203	0.0349
3.	0.0051	1.0164	0.0181
4.	0.0005	0.1143	0.4339
5.	0.0198	1.3201	0.5895
6.	0.0126	0.4355	0.2688
7.	0.0006	0.4051	0.1081
8.	0.0096	0.8315	0.0025
9.	0.0136	0.7706	0.1815
10.	0.0011	0.3746	0.0154
11.	0.0003	0.9229	0.2283
12.	0.0076	0.4355	0.3850
13.	0.0131	0.375	0.0022
14	0.009	0.831	0.268

Fig. 6. Quantitative test results – salivary metals

The salivary amylase activity for the 18 analysed saliva samples is presented in table 5.

Table 5. “Salivary Project” the salivary amylase activity

Nr. crt.	**Samples**	**Control extinction**	**Sample extinction**
1.	S1 **- L1**	0,045	0,440
2.	S2**- L2**	0,045	0,510
3.	S3 **– L3**	0,045	0,610
4	S4 **– L3**	0,045	0,615
5	S5 **- L1**	0,045	0,442
6	S6 **- L2**	0,045	0,514
7	S7 **- L2**	0,045	0,515
8	S8 **- L1**	0,045	0,444
9	S9 **– L3**	0,045	0,612
10	S10 **– L3**	0,045	0,615
11	S11 **- L1**	0,045	0,443
12	S12 **- L2**	0,045	0,517
13	S13 **– L3**	0,045	0,612
14	S14 **– L3**	0,045	0,615
15	S15 **- L1**	0,045	0,442
16	S16 **- L2**	0,045	0,518
17	S17 **- L2**	0,045	0,516
18	S18 **- L1**	0,045	0,441

The study is on-going, allowing us to approach in parallel the saliva 1. cellular component evaluation /host *vs* microbiocenosis/ 2. physico-chemical properties 3. immunologic properties, in different categories of patients.

Some advantages in our protocol, are: a very good selection of the patients /colaborative/ the rapid start of saliva testing procedures (the samples get to the lab in maximum 60 minute after collection) and having the opportunity to use the atomic absorbtion spectophotometry for analysis based on the low threshold and ability of providing levels of multiple metals simuntaneously.

The study design also maintains the possibility of enlarging the panel of salivary determinants, such as: ATP-ase, lysosyme testing, and other inorganic substances to be evaluated, according to individual features.

To get a correct and pertinent general data-base, we still work in standardization of initial qustionnaire completion, with key-words, enabling the informatics team to stock, analyze and interprete different types of variables.

One of our domain to be developed is the animal model /Wistar rat/– the essential step in innovative therapeutic products /plants extracts-bioadhesive materials/ use, the last step in our study design.

CONCLUSIONS

1. Saliva collection is an easy noninvasive, stress-free procedure
2. Once again is underlined the importance of quality standards appplication regarding preanalytic and analytic phases
3. Dental caries is a multifactorial process that depends on the interaction of host, substrate, microbiocenosis and salivary factors, in **L2** the presence of C. albicans proves its acidogenic and heterofermentative role
4. The potential use of salivary cadmium measurements for the biological monitoring of occupational cadmium exposure, in different Bihor county area, in relation with the Public Health Agency and Environment Protection Agency pollution records
5. The computing-assisted model allows the salivary patterns identification - using a modern tool „images-capture" and variable analyses
6. The computing-assisted model, represents also the basement on local and general pathologic conditions /inflammation, chronic diseases, tumoral pathology, toxicology etc/ interpretation
7. Using the salivary patterns, can be developed preventive/curative therapeutic schemes

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COMPUTATIONAL MODEL FOR ADSORBTION OF CALCIUM AND MAGNESIUM IONS ON HYDROXYAPATITE

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ABSTRACT

The strength of the calcium and magnesium ion bonding with the surface of hydroxyapatite was investigated using computational methods. A virtual model of the crystalline net was designed, starting from standard structure, and a binding site for the cation was taken into consideration. Using computational semiempirical methods the stability of the structure was assessed, and results were compared in order to understand the magnitude of energetic factors implied in bonding competition of calcium versus magnesium, in the process of forming the cristalline hydroxyapatite dopped with magnesium ions. No hydration-dehydration processes were considered at this stage.

Key words: hydroxyapatite, calcium, magnesium, bone tissue

INTRODUCTION

Calcium phosphate and hydroxyapatite based ceramics are currently used in medicine as replacements for human bones (biomaterials). This bone substitute materials are of large interests due to their reliability in grafts industry, where sampling trauma, and biological hazards are of great concern.

Bone reconstruction technology require synthetic compounds for treatment of bone fractures, arthritis, osteoporosis, tumors, bone infections and even spine surgery (Popescu et al., 1980).

Most frequently used synthetic products are based on a mineral fraction (hydroxyapatite and calcium phosphates) colonized with stem cells, natural growing factors and morphogenetic bone proteins. The hydroxyapatite crystallites in natural bone tissue contain certain amounts of impurities, in particular Mg^{2+}, which substitutes for Ca^{2+}.

Understanding the processes that take place on the surface of the synthetic bone replacement materials can improve the quality of the end-products, and open ways for new compounds.

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MATERIALS AND METHODS

A mathematical model has been designed for the hydroxyapatite crystalline net, one that best describes spatial models for the constituent atoms of $Ca_{10}(PO_4)_6(OH)_2$.

Hydroxyapatite crystallizes in hexagonal or monoclinic system, $P6_3/m$ or 2/m symmetry group. Elementary cell dimensions for $P6_3/m$ symmetry group: a=b=9.3973 c=6.8782 Z=2

Starting structure was considered a .cif file from American Mineralogist (http://rruff.geo.arizona.edu/AMS/result.php?mineral=Hydroxylapatite – 2010, August), with standard geometry already available (hexagonal system, $P6_3/m$ symmetry group). A crystalline net was generated from smallest repetitive unit, by use of the Mercury (http://www.ccdc.cam.ac.uk/products/mercury - 2010, August) program (Fig. 1).

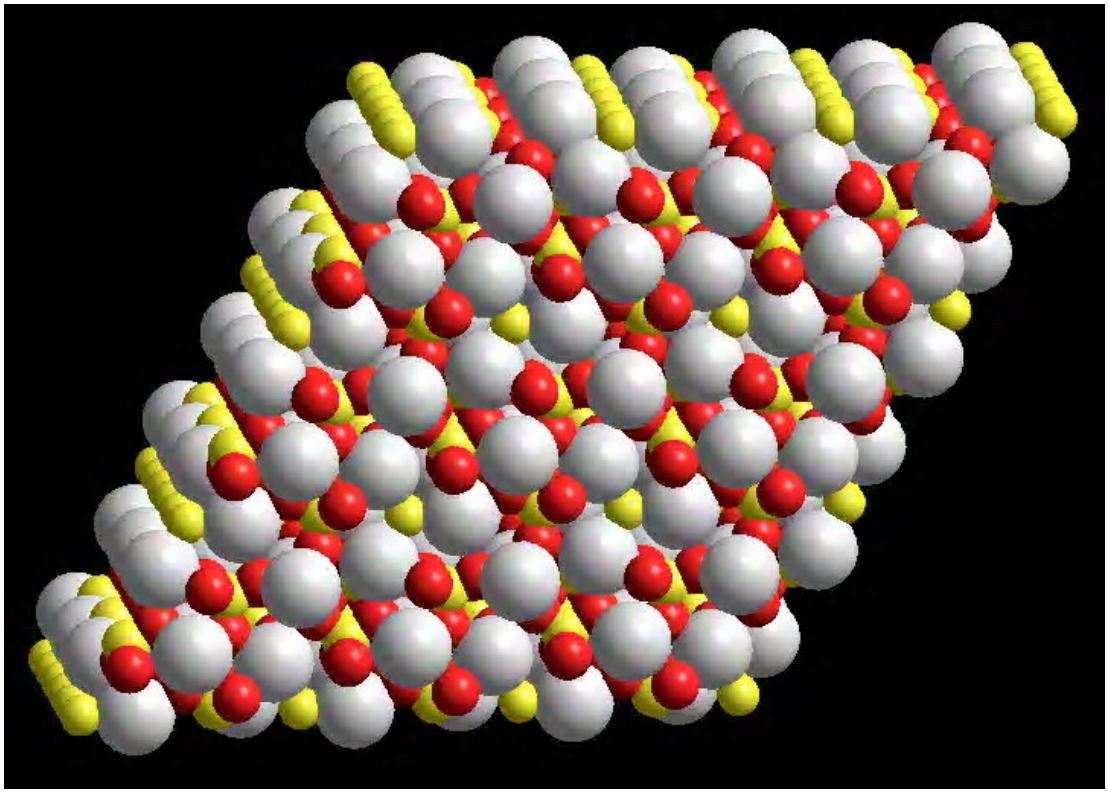

Fig. 1. Crystalline net for hydroxyapatite

The model can be used to study the interactions that take place on the crystalline surface (e.g. cation bonding competition), and factors that influence crystal growing (e.g. surface electrostatic potential, bonding of crystalline habitus modifiers, hydration-dehydration processes).

A binding site containing one surface Ca^{2+} ion was isolated and the structure was imported into HyperChem (HyperChem™ Release 5.11 Professional for Windows, Hypercube, Inc. 1999, Gainsville FL, USA, www.hyper.com program for energetic calculations (Fig. 2).

For computational purposes, semiempirical PM3 method (implemented in the HyperChem package) was chosen, for speed and parametrization considerations. The heat of formation for the structure was calculated by single point method.

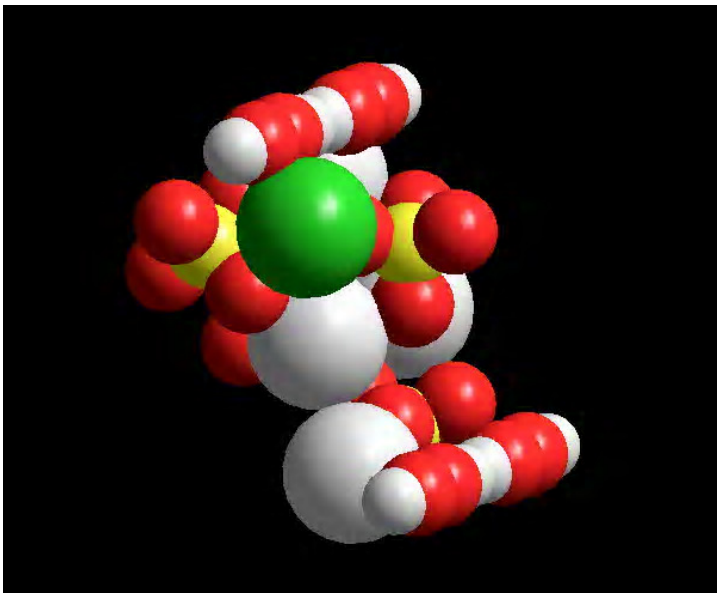

Fig. 2. Calcium ion in the binding site (green colour)

RESULTS AND DISCUSSIONS

Single point calculations were performed for the structure with Ca^{2+} and respectively Mg^{2+} in the binding site, and without any cation (empty binding site).

Heat of formation is a measure of stability of a chemical structure; lower value for heat of formation means greater stability for structure in question. For comparison reasons, absolute values have little importance, instead general trend and order of magnitude are of interest.

For the present case (Ca^{2+} versus Mg^{2+} as competitive ions) the results are as follows (heat of formation, in kcal/mol):

Hydroxyapatite fragment (empty binding site): -483.7 kcal/mol
Hydroxyapatite with Mg^{2+}: -656.8 kcal/mol
Hydroxyapatite with Ca^{2+}: -1152.6 kcal/mol

CONCLUSIONS

Results show that Ca^{2+} complex with hydroxyapatite is more stable than Mg^{2+} correspondent, a confirmation of experimental observations, so that when both ions are present, the calcium complex is more likely to be formed.

Also, since the Mg^{2+} complex has a lower energy than the lone hydroxyapatite fragment, the magnesium ion will have a tendency to bind to the formed crystal surface, competing with calcium ion. The competition is still destructive for the cristalline net, since the magnesium ion with a different ionic radius will induce lattice strain in crystalline matrix.

The amount of magnesium can be influenced by Mg^{2+} concentration, and thus calcium phosphates dopped with different amounts of magnesium can be obtained.

Such synthetic crystalline structures resemble the natural bone tissue, and can be used as biomimetic materials in bone reconstruction processes.

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PATHOBIOCHEMICAL ASPECTS OF IRON, COPPER, ZINC AND TOTAL ANTIOXIDANT STATUS IN AMYOTROPHIC LATERAL SCLEROSIS

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ABSTRACT

The brain has a number of characteristics that make it especially susceptible to free-radical-mediated injury. Brain lipids are highly enriched in polyunsaturated fatty acids and many regions of the brain, for example, the substantia nigra and the striatum, have high concentrations of iron. Both these factors increase the susceptibility of brain cell membranes to lipid peroxidation. Because the brain is critically dependent on aerobic metabolism, mitochondrial respiratory activity is higher than in many other tissues, increasing the risk of free radical 'leak' from mitochondria; conversely, free radical damage to mitochondria in brain may be tolerated relatively poorly because of this dependence on aerobic metabolism. The aim of this study was to evaluated the concentrations of copper, zinc, iron and Total Antioxidant Status in a neurodegenerative disease; Amyotrophic Lateral Sclerosis (ALS).

Key words: Total Antioxidant Status (TAS), Copper, Zinc, Amyotrophic Lateral Sclerosis (ALS)

INTRODUCTION

Neurodegenerative disease (greek *νέυρο-, néuro-*, "nerval" and latin *dēgenerāre*, "to decline" or "to worsen") is a condition in which cells of the brain and spinal cord are lost. There is a deterioration of neurons or their myelin sheath, which over time will lead to dysfunction and disabilities resulting from this. The various neurodegenerative diseases (diseases in which neurons degenerate and die) have different symptoms, affect different parts of the brain, and have different causes. All of these diseases (Parkinson disease, Alzheimer disease, ALS, Friedrich ataxia, Huntington disease, prion disease) have some common features as impaired mitochondrial function, increased oxidative damage, and defects in the ubiquitin - proteasome system, the presence of abnormal, aggregated proteins, changes in iron metabolism and some involvement of excitotoxicity and of inflammation.

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Amyotrophic lateral sclerosis (ALS) is the most common neurodegenerative disease of the motor neuron system involving the lower and the upper motor neurons in spinal cord, brain stem, and cerebral cortex. Average age of onset of ALS is 57 (65) years. Clinical symptoms at the beginning are painless, muscle weakness and impaired muscle tone, leading to atrophy. Problems with speech and swallowing follow. The disease is chronic and progressive, often leading to death within a few years of its appearance. Males are affected almost twice as often as females. The incidence of ALS in populations of European descent is approximately two per 100 000 population per year, therefore the prevalence is estimated at six per 100 000 population. 19% or more of ALS cases are sporadic. Increased oxidative damage occurs in ALS, but its importance to the disease pathology is unclear.

Iron is one of the common metal elements.

It is very largely bound to proteins not free in cells. It was very readily available from the primitive reducing sea as ferrous ion but, as oxygen pressure rose more than two billion years ago, iron became ferric ions in solution, which precipitated, and availability became much reduced. Consequently, all aerobic organisms have cleverly devised scavenging systems for iron. The essential nature of the element derives from its use as a catalyst. In its protein, combinations it is found bound in iron–sulphur proteins, in heme proteins, and in proteins bound simply to nitrogen and oxygen side chains. These proteins are largely engaged in oxidation or reduction catalysts, in the transport of electrons, as carriers (haemoglobin and myoglobin), as sensors for CO, NO, and O_2, in DNA synthesis from RNA, and as storage buffers for iron. There is in fact a very extensive network of iron proteins essential in all cells but very noticeable in the bioenergetics of both chloroplasts and mitochondria. There is for this metal element a series of concentration controls linked through transcription factors to DNA. It may be that the overall expressions of many functional parts of a cell are linked to the concentration of free ferrous ions in the cell cytoplasm.

However, the storage of iron is in a ferric ion precipitate bound in a protein, ferritin.

There may be no life without zinc.

Next to iron in importance amongst trace elements is zinc. Unlike iron, it was restricted in its availability to primitive life since it has an insoluble sulphide. As sulphur, in the form of H_2S, became oxidised to sulphate, so zinc was liberated, and it is now quite a common element in the sea. Zinc is not like iron in its functions. It does not take part in oxidation or reduction reactions but is a good acid catalyst. Hence, it finds use in organic chemistry as well as in organisms. In cells, its acidic function is used not only in a wide range of degradative enzymes – peptidases, nucleases, and saccharases, and in hydration reactions – but also in RNA/DNA synthetases. Zinc has a distinct role in the nucleus of eukaryotes in proteins called zinc fingers, which act as transcription factors especially involving sterol, thyroxin, retinoic acid, and related hormones. Thus, it is important in homeostasis and in organism metamorphic transformations such as the transition through puberty.

In medicine and biology, zinc has several connotations. It is an essential micronutrient, a component of enzymes and other proteins and a very toxic pollutant as well.

To neuroscientists, zinc is also an ionic signal; Zn^{2+} enters cells through gated channels and moves among various organelles and storage depots within cells, modulating protein function by binding to and detaching from zinc-dependent proteins. Excess of free zinc is toxic.

Yinc ion (Zn^{2+}) is selectively stored in, and released from, the presynaptic vesicles of a specific type of neurons in the mammalian brain. This zinc – releasing

neurons also releases glutamate, so the term “gluzinergic” has been proposed to describe them. There is now strong evidence that free zinc, normally very low in cells, is used at considerable concentrations in certain parts of the brain as a transmitter and in the reproductive tract of males.

Copper is probably not a universal requirement for life. The sulphides of copper are extremely insoluble and primitive anaerobic archaea probably did not use it. Later oxidation of sulphide generated available copper and in general, aerobes employ it as an oxidative catalyst. This use is mainly confined to extracellular or periplasmic compartments of cells since free copper itself is very poisonous internally, where it is probably no more than 10^{-15} M. The locations of the sites of action of copper proteins contrast strongly with those of iron as seen in the different cell compartments in which the two are used. A particular function of copper is in the crosslinking of extracellular matrices, which helps to stabilize multicellular organisms e.g. the final forms of collagen, lignin, and chitin. The homeostasis of copper in cells appears to be managed by a class of proteins, metallothineins, which also control the levels of free zinc. Uptake and rejection of copper requires cellular pumps and several disadvantageous inherited conditions arise from mutations in these pumps.

Growing evidence suggests that the generation of oxidants does not result simply from an accidental disruption of aerobic metabolism, but rather from an active process crucial for the nonspecific immune defences of the brain. While essential for survival, these processes may be inappropriately activated to cause neuro-degeneration.

Neurons are highly susceptible to oxidative stress, which can induce both neuronal necrosis and apoptosis. Oxidants may also have more subtle roles in compromising the integrity of the blood brain barrier and in producing reactive changes in astrocytes that further propagate injury. Moreover, oxidative stress appears to provide a critical link between environmental factors, such as exposure to pesticides, herbicides, and heavy metals, and endogenous and genetic risk factors in the pathogenic mechanisms of neurodegeneration, particularly in Parkinson disease.

A better understanding of the role of oxidants in neurodegeneration still holds a largely unfulfilled potential to reduce the burden of both acute and chronic neurodegeneration.

MATERIALS AND METHODS

Blood was taken from 11 patients with ALS, and 19 controls. All of patients were aged, between 31-70 years. The diagnosis ALS was made on clinical findings. Blood was taken from an antecubital vein into a sterile vacutainer. Hibitane skin sterilisation, minimal venous stasis. 19 subjects defined as being free of major medical or surgical illness within 5 years and leading an active and independent life were used as controls in this study. All of controls were aged, between 40-69 years.

Total Antioxidant Status (TAS) was measured spectrofotometrically, Randox Laboratories Ltd. U.K. reagents were used, standard TAS Lot. No. 224NX, 1.87 mmol/L concentrations, human serum control Lot 228NX, target value 1.88 mmol/L, range 1.50-2.26 mmol/L. A Jasco V-530 spectrophotometer (Jasco Corporation Tokyo-Japan) with validation program was used. 1.0 cm High Precision Cells Hellma GmbH & Co.KG Mulheim-Germany, certificated. Wavelength: 600 nm, Temperature: 37^0C. The assay principle is that metmyoglobin reacts with H_2O_2 to form the radical species, ferrylmyoglobin. A chromogen (2,2’-azino-di-[ethylbenzthiazoline sulfonate];

ATBS is incubated with ferrylmyoglobin to produce the radical cation species ATBS. This has a relatively stable blue-green colour, which is measured at 600 nm. Antioxidants in the added sample cause suppression of this colour production to a degree that is proportional to their concentration.

The SH groups were measured spectrofotometrically, using a particular Ellman method according Suzuki (1990), reversed Cavrini (1989) method and HPLC.Uric acid, copper, zinc, iron, cholesterol, triglycerides, phospholipids, bilirubin, albumin, C-reactive protein, were analysed with Hitachi 717 Boehringer Mannheim (Germany) automatic analyser, using Futura System (Italy) reagents.

The results were expressed as mean ± SD, range of variation and median by using STATISTICA 8.0 for Windows.

RESULTS AND DISCUSSIONS

We present the results of our investigations in the next tables and graphics.

From statistical point of view, we noted a significant difference ($p < 0.001$) from females and males in the control group at uric acid, cholesterol, HDL-cholesterol, LDL-cholesterol and Zinc levels.

This difference is possible to be characteristic only for this control group but it is necessary to point out that the subjects were from the same areal, with similar mod of nutrition. The measure of central tendency, the *median* and the *mean* the particularly informative measure of the "central tendency" of the variable if it is reported along with its confidence intervals in this group are in proximity. In this case, it is possible to assume that these differences are not consequences of the nutrition mode. The exogenous factors were the same.

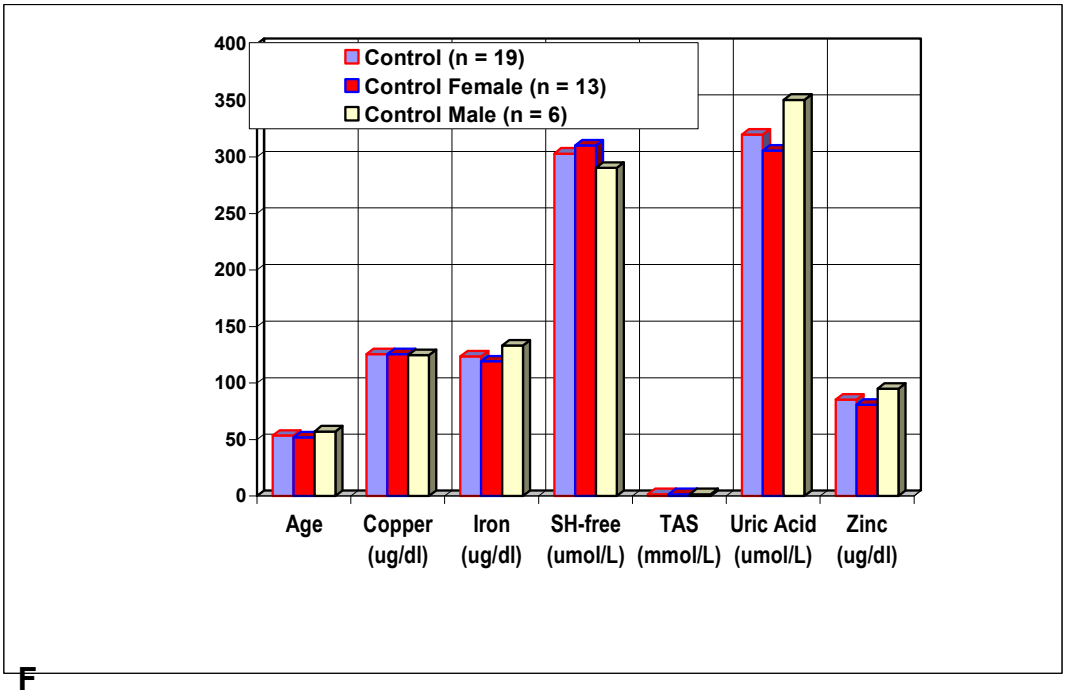

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Fig. 1. Graphical expression of mean values in control group.

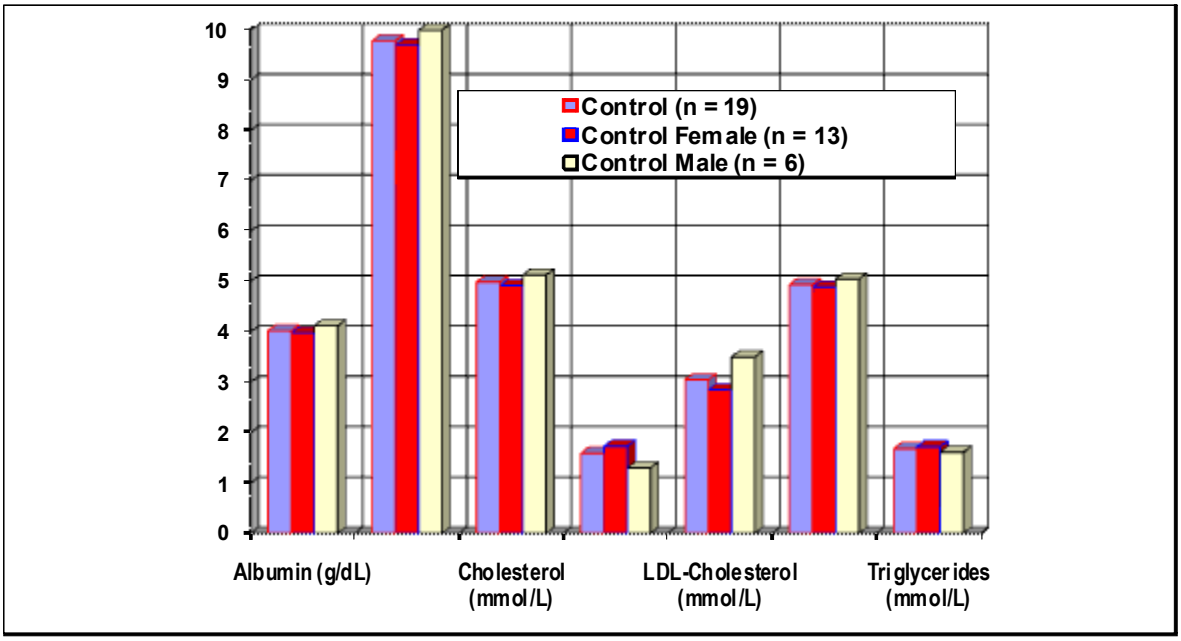

Fig. 2. Graphical expression of mean values in control group

Table 1.The characterisation from biochemical point of view of the females control group.

Specification	Control F (n=13)		
	Range	Median	Mean ± SD
Age (years)	41 - 69	52.00	51.69 ± 7.44
Albumin (g/dL)	3.40 – 4.25	4.15	3.98 ± 0.29
Bilirubin (µmol/L)	6.25 – 11.25	10.48	9.70 ± 1.85
Cholesterol (mmol/L)	4.65 – 5.30	5.00	4.92 ± 0.21
Copper (µg/dl)	80.00 – 145.50	128.10	125.37 ± 17.49
CRP (mg/L)	5.45 – 9.50	5.95	6.82 ± 1.45
Iron (µg/dl)	95.44 – 165.50	108.40	119.13 ± 23.53
HDL-Cholesterol (mmol/L)	0.99 – 2.01	1.80	1.73 ± 0.29
LDL-Cholesterol (mmol/L)	2.64 – 3.34	2.84	2.85 ± 0.18
Phospholipids (mmol/L)	4.45 – 5.20	5.00	4.89 ± 0.24
SH – Free (µmol/L)	244.00 – 335.00	333.00	310.14 ± 37.07
TAS (mmol/L)	1.40 – 1.78	1.60	1.61 ± 0.14
Triglycerides (mmol/L)	1.25 – 1.98	1.75	1.72 ± 0.18
Uric Acid (µmol/L)	294.30 – 320.15	299.10	305.63 ± 10.81
Zinc (µg/dl)	66.57 – 100.00	80.45	80.72 ± 11.38

Table 2. The characterisation from biochemical point of view of the males control group.

Specification	Control M (n=6)		
	Range	Median	Mean ± SD
Age (years)	42 - 69	55.50	57.17± 10.15
Albumin (g/dL)	3.60 – 4.60	4.22	4.12 ± 0.37
Bilirubin (µmol/L)	7.00 – 15.80	9.03	9.99 ± 3.61
Cholesterol (mmol/L)	4.95 – 5.30	5.11	5.13 ± 0.13
Copper (µg/dl)	89.45 – 140.10	135.4	124.58 ± 21.23
CRP (mg/L)	5.45 – 9.00	7.13	7.03 ± 1.24
Iron (µg/dl)	100.20 – 160.20	145.20	133.05 ± 25.84
HDL-Cholesterol (mmol/L)	0.99 – 1.60	1.32	1.30 ± 0.31
LDL-Cholesterol (mmol/L)	3.24 - 3.76	3.49	3.50 ± 0.22
Phospholipids (mmol/L)	4.75 – 5.20	5.09	5.05 ± 0.17
SH – Free (µmol/L)	244.00 – 335.20	291.62	290.47 ± 48.07
TAS (mmol/L)	1.41 – 1.80	1.56	1.60 ± 0.17
Triglycerides (mmol/L)	1.45 – 1.70	1.65	1.61 ± 0.11
Uric Acid (µmol/L)	300.20 – 398.05	355.29	350.59 ± 39.28
Zinc (µg/dl)	90.45 – 100.40	90.45	94.98 ± 4.11

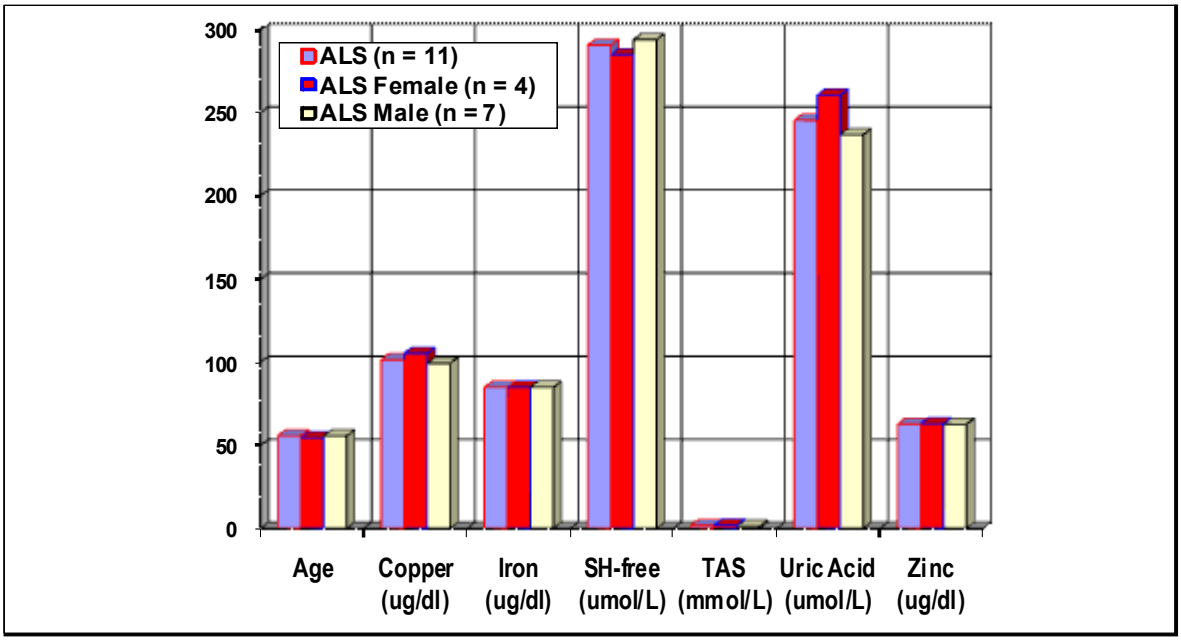

Fig. 3. Graphical expression of mean values in ALS group

Table 3. The characterisation from biochemical point of view of the females ALS group.

Specification	Amyotrophic Lateral Sclerosis F (n=4)		
	Range	Median	Mean ± SD
Age (years)	50 – 65.00	51.50	54.50 ± 7.14
Albumin (g/dL)	3.50 – 4.29	3.88	3.89 ± 0.34
Bilirubin (µmol/L)	9.70 – 13.00	12.17	11.76 ± 1.46
Cholesterol (mmol/L)	2.95 – 6.01	4.62	4.55 ± 1.25
Copper (µg/dl)	98.00 – 115.00	104.00	105.25 ± 8.62
CRP (mg/L)	7.28 – 9.12	7.83	8.01 ± 0.84
Iron (µg/dl)	80.17 – 91.00	84.86	84.86 ± 5.53
HDL-Cholesterol (mmol/L)	0.75 – 3.45	1.05	1.57 ± 1.26
LDL-Cholesterol (mmol/L)	2.64 – 3.34	2.84	2.85 ± 0.18
Phospholipids (mmol/L)	4.55 – 4.98	4.66	4.71 ± 0.20
SH – Free (µmol/L)	220.15 – 352.50	283.50	284.91 ± 67.00
TAS (mmol/L)	1.60 – 1.98	1.92	1.86 ± 0.17
Triglycerides (mmol/L)	0.51 – 2.57	1.01	1.28 ± 0.90
Uric Acid (µmol/L)	161.25 – 341.19	270.00	260.61 ± 81.12
Zinc (µg/dl)	59.05 – 66.40	62.92	62.82 ± 3.59

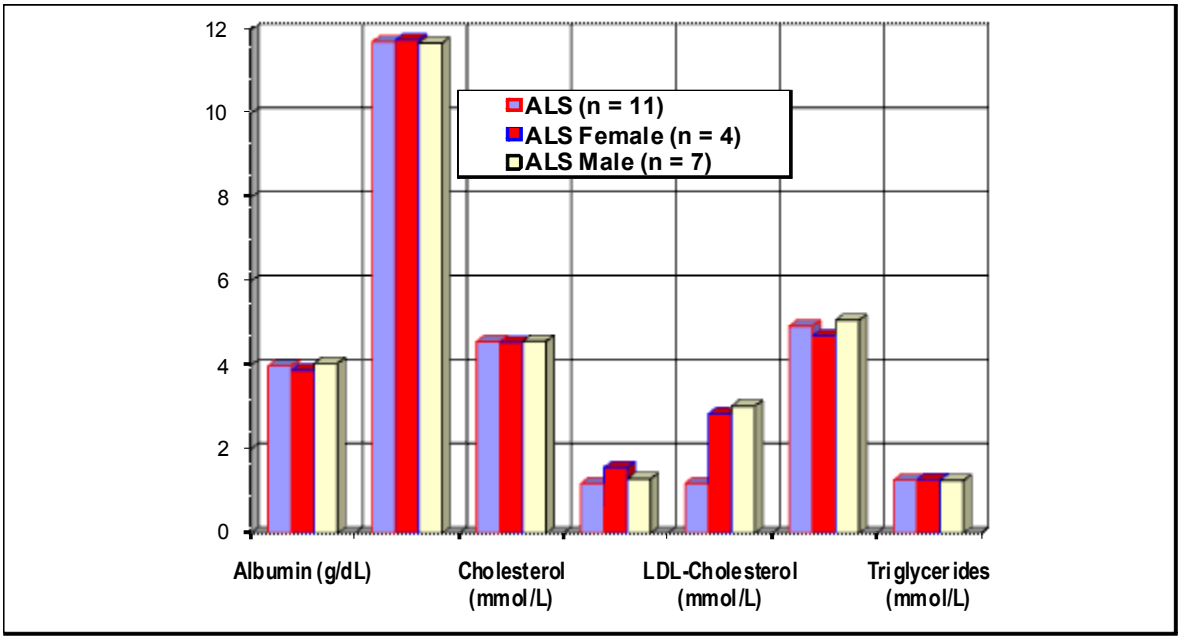

Fig. 4. Graphical expression of mean values in ALS group

Table 4.The characterisation from biochemical point of view of the males ALS group

Specification	Amyotrophic Lateral Sclerosis M (n=7)		
	Range	Median	Mean ± SD
Age (years)	31.00 – 70.00	56.00	55.57 ± 12.04
Albumin (g/dL)	3.42 – 5.00	3.97	4.05 ± 0.64
Bilirubin (µmol/L)	10.69 – 13.08	11.49	11.68 ± 0.83
Cholesterol (mmol/L)	3.65 – 5.36	4.57	4.58 ± 0.52
Copper (µg/dl)	65.00 – 120.00	99.00	99.28 ± 18.54
CRP (mg/L)	7.00 – 9.20	7.40	8.06 ± 1.00
Iron (µg/dl)	75.05 – 92.50	85.08	84.90 ± 5.88
HDL-Cholesterol (mmol/L)	0.52 – 1.60	1.32	1.30 ± 0.31
LDL-Cholesterol (mmol/L)	2.75 – 3.90	2.81	3.03 ± 0.41
Phospholipids (mmol/L)	4.95 – 5.20	5.03	5.08 ± 0.10
SH – Free (µmol/L)	224.00 – 358.00	334.00	294.03 ± 65.20
TAS (mmol/L)	1.57 – 1.95	1.85	1.82 ± 0.12
Triglycerides (mmol/L)	0.70 – 2.26	1.27	1.26 ± 0.51
Uric Acid (µmol/L)	161.25 – 364.35	228.00	236.85 ± 61.70
Zinc (µg/dl)	50.22 – 80.45	60.50	62.66 ± 9.91

Analysing our result it is possible to point out:

- There is a decrease ($p < 0.01$) of Cu, Zn, and Fe levels in serum from females affected by amyotrophic lateral sclerosis when compared with the concentrations from controls (females) on the other hand we noted an elevation of Total Antioxidant Status.
- There is a decrease ($p < 0.01$) of Cu, Zn, and Fe levels in serum from males affected by amyotrophic lateral sclerosis when compared with the concentrations from controls (males) on the other hand we noted an elevation of Total Antioxidant Status.

CONCLUSIONS

Many redox-sensitive proteins are involved in regulating apoptotic pathways, suggesting that the redox environment of the cell is important. The production of Reactive Oxygen Species (ROS), in particular, has been associated with programmed cell death in many pathological contexts including stroke, inflammation, ischemia, lung edema, and neurodegeneration.

Several chemical and physical treatments capable of inducing apoptosis are also known to generate oxidative stress. The major physiological source of ROS in mammals are the mitochondrion, where oxygen is reduced to water. A crucial event associated with the intrinsic pathway is the uncoupling of oxidative phosphorylation in the mitochondria and the dissipation of mitochondrial transmembrane potential, a decrease in ATP, and an increase in ROS.

The resultant phenotypes are concentration dependent; at relatively low levels, ROS function as signalling molecules promoting proliferation and survival, whereas higher levels of ROS are apoptotic while even higher levels are necrotic. ROS-mediated apoptosis causes disruption of the mitochondrial membrane potential and permeability transition leading to cellular dysfunction; ATP synthesis is blocked,

redox molecules including NADH, NADPH, and GSH are oxidized, and ROS levels increase. In the death receptor pathways, ROS accumulates prior to all morphological and biochemical alterations associated with apoptosis. Antioxidant treatments prevent death-receptor-mediated apoptosis.
In most cases, ROS triggers programmed cell death by oxidatively altering cellular proteins and other components or by directly activating the mitochondrial pathway.
Apoptotic effectors, particularly caspases, are redox sensitive.

The inorganic chemistry of metals is widely utilized in various biological processes such as enzyme reactions, signal transductions, electron transfer, and oxygen transport. Transition metal ions in particular play critical roles as electron transfer intermediates in various redox reactions. Organisms must acquire metals from the environment and incorporate them into metalloproteins by the post-translational addition of metal or metal-containing prosthetic groups. However, excess metal accumulation and their release in free reactive forms can be toxic. Since both deficiency and excess lead to serious problems in organisms, regulation of metal metabolism, including uptake, trafficking, assembly into metalloproteins, and detoxification, is clearly important. Recent progress in elucidating mechanisms for metal homeostasis has revealed underlying principles of metal metabolism and implicates metals in development, growth, and disease. Since disorders in metal metabolism are linked to a number of health problems, studies on metal metabolism can have important clinical implications.

Redox-active metals mediate electron transfers in various biochemical reactions. The catalytic centres of many enzymes contain Cu, Zn, Fe, heme, or iron–sulphur clusters that are essential for function. For example, energy generation by mitochondrial oxidative phosphorylation depends on Cu and heme incorporation into proteins, such as cytochrome *c* oxidase. Cu- and Zinc-containing superoxide dismutase (Cu, ZnSOD) utilizes Cu in the detoxification of $O2^{\cdot-}$. Aconitase in mitochondrial citric acid cycle is an example of a Fe–S centre enzyme. Cu-containing enzymes play essential roles in the synthesis of catecholamine, a neurotransmitter. Furthermore, haemoglobin in red blood cells carries a major portion of Fe in mammals, and oxygen transport by heme in red blood cells is essential for respiration.

Nutritional metal deficiency and genetic diseases of metal metabolism have further provided striking evidence that metals are critical trace elements in a number of other physiological processes.
While metals are essential nutrients, their excess accumulation is toxic. Transition metal ions readily catalyse reactions that result in the production of hydroxyl radicals through the Fenton and Haber–Weiss reactions.

Aberrant Cu, Zn and/or Fe metabolism is implicated in multifactorial human disorders such as neurodegenerative diseases, cardiovascular diseases, and cancer. For example, Cu has been implicated in the ethology of Alzheimer's disease, which is characterized by accumulation of *β*-amyloid (A*β*), a proteolytic product of the amyloid precursor protein (APP). APP binds Cu to reduce Cu (II) to the more reactive Cu (I). The binding of Cu to A*β* elevates A*β* aggregation. Cu is highly concentrated within senile plaques, the histopathologic hallmarks of Alzheimer's disease that are generated by the deposition of A*β*. Deposition of Fe in the brain is also a common feature of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.

Although it is not known whether Fe and Cu deposition is a cause or consequence of these diseases, Fe and Cu toxicity likely plays an important role in progression of neuronal damage observed in these diseases.

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STUDIES ON THE USE OF STERILE FROM COAL EXPLOITATION IN VIEW OF DUMPS REMEDIATION AND STABILIZATION

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ABSTRACT

Sterile dumps near mines often bring meaningful changes in the landscape, taking the size of hills. They often endanger the surroundings. By applying a suitable re-cultivation, mining land can revert to their previous uses or may gain new purposes. In addition, in mining areas the re-cultivation is made depending on the intended purpose and it can be arranged a pleasant landscape for residents. In the present paper is studied the possibility of stabilization and remediation of sterile from the mines and fly ash dumps, by cultivation with barley as bio-indicator plant. The sterile presents the characteristics of calcium peat and due to the low humus (organic matter) and macronutrients content, does not present fertilizing characteristics. It was studied its use as a basis, mixed with other wastes (fly ash) in view of their stabilization and remediation, with the final goal of their storage. The chemical analysis showed that both sterile and fly ash have a high metal content (Cr, Cd, Cu, Ni, Pb, Zn), which raises the need for application of remediation methods. For stabilization was cultivated barley on different mixtures sterile – fly ash: only sterile, 1/2 sterile + 1/2 ash, 1/3 sterile + 2/3 ash, two different layers of sterile and ash of equal heights. After bio-accumulation in barley was found that: the cultivation of the sterile (without ash) leads to high extraction degrees of heavy metals; among the sterile–ash mixtures, the highest extraction degrees were reached for the 2/3 sterile + 1/3 ash mixture; the stabilization of dumps by building layers is not recommended because the reached extraction degrees are lower; the lowest extraction degree was for nickel (~50%), and the highest for zinc (~90%).

Key words: sterile, remediation and stabilization of dumps

INTRODUCTION

Some human activities such as mining operations determine the lack of vegetation in some areas due to high levels of metals concentration. Due to their role in the living organisms, as macro and micronutrients, metals carry out very important functions. Some of the elements present in these soils are macro respectively micronutrients very important for plant growing and animal nutrition if the concentration remains under a limit value. If the concentration is higher, they can be extremely toxic for plant growing and for the animals and human health, especially heavy metals (Iovi et al., 2000; Fageria et a., 2002).

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By applying of an appropriate re-cultivation, mining areas can be reverted to their previous purposes or may gain new uses. Re-cultivation of sterile dumps depends largely on the suitability for cultivation of the lithological material resulting from excavation but also depends on how development works are carried on (Pietraru, 1982; Neag, 1997; Rădulescu, 2003; Burtică et al., 2005).

In the present paper is studied the possibility of stabilization and remediation of sterile from the mines and fly ashes dumps. At the same time for the sterile from the exploitation of coal leaching tests were performed in view of its storage in special places. A series of chemical and physical-chemical properties of the sterile were also determined in order to use it as fertilizer. The chemical analysis showed that both sterile and fly ash have a high metal content (Cr, Cd, Cu, Ni, Pb, Zn), which raises the need for application of remediation methods. For stabilization was cultivated barley as bio-indicator plant.

MATERIALS AND METHODS

In order to establish the waste class in which the sterile fits, the leaching test was performed according to the Romanian legislation published in Monitorul Oficial Nr. 194 bis (Martie 8, 2005). For this purpose sterile samples were mixed with water in two proportions: L:S = 10:1 and L:S = 2:1, respectively. The suspensions were stirred with 200 rpm for 24 h using an IKA RCT basic magnetic stirrer and then filtered.

The chemical and physical-chemical properties of the sterile were determined according to the Romanian legislation (Muntean et al., 2009).

Sterile and ash samples were mixed in different proportions, arranged in vegetation vessels, and cultivated with barley as a bio-indicator plant. The vessels were kept 30 days in laboratory, and watered regularly with drinking water. In view of chemical analysis, after 30 days the plants were harvested and the stem and the root were separated. The plant parts were dried at room temperature (20 °C) and then heated in an oven at 550°C for six hours.

In order to determine the metals content of the sterile, ash and plant parts, the samples were brought into solution by boiling until almost dry with a mixture of concentrated hydrochloric acid and concentrated nitric acid HCl : HNO_3 = 1 : 3. The residue was treated with water and filtered.

The concentration of metal ions in the solutions was determined by means of atomic absorption spectrometry, using a Varian SpectrAA 280 Fast Sequential Atomic Absorption Spectrometer with an air-acetylene flame.

RESULTS AND DISCUSSION

1. Waste class

Experimental data obtained from the leaching test are presented in Table 1. One may notice that the sterile from coal exploitation fits into the non-hazardous waste class and can be accepted for storage in specially designated waste warehouses.

Table 1. Results of the leaching test

Parameter	L/S=2:1		L/S=10:1	
	Maximum admitted value (mg/kg d.m.*)	Experimental value (mg/kg d.m.*)	Maximum admitted value (mg/kg d.m.*)	Experimental value (mg/kg d.m.*)
As	0.4	0.2	2	1.2
Cd	0.6	SLD	1	SLD
Cr_{total}	4	SLD	10	0.24
Cu	25	0.05	50	0.13
Hg	0.05	0.02	0.2	0.12
Ni	5	0.07	10	3.25
Pb	5	0.38	10	1.00
Zn	25	SLD	50	6.68
Chloride	10000	7895	15000	12456
Sulphate	10000	6897	20000	17985

* d.m. – dried material

2. Chemical and physical-chemical properties of the sterile

Experimental data regarding the chemical and physical-chemical characterization of the sterile are presented in Table 2. These data show that:

- the sterile is alkaline;
- the humus content is low and therefore the sterile is low in organic matter;
- the sterile has a high content of exchangeable bases;
- it has a low hydrolytic acidity;
- it has a medium carbonates content;
- the density of the sterile is typical for peaty soils (1,8-2 g/cm^3);
- the sterile has a low content of macronutrients (N, P, K, Ca, Mg).

Table 2. Chemical and physical-chemical properties of the sterile

Investigated parameters	Experimental value
pH	8.58
Humus (%)	0.084
Exchangeable bases (me/100 g sterile)	44.6
Hydrolytic acidity (me/100 g sterile)	0.03
Carbonate (g/kg d.m.)	20-80
Density (g/cm^3)	1.89
Moisture (%)	3.6
Total nitrogen (%)	0.07
Total phosphorus (mg/kg d.m.)	12.2
Potassium (mg/kg d.m.)	36
Calcium (g/kg d.m.)	3.78
Magnesium (mg/kg d.m.)	546

One may conclude that the sterile presents the characteristics of calcium peat, is slightly alkaline in nature and has the specific density. At the same time, due to the low humus (organic matter) and macronutrients content, the sterile does not present fertilizing characteristics. Next, we will study its use as a basis, mixed with other wastes (fly ash) in view of their stabilization and remediation, with the final goal of their storage.

3. Heavy metals content of sterile and fly ash

Experimental data regarding the initial heavy metals content of the sterile and fly ash are presented in Table 3. Data show that both sterile and fly ash have a high metal content, which raises the need for application of remediation methods.

Table 3. Initial heavy metals content of the sterile and fly ash

Metal	Maximum admitted value (mg/kg d.m.)	Heavy metal content (mg/kg d.m.)	
		Sterile	Fly ash
Cr	30	28.8	92.1
Cu	20	91.9	24.0
Cd	1	22.1	5.79
Ni	20	260	83.6
Pb	20	269	93.0
Zn	100	1502	65.3

4. Studies regarding the remediation and stabilization of sterile and fly ash through bio-accumulation

For the studies, the bio-indicator plant (barley) was cultivated on different mixtures sterile – fly ash: only sterile, 1/2 sterile + 1/2 ash, 1/3 sterile + 2/3 ash, two different layers of sterile and ash of equal heights.

The mass loss of the plant parts (root, stem) during thermal treatment at 550 °C was between 30 and 80%. After analyzing the plant parts, the highest content of copper, chromium, nickel and zinc was found in the roots as a result of their absorption capacity, and the highest lead and cadmium content was found in the stems.

Figure 1 presents the experimental data regarding the extraction degree of heavy metals from the sterile and fly ash mixtures after bio-accumulation in barley.

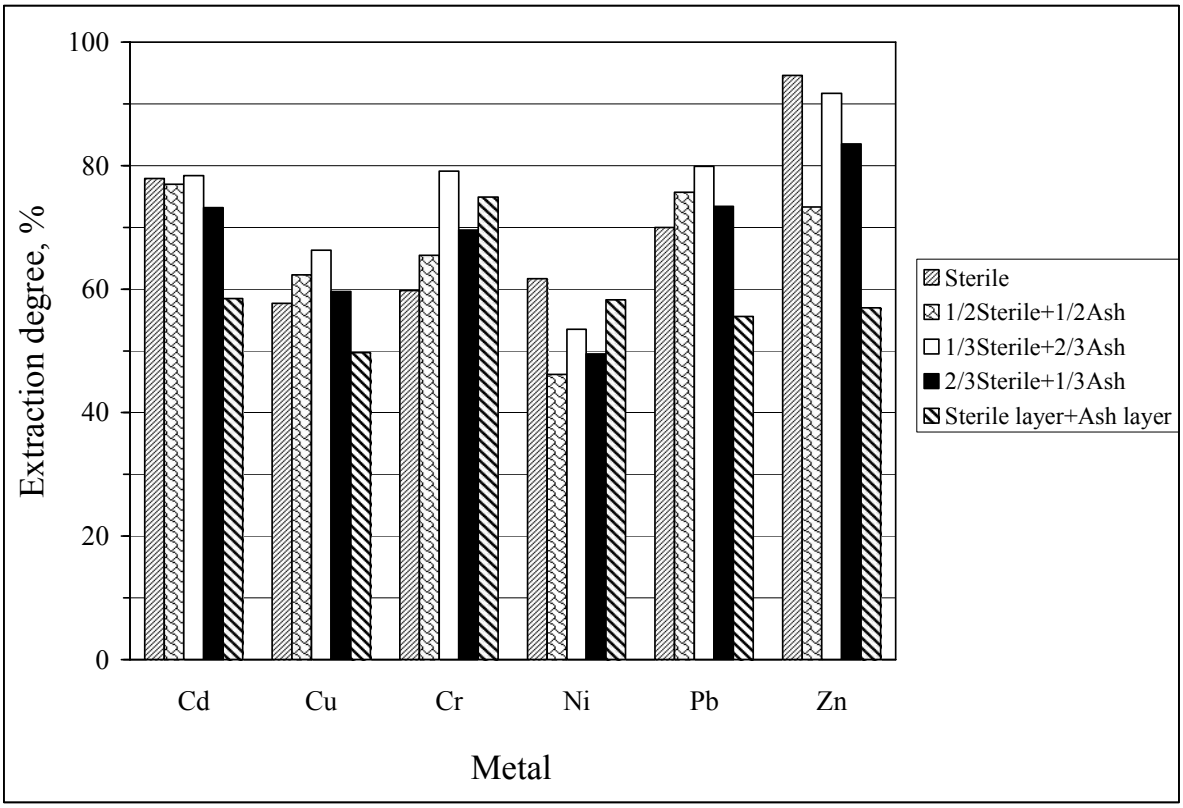

Fig. 1. Extraction degree of heavy metals from the sterile and fly ash mixtures

Experimental data show that:

- the cultivation with the bio-indicator plant of the sterile (without ash) leads to high extraction degrees of heavy metals;
- among the sterile–ash mixtures, the highest extraction degrees were reached for the 2/3 sterile + 1/3 ash mixture;
- the stabilization of dumps by building layers is not recommended because the reached extraction degrees are lower;
- the lowest extraction degree was for nickel (~50%), and the highest for zinc (~90%).

CONCLUSIONS

The present paper present studies on the possibility of stabilization and remediation of sterile from the mines and fly ash dumps, by cultivation with barley as bio-indicator plant.

The sterile from coal exploitation was submitted to the leaching test and was found that it fits into the non-hazardous waste class and can be accepted for storage in specially designated waste warehouses. The sterile presents the characteristics of calcium peat and due to the low humus (organic matter) and macronutrients content, does not present fertilizing characteristics. It was studied its use as a basis, mixed with other wastes (fly ash) in view of their stabilization and remediation, with the final goal of their storage.

The chemical analysis showed that both sterile and fly ash have a high metal content (Cr, Cd, Cu, Ni, Pb, Zn), which raises the need for application of remediation methods. For stabilization was cultivated barley on different mixtures steril –fly ash:

only sterile, 1/2 sterile + 1/2 ash, 1/3 sterile + 2/3 ash, two different layers of sterile and ash of equal heights.

After cultivation, the plant parts (roots, stem) were analyzed. The highest content of copper, chromium, nickel and zinc was found in the roots, and the highest lead and cadmium content was found in the stems.

The experimental data regarding the extraction degree of heavy metals from the sterile and fly ash mixtures after bio-accumulation in barley showed that: the cultivation with the bio-indicator plant of the sterile (without ash) leads to high extraction degrees of heavy metals; among the sterile–ash mixtures, the highest extraction degrees were reached for the 2/3 sterile + 1/3 ash mixture; the stabilization of dumps by building layers is not recommended because the reached extraction degrees are lower; the lowest extraction degree was for nickel (~50%), and the highest for zinc (~90%).

One may conclude that the stabilization and the remediation of the sterile and fly ash through bio-accumulation of heavy metals in bio-indicator plants has led to good results. The method can be applied widely, but it requires at least 2 consecutive years of intensive cultivation.

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HOMEOSTASIS CHANGES INDUCED BY *CIS*-PLATINUM ON THE SERUM NON-PROTEIN NITROGENOUS METABOLITES IN EXPERIMENTAL ANIMALS

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ABSTRACT

The effects of cis-diamminedichloroplatinum (noted cis-platinum or cDDP) on the homeostasis of non-protein nitrogenous metabolites, i.e. urea, creatinine and uric acid were studied. Among these metabolites in pregnant female rats blood serum urea, creatinine and uric acid were determined while in amniotic fluid and in the fetuses pooled blood serum only urea and creatinine. Cis-platinum, an oncostatic drug, was injected intraperitoneally in female rats on day 14 of pregnancy (2.5 and 5.0 mg/ kg body weight) and its effects were studied on day 20 of pregnancy both in mothers and fetuses, when the pregnant animals were killed. Homeostasis changes were revealed with respect to blood non-protein nitrogenous metabolites. In maternal blood serum the increase of urea and creatinine as well as the decrease of uric acid concentration was observed. The concentration of urea and creatinine in the amniotic fluid and pooled fetal blood serum showed also an increase, but less marked than in the maternal blood samples. These data reveal a dyshomeostasis as a consequence of disturbances in protein metabolism which affect also the non-protein nitrogenous metabolites. The effect may be correlated with the known toxicity of cDDP.

Key words: *cis*-platinum effects; homeostasis changes; serum non-protein nitrogenous metabolites

INTRODUCTION

In the antitumoral chemotherapy there are used various chemical substances such as: alkylating agents, e.g. cyclophosphamide, chlorambucil, nitrosourea a.o.; antimetabolites, e.g. 5-flurouracil, 6-mercaptopurine, methotrexate a.o.; steroid hormones, e.g. estrogens and androgens; antibiotics, e.g. actinomycin D, bleomycin

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a.o.; alkaloids, e.g. vincristine, vinblastine a.o. (Salmon and Apple, 1976; Waring, 1981; Garban et al., 1987).

During the investigation of the effects of platinum coordination complexes in electric field on bacterial growth (Rosenberg et al., 1969) a new class of chemotherapeutics was discovered. Among these platinum based chemotherapeutics cis-platinum is the most important both from chemical and pharmacological point of view. It is an inorganic coordination compound synthetized by Peyrone in 1845 (cited by Theophanides, 1980). The separation of cis- and trans-isomers was made by Werner in 1889 (cited by Riley and Sternson, 1985).

Studies on the antitumoral action of *cis*-platinum (cDDP) imply not only the knowledge of pharmacokinetic, biochemical but also toxicological aspects (Slater et al., 1976; Reedijk et al., 1984; Garban et al., 1988).

It is known that the cytostatic drugs beside their main positive effect on neoplasic tissues have toxic activity on various organs among which kidneys. In this context present paper deals with the in vivo experiments concerning cis-platinum action on the serum non-protein nitrogenous metabolites (i.e. urea, creatinine and uric acid) homeostasis in pregnant female rats, amniotic fluid and in fetuses.

MATERIALS AND METHODS

The experimental model. Experiments were performed on nulliparous female rats (Wistar strain) maintained in pathogen-free conditions, at 22-25^{o}C room temperature, at 55-65% realative air humidity and weighing 200+10 g as well as on their fetuses. The experimental animals were fed on normal rhythm and standard breeding foos and water. After mating, the pregnant animals were divided in three groups: one control group (C) injected intraperitoneally (i.p.) on day 14 of pregnancy with physiological saline and two experimental groups (E_1 and E_2) injected intraperitoneally (i.p.) with cis-platinum in solution containing cis-platinum in doses of 2.5 and 5.0 mg/kg b.w. respectively (on the same day). Each group consisted of 10 animals.

The pregnant animals (control and treated) were killed on day 20 of pregnancy between 8.00 - 9.00 a.m. - important condition for the chronobiological rhythm of metabolic processes (Hrushesky and Bjarnason, 1983; Gârban et al., 1986).

After Ketanest narcosis and laparatomy the uterine horns were opened and the conceptuses exteriorized. Amniotic fluid was extracted by the puncture of bulging amniotic sac. Fetal blood samples were obtained by heart micropuncture using a fine glasspipette. As the amount of blood obtained from individual puncture was rather small, blood samples of a litter was pooled. Fetal blood serum was collected only from the offsprings of 6 mothers from each group (C, E_1, E_2). The number of pregnant female rats was noted by n_1 and of the fetuses n_2. In the second step blood samples were collected from the mothers by puncture of the vena cava caudalis for biochemical determinations. General data on the experimental conditions are given in Table 1.

Biochemical investigations. From the maternal blood serum, amniotic fluid and pooled fetal blood serum (from the fetuses of 6 pregnant females from each group) urea and creatinine were determined. Urea was determined by enzymatic method with urease and creatinine by spectrophotometric method with picric acid (Jaffé's reaction). Uric acid, only from maternal blood, by Heilmayer method with phosphotungstic reagent was dosed.

Table 1. Synopsis of experimental design

Group	No.of pregnant females	No.of conceptuses		Adm. subst.	Dosage mg/kg b.w.	Way of admin.	Day of pregnancy	
		Total	Living				Treatm.	Killing
C	10	74	73	physiol. saline	-	i.p.	14	20
E_1	10	85	81	cDDP active subst. in sol.	2.5	i.p.	14	20
E_2	10	91	76		5.0	i.p.	14	20

Statistical evaluation. Mean values, standard deviation (SD) and t_{exp} by t-test (Student) were established. For calculating statistical parameters we used the methods of the classic mathematic statistics. Finally having t_{exp} (and using t_{calc}) confidence probability (P) for various experimental data were determined.

RESULTS AND DISCUSSIONS

According to literature data, renal, gastrointestinal and neural toxicity are the main toxic side effects induced by cDDP (Slater et al., 1977; Van der Vijgh et al.,1983), being considered the main cause of lethality.

Knowing that Pt accumulates in the kidney cortex and the medulla both in animals (Van der Vijgh et al., 1983) and in humans (Stewart et al., 1985) the determination of non-protein nitrogenous metabolites were studied in this research in order to obtain clearer data concerning the pharmacological effects on the kidney function.

Our results regarding serum urea, creatinine and uric acid in pregnant female rats are given in Table 2 .

Table 2. Non-protein nitrogenous compounds in blood serum of pregnant female rats

Specification	n	Urea (mg %)	Creatinine (mg %)	Uric acid (mg %)
		X ± SD	X ± SD	X ± SD
C	10	36.10 ± 4.01	1.02 ± 0.07	2.73 ± 0.23
E_1	10	40.60 ± 5.13	1.16 ± 0.19	2.52 ± 0.33*
$\Delta X_1 = X_C - X_{E1}$		+ 4.50	+ 0.14	- 0.21
E_2	10	46.70 ± 5.67*	1.28 ± 0.23*	2.28 ± 0.21*
$\Delta X_2 = X_C - X_{E2}$		+ 10.60	+ 0.26	- 0.39

* $0.95 < P < 0.99$

Values of urea and creatinine in amniotic fluid and shown in Table 3.

Table 3. Non-protein nitrogenous compounds in amniotic fluid of pregnant female rats

Specification	n	Urea (mg %)	Creatinine (mg %)
		X ± SD	X ± SD
C	10	35.45 ± 3.05	0.95 ± 0.05
E_1	10	38.70 ± 5.16	1.05 ± 0.12
$\Delta X1 = X_C - X_{E1}$		+ 3.25	+ 0.10
E_2	10	43.10 ± 4.98*	1.09 ± 0.15*
$\Delta X2 = X_C - X_{E2}$		+ 7.55	+ 0.12

* $0.95 < P < 0.99$

Our findings evidence an increase of serum urea and creatinine in E_1 and E_2 groups, direct proportional with the increasing administered dose. This augmentation attests the nephrotoxic effect of cDDP. In previous experiments (Gârban et al., 1987) the same dose of cDDP induced a more marked increase of both metabolites., a difference possibly due to a larger period of action (between days 7-20 of pregnancy), a more severe lesion of the renal function. The observed homoestasis changes evidenced that the effect of cDDP is time-dose dependent. As to the uric acid concentration a decrease in the experimental groups were observed.

Creatinine is a breakdown product of creatine - an important component of muscle. Creatinine can be converted to the ATP molecule, which is a high-energy source. The daily production of creatine and subsequently creatinine, depends on muscle mass, which fluctuates very little.

Urea is a substance secreted by the liver, in the urea cycle as a waste product of the digestion of protein and removed from the blood by the kidneys. Urea itself is not a toxic substance. However, BUN is a marker for other nitrogenous waste.

The most common cause of an elevated serum urea and creatinine concentration is poor kidney function, although a serum creatinine level is a somewhat more specific measure of renal function.

Literature data mention the increase of blood urea nitrogen - BUN in rats (Slater et al., 1977) and of serum creatinine in humans (Steward et al., 1985; Corder et al., 1977) under the action of cDDP.

A peculiar aspect was revealed by the urea and cretinine content in the amniotic fluid of the pregnant females. Their values were increased in treated experimental animals (significant in group E_2), but this increase is less marked than in the blood serum.

According to Gresham et al. (1972) and Boylan et al. (1985) the fetal urine, one of the contributors to amniotic fluid, shows a higher urea concentration than the fetal plasma (determinations made on guinea pigs, sheeps and lambs).

Analytical data concerning the urea and creatinine concentration in the pooled fetal serum are presented in Table 4.

Table 4. Non-protein nitrogenous compounds in pooled blood serum of fetuses

Specification	n_1/n_2	Urea (mg %)	Creatinine (mg %)
		X ± SD	X ± SD
C	5/45	33.25 ± 3.39	0.96 ± 0.04
E_1	5/45	36.10 ± 4.86	1.05 ± 0.08
$\Delta X_1 = X_C - X_{E1}$		+ 2.85	+ 0.09
E_2	5/45	38.30 ± 6.77*	1.07 ± 0.11*
$\Delta X_2 = X_C - X_{E2}$		+ 5.05	+ 0.11

* $0.95 < P < 0.99$

Note: n_1 – number of mothers; n_2 – number of fetuses

As seen in groups E_1 and E_2, both urea and creatinine are significantly augmented. The dose-effect relationship reveals a direct proportionality. In the case of fetuses it is known that in early pregnancy the various metabolites, precursors of anabolic processes specific in fetal developmen, which underwent previously the action of cDDP, are transported to the placenta by maternal blood. The transfer of metabolites in the moment of our experiments (day 14 of pregnancy) is realised mainly through the chorioallantoic placenta while in advanced pregnancy cis-platinum crosses the placental barrier.

According to Longo – cited by Assoli (1972), the nitrogenous end products of amino acid and protein metabolism including urea and creatinine are present in almost equal concentrations in maternal and fetal blood serum, indicating no active transfer or marked barrier to diffusion of these substances. In this study the lower values of fetal serum urea and creatinine as compared to values found in maternal blood may be explained by the decrease of total serum proteins, due to cDDP action on DNA synthesis (one of the possible sources of metabolites) and by the relatively lower rate of catabolic processes in the developing fetal organism.

The biochemical dyshomeostasis evidenced by our analytical data reveals the renal injury manifested by changes in protein metabolism.

CONCLUSIONS

1. Non-protein nitrogenous compounds in pregnant female rats serum revealed a dyshomeostasis consisting in increased serum urea and creatinine concentration in treated animals. Uric acid from maternal blood serum showed decreased values. Among analytical values (increased or decreased) and the concentration of the administered cDDP there is a direct proportional relationship.

2. In the amniotic fluid the concentrations of urea and creatinine were also increased, but more diminished than that in the maternal blood serum.

3. In fetuses - at day 20 of gestation of rats - the non-protein nitrogenous metabolites (urea and creatinine) from pooled fetal serum revealed augmented values. The obtained data attest that cis-platinum produce renal injuries.

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SPECTROPHOTOMETRIC DETERMINATION OF IRON IN SOIL SAMPLES BY STANDARD ADDITION METHOD

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ABSTRACT

A spectrophotometric method using 1, 10 - phenanthroline has been used for the determination of iron mobile forms concentration in soil. In order to increase the sensitivity of the analytical determination, we used the method of the standard addition. The method of the standard addition may be applied when the chemical compounds to be determined are in low concentration and the chemical matrix is not constant in the samples to be analyzed, like in natural waters and biological fluids. The soil extracts were prepared using four different extractants: 0.05 M EDTA solution, water, 0.01 N $CaCl_2$ solution, and Morgan reagent. The method has been shown to be inadequate for the determination of total iron in soil, because of the low pH of extracts. The method is simple, sensitive, selective, inexpensive, and can be used for determination of iron in soil samples. The results were in good agreement with those obtained using atomic absorption spectroscopy for neutral and slight basic soil extracts.

Key words: standard addition method, iron, soil, molecular absorption spectrometry

INTRODUCTION

Iron is the fourth most abundant element on earth, mostly in the form of silicates, oxides and hydroxides, forms that are not readily available for plant use (Cuerinot and Yi, 1994). The content of iron in the soil varies from a fraction of per cent to several per cent, and its concentration and distribution depend largely on the type of soil (Jankiewick and Ptaszynksky, 2002).

In soil, iron exists as ferrous and ferric ions; the form in which predominates depend on the pH, organic matter and the aeration of the soil. The reduced valence state prevails under low oxygen supply and relatively higher moisture level conditions, while the oxidized valence state prevails under oxidizing conditions. At pH values common in soils, the oxidized state form highly stable complexes with organic matter (Kidanu *et al*., 2009). In alkaline soils, short-term anoxia leads to the reduction of Fe(III) to Fe(II), form which is more readily available for plant uptake than Fe(III) (Zudu-Sasse and Schaffer, 2000; Cuerinot and Yi, 1994). Both excess and deficiency

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of iron in the soil adversely affects plant growth and development (Bartholomeus et al., 2005).

A broad variety of analytical techniques have been used to determine trace and essential elements in soils and environmental samples: atomic absorption spectrometry with flame (FAAS) and graphite furnace (GFAAS), inductively coupled plasma optical emission spectrometry (ICP-OES), mass spectrometry (ICP-MS), etc. (Bartholomeus et al., 2007; Olatunji, 2008; Kara et al., 2009). Among other methods of analysis, molecular absorption spectroscopy in the visible may be also used for determining iron in soil samples (Ahmed and Roy, 2009). Spectrophotometry is essentially a trace analysis technique and is one of the most powerful tools in chemical analysis.

The method of standard addition (SAM) is used in instrumental analysis to determine concentration of a substance in an unknown sample by comparison to a set of samples of known concentration, similar to using a calibration curve. Standard addition can be applied to most analytical techniques and is used instead of a calibration curve to solve the matrix effect problem. The standard additions method is used to determine the concentration of an analyte that is in a complex matrix such as biological fluids, soil samples, etc. (Harris, 2003)

Previous we improved and utilized the molecular absorption spectroscopy in the visible using standard addition method for the determination of some metals in water samples (Dumbrava and Birghila, 2009).

The aim of this work was to develop the standard addition method as an accessible, reproducible and reliable analytical procedure for the determination of iron in soil, an alternative for more expensive methods. The method was tested on neutral and slight basic soil extracts (obtained using as extractans EDTA solution, water, $CaCl_2$ solution, Morgan reagent) for determination of iron mobile forms. We tried to determine also the total iron concentration but, because the pH of extracts, some of the extracts components precipitated during the extracts processing and the results were not reproducible.

To evaluate the molecular absorption spectrometry (using standard addition method) as a method for determination of iron mobile forms in soils, the results obtained with this method were compared with those obtained by flame atomic absorption spectrometry (FAAS).

MATERIALS AND METHODS

1. *Soil sampling and extraction.* The untreated and unpolluted soil samples were collected from a private garden (0 - 10 cm depth) of Constanta, Romania.

The samples were thoroughly mixed, air - dried under room temperature and ground to pass a 2 mm sieve. For determining total iron content, the samples were mineralized with a mixture of concentrated HNO_3 and HCl 1:3. In order to establish the mobile iron concentration, we used four different extractants: 0.05 M disodium EDTA solution (at pH=7.0 with NH_3), distillate water, 0.01 M $CaCl_2$ solution, and Morgan reagent (CH_3COONa + CH_3COOH solution, pH = 4.8) (Dumbrava *et al.*, to be published).

All chemicals were of analytical reagent grade.

2. *Determination of iron concentration in soil extracts.* Iron concentration in aqueous extracts was measured using the 1,10 – phenantroline reaction, based on the coloured complex formed by Fe(II) in the presence of 1, 10 - phenantroline. The colour was fully developed after 20 min and absorbance was measured at 510 nm in a 1 cm long quartz cuvette using a Jasco V 550 spectrophotometer.

1, 10 – phenantroline is a chelating agent and in a neutral or slightly alkaline medium forms with iron(II) a orange-red complex ion (Tesfaldet and Staden, 2004). The hydroxylamine hydrochloride was used to reduce the Fe(III) to Fe(II). The reaction occurs in the presence of the sodium acetate, which maintains the solution pH between 6 and 9, in order to prevent the precipitation of cations in the form of hydroxides.

All the analyses were made by triplicate, the mean values being reported.

RESULTS AND DISCUSSIONS

The iron concentrations in the analyzed soil samples and the parameters obtained using standard addition method are presented in Table 1.

Table 1. Iron concentration in the soil and different parameters obtained using standard addition method.

Extractant used	concentration (mg Fe/kg dry mass)	concentration (mg Fe/100mL)	$\bar{x}$	S.D.	R.S.D. (%)
H_2O	48.26	0.04826	0.04826	0.00043	0.89
EDTA	66.30	0.06630	0.06630	0.00003	0.45
$CaCl_2$	2.88	0.02880	0.02880	0.00029	2.01
Morgan reagent	3.56	0.03560	0.03560	0.00031	1.74

The precision of the standard addition method was evaluated by determining different concentrations of iron (each analyzed n = 10 times). The relative standard deviation (n = 10) was 2% - 0.5%, indicating that this method is precise and reproducible. The precision in terms of relative standard deviation of the present method are very reliable for the determination of iron in real samples down to mg/kg levels in soil.

As we can see, the amount of iron depends on the extractant used, the levels being between 2.88 mg/kg dry soil (for extraction with $CaCl_2$ solution) and 66.30 mg/kg dry soil (for extraction with NH_4-EDTA solution). The results are in good agreement with the theoretical approach; using EDTA as extractant, iron concentration (iron mobile forms) is higher, probably because its ability to mobilize metal cations more efficiently than water. The $CaCl_2$ solution and Morgan reagent were used for the extraction of bioavailable iron, which is only a fraction of iron mobile forms.

The results obtained using addition standard method were compared with that determined by atomic absorption spectrometry (Table 2).

Table 2. Comparison between iron concentration in soil samples determined by spectrophotometric (standard addition method) and atomic absorption techniques.

Extractant	Iron content (mg/kg dry mass)	
	Spectrophotometric method	Atomic absorption
water	48.26	56.13
EDTA	66.30	75.81
$CaCl_2$	2.88	3.69
Morgan reagent	3.56	4.62

In all analyzed extracts the content of iron determined by the spectrophotometric method was lower than the content determined by atomic absorption spectrometry method. This fact may suggest that Fe(III) was not entirely reduced to Fe(II), especially if it is found in coordinative compounds. The small quantities of iron extracted with $CaCl_2$ solution and Morgan reagent requires an improvement of spectrophotometric method for bioavailable iron determination.

CONCLUSIONS

The determination of iron mobile forms by spectrophotometric method, using the standard addition may be a good alternative for more sophisticated and expensive methods.

The iron mobile forms concentrations determined using spectrophotometric method are lower (with 10-22%) than obtained with atomic absorption spectrometry. The highest differences appear for the bioavailable iron, extracted with $CaCl_2$ 0.01 M solution and Morgan reagent, probably because of the low values of concentrations; the spectrophotometric method should be optimized for determination of bioavailable iron in soil samples.

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INFRARED SPECTRA OF SOME FIRST ROW METAL COMPLEXES, CONTAINING 1, 4-BIS (3-AMINO-PROPYL)PIPERAZINE (L') AS LIGAND.

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ABSTRACT

In the field of coordination chemistry the Schiff bases represents a very usefull class of compounds due to their versatility to act as ligands with metal ions. The characteristic bond of the Schiff bases, C=N, has reversible nature which allows by hydrolysis, obtaining the initial corresponding aldehyde and amine compounds (Boiocchi et al., 2007; Nenitescu, 1966; Becker et al., 1982; Hendrickson et al., 1976; Cseh et al., 2008; Mukherjee et al., 2009). Present study reveals that during the reaction of the Schiff base ligand N, N'-bis[3(4-dodecyloxy-benzylideneamino)-propyl]-piperazine (L) with the copper and nickel perchlorate, the imino bond is destroyed. In order to explain this behaviour, the influence of the metal ions nature was studied. The nature of the obtained complexes $CuL'(ClO_4)]\cdot ClO_4\cdot 4H_2O$*(1) and* $[NiL'(ClO_4)]\cdot ClO_4\cdot H_2O$*(2) has been established on the basis of results of elemental analysis, atomic absorption spectroscopy (AAS), electric molar conductivity, electronic (UV-Vis) and infrared (IR) spectroscopy (Bucovicean et al., 2010). In this paper we report only infrared spectra of the copper(II) (1) and nickel(II) (2) complexes containing (L') as ligand, where L'=1,4-Bis(3-aminopropyl)piperazine, stressing the importance of information brought by this spectral method.*

Key words: Schiff base ligand; imino bond cleavage; copper(II) complex; nickel(II) complex; 1,4-Bis(3-aminopropyl)piperazine; perchlorate counter anion;

INTRODUCTION

Infrared spectroscopy is one of the classical methods for structure determination of molecules. This standing is due to its sensitivity to the chemical composition and architecture of molecules (Barth, 2007). Fourier transform infrared (FTIR) spectrometry is a well established technique that provides highly specific molecular information of a wide range of compounds used in different fields (Kuligowski et al., 2008). In recent years, it becomes more and more that the reports about FT-IR is used in traditional Chinese medicine on some aspects, such as identifying counterfeits, controlling qualities, forecasting stability, etc (Liu et al., 2006). The high information content in an infrared spectrum carries over also to biological systems (Barth, 2007).

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The piperazine nucleus is often found embedded in chemotherapic agents exhibiting a wide range of biological activities (anthelmintic, antiprotozoal, bactericidal, fungicidal, antiviral, and antitumour properties (Filosa et al., 2007). Nanomolar concentrations of some piperazine derivatives were discovered to inhibit acute human immunodeficiency HIV virus infections and suppress the production of virus from chronically and latently infected cells containing integrated proviral (Balaban et al., 2008). Among these, Cu^{2+} cation is an essential trace element for humans, and this ion plays an important role in the maintenance of homeostasis in living organisms. An alteration in its cellular homeostasis is connected to serious neurodegenerative diseases, such as Menkes and Wilson diseases, familial amyotropic lateral sclerosis, Alzheimer's disease, and prion diseases (Li et al., 2009). We describe here infrared spectra of new copper(II) (1) and nickel(II) (2) complexes containing 1,4-bis(3-aminopropyl)piperazine as the ligand, obtained as a result of hydrolytic cleavage of the imino bond of Schiff base N,N'-bis[3(4-dodecyloxy-benzylideneamino)-propyl]-piperazine by complexation. Also we study the influence of metal ions on the stability of Schiff base N,N'-bis[3(4-dodecyloxy-benzylideneamino)-propyl]-piperazine.

MATERIALS AND METHODS

All the reagents and solvents were purchased from Merck, Aldrich, and used without further purification.

The complexes characterization was performed by elemental analysis, electric molar conductivity, UV-VIS and IR spectroscopy (Bucovicean et al., 2010).
IR spectra were recorded in KBr pellets on a Jasco FT/IR-430 spectrophotometer, in the 400-4000 cm^{-1} range.

Synthesis

Both copper(II) (1) and nickel(II) (2) complexes have been obtained using the following recipe: A solution of $M(ClO_4)_2.6H_2O$ (0.1 mmol) dissolved in 5 ml tetrahydrofurane was added to a solution containing the Schiff base N,N'-bis[3(4-dodecyloxy-benzylideneamino)-propyl]-piperazine (L) (0.1 mmol) dissolved in 10 ml tetrahydrofurane. The mixture was refluxed for 4h, when a precipitate was formed. The solid formed was filtered, washed with tetrahydrofurane an dried at room temperature. Yield: 73-81 % (Bucovicean et al., 2010).

RESULTS AND DISCUSSIONS

Starting from Shiff base ligand N, N'-bis[3(4-dodecyloxy-benzylideneamino)-propyl]-piperazine (L), and metallic salts: copper(II) and nickel(II) perchlorate hexahydrate, the formation of a rigid core by complexation of the aminic groups failed due to the destruction of the imino bond. Thus the complexes $CuL'(ClO_4)]\cdot ClO_4\cdot 4H_2O$(1) and $[NiL'(ClO_4)]\cdot ClO_4\cdot H_2O$(2) containing 1,4-Bis(3-aminopropyl)piperazine (L') were obtained (Bucovicean et al., 2010).

Complex compounds and related reactions are sketched in Fig. 1

Examination of the infrared spectra of the complexes (1) and (2) shows the characteristic bands of the 1,4-bis(3-aminopropyl)piperazine (L') ligand. However some changes demonstrate the coordination to the metal ion. Thus, in the spectrum of complex (1), the bands assigned to the ν(NH) stretching asymmetric and symmetric vibrations of NH_2 group, are shifted towards lower frequencies at 3313 and 3268 cm^{-1}, compared to the free ligand (3369 and 3283 cm^{-1}).

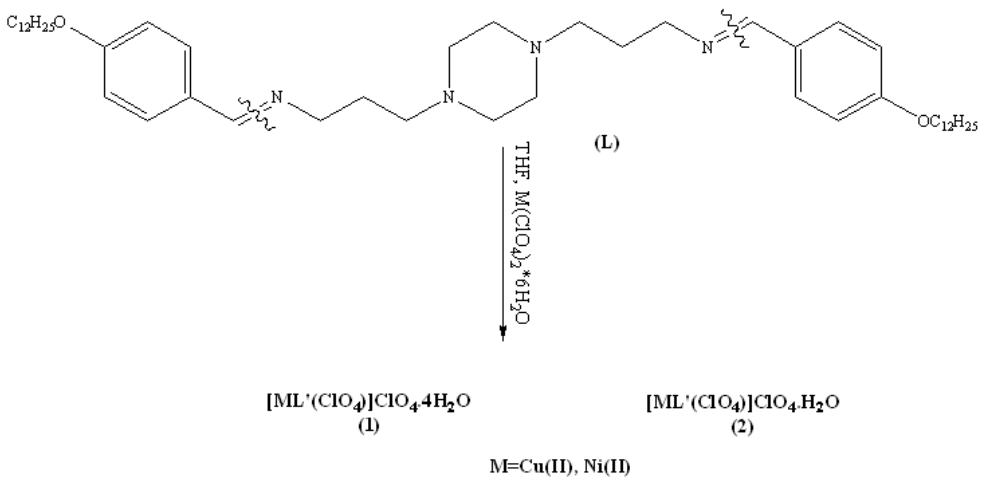

Fig. 1 Reagents and conditions: (1) N, N'-bis[3(4-dodecyloxy-benzylideneamino)-propyl]-piperazine (L), tetrahydrofurane solution (THF), $Cu(ClO_4)_2 \cdot 6H_2O$, $Ni(ClO_4)_2 \cdot 6H_2O$, 1,4-Bis(3-aminopropyl)piperazine (L') (Bucovicean et al., 2010).

In the infrared spectrum of complex (2) can be also observed the ν(NH) bands shifted towards lower frequencies, namely at 3297, 3175 cm^{-1} (Medeleanu and Milea, 1998; Boiocchi et al., 2004). The shift of these bands towards lower frequencies compared to that of the free ligand, indicates their involvement in coordination of the metal center. The bands attributable to ν(C-H) of N-CH_2 modes of the piperazinic moiety disappear, in the case of both the complexes (1) and (2) suggesting the involvement of piperazine nitrogen atoms in coordination of the metal ion, when acts as a tetradentate ligand and adopts a *bath* conformation (Medeleanu and Milea, 1998). The relatively weak bands around 3000 cm^{-1} observed in the spectra of complexes (1) and (2) are assigned to the ν(CH_2) mode of the propylene groups.

The metal-nitrogen vibrations can be assigned at 648 cm^{-1} and 690 cm^{-1}, respectively, for complexes 1 and 2 (Nakamoto, 1963).

The strong absorption bands at 1099, 1023, 914 cm^{-1} and 621 cm^{-1} are attributable for monodentate and ionic perchlorate groups, for complex (1). In the case of complex (2) the bands attributable for bidentate and ionic perchlorate can be observed at 1268, 1142, 1087, 941 cm^{-1} and 1108 cm^{-1} (Medeleanu and Milea, 1998; Nakamoto, 1963; Cotton, 1972).

The broad bands at 3734, 3617 cm^{-1}(for 1) and 3735, 3614 cm^{-1} (for 2) in the spectra of complexes (1) and (2) can be attributed to stretching vibrations (ν_{OH}) of water molecules (Medeleanu and Milea, 1998).

CONCLUSIONS

1. Infrared spectra of the obtained compounds confirm the imino bond cleavage of Schiff base ligand N,N'-bis[3(4-dodecyloxy-benzylideneamino)-propyl]-piperazine (L) and formation of the copper(II) (1) and nickel(II) (2) complexes containing 1,4-bis(3-aminopropyl)piperazine as the ligand.

2. In both cases, infrared spectra show that the ligand L' acts as a tetradentate, through N_2 set of donor atoms of piperazine and one or two oxygen atoms from perchlorate group, when adopts a *bath* conformation. Their physico-chemical characterization, indicates the obtaining of two new mononuclear complexes.

3. The present study shows that the metal ions nature is not responsable for thehydrolitic cleavage of the Schiff base N, N'-bis[3(4-dodecyloxy-benzylideneamino)-propyl]-piperazine. We assume that the presence of the trace amounts of water arising from copper(II) and nickel(II) perchlorate hexahydrate destabilises the imino bond with the formation of the complexes $[CuL'(ClO_4)]\cdot ClO_4\cdot 4H_2O$ (1) and $[NiL'(ClO_4)]\cdot ClO_4\cdot H_2O$ (2) where L' =1,4-Bis(3-aminopropyl)piperazine.

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DETECTION AND CHARACTERIZATION OF FREE RADICALS IN SOME GAMMA IRRADIATED DRUGS AND FOODS BY EPR SPECTROSCOPY

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ABSTRACT

Electron Paramagnetic Spectroscopy (EPR) was used to investigate the γ-radiation damage in the microcrystalline powder form of some drugs as antiemetics (Metoclopramide and Odansetron), cytostatics (Mercaptopurine and Azathioprine), beta blockers (Atenolol, Pindolol), some foodstuffs spices (black and white pepper and sweet and hot pepper) and dried fruits (banana and pineapple). The absorbed dose of drugs was 15 kGy corresponding to an average dose used in the sterilization process ESR measurements proved that both of them contained various stable paramagnetic species after irradiation. Some spectroscopic properties and suggestions concerning possible structure of the radicals are discussed in this paper

Key words: EPR, antiemetics, cytostatics, beta blocker, spices, dried fruits

INTRODUCTION

The study of free radicals induced by high-energy ionizing radiation in drugs and foodstuffs by Electron Paramagnetic Resonance (EPR), are increasing due to applications in the medical sterilization and hygienic quality of foods (Farkas, 1989; Saint-Lebe and Rafii, 1995; Barbarin et al., 1996). If the free radicals are produced exclusively by radiolysis, and it is relatively stable with respect to time, then it can be used as indicators for radiation exposure, as long as it is possible to distinguish this category of free radicals, from other signals in the EPR spectra.

The main aspect of the irradiation with gamma radiation is the tolerance of the product with radiation. During use of this type of radiation, high-energy photons bombard the product, causing electron displacement within giving rise to free radicals. Chemically (Rosenthal, 1993), free radicals (*R*) can be formed as a direct result of radiolysis ($RH \rightarrow R\bullet + H\bullet$), dissociation of radical cations ($RH^+ + RH \rightarrow R\bullet + \dot{R}H_2^+$), reactions between electrons and molecules ($A + e^- \rightarrow A^-$) and also as a consequence of ion-molecule reactions ($RX + e^- \rightarrow R\bullet + X^-$).Theoretically, the free radicals may reform the original bond, may react to form secondary radicals or may persist depending on their ability to

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move into a position for bimolecular reaction, which predetermine the pathways for decay and hence, the lifetime (environment factors that alter their activity). If the process of radical formation occurs in a constrained matrix, such as dry polycrystalline or amorphous material, the free radicals are trapped and stabilized. Therefore, the concentration of free radicals depends on the nature of the material irradiated, the radiation dose, and the time interval between irradiation treatment and radical measurement.

In this paper, Electron Paramagnetic Spectroscopy (EPR) was used to investigate the free radicals in γ-radiation damage in solid form of some drugs as antiemetics (Metoclopramide and Odansetron), cytostatics (Mercaptopurine and Azathioprine), beta blockers (Atenolol, Metroprolol, Pindolol,Verapamil), and some foodstuffs spices (black and white pepper)

MATERIALS AND METHODS

Microcrystalline powder of samples was exposed to γ-radiation from a ^{60}Co source (GAMMA CHAMBER 900) in ambient conditions. The absorbed dose of drugs was15 kGy corresponding to an average dose used in the sterilization process. ESR spectra were recorded with Bruker EMX spectrometer, operating in the X-band (9.1 GHz – 9.6 GHz) equipped with a computer acquisition system. The computer simulation analysis of the spectra was made by using programs that are available to the public through the Internet, for obtaining the magnetic characteristic parameters (http://epr.niehs.nih.gov/ - 2010).

RESULTS AND DISCUSSIONS

Antiemetics (Metoclopramide and Odansetron*)*.

The EPR spectra of γ-irradiated solid metoclopramide and odansetron in solid state of both samples corresponding to each dose are dominated by a broad central signal with specific characteristics given by chemical structures (Fig.1).

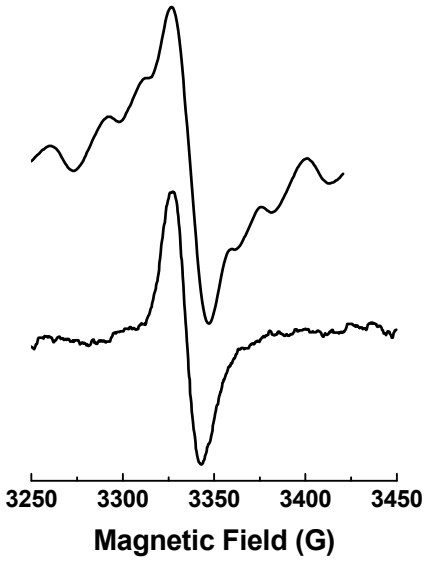

Fig. 1. EPR spectra of odansetron

The values of the g-factor are characteristic for carbon- or nitrogen-centered radicals (Damian, 2003). The spectra of metoclopramide samples exhibit a broad signal without hyperfine structure, centered on the g=2.0047 and a width line of 20 G. A prevalent free radical compatible with these parameters can be a radical in which no hyperfine interactions occur and it is proposed to be a radical of type $R-\dot{C}OO^{-}$, formed by breaking chemical bond between amidic carbon and amidic nitrogen in presence of some hydroxyl radicals from irradiated water molecules. The presence of hyperfine structure of EPR spectra of γ-irradiated odansetron is due to its more complex chemical structure. The most probable changes are due to reorientation of imidazolic group versus carbazolic group and breaking the bond between carbon and nitrogen. Such, the free radicals generated by irradiations, can be localized in different local conformations of molecular structures giving rise to nonequivalent magnetic species.

Cytostatics (Azathioprine and Mercaptopurine).

The broad signal observed for Azathioprine is characteristic for free radical trapped in a solid matrix (Fig.2).

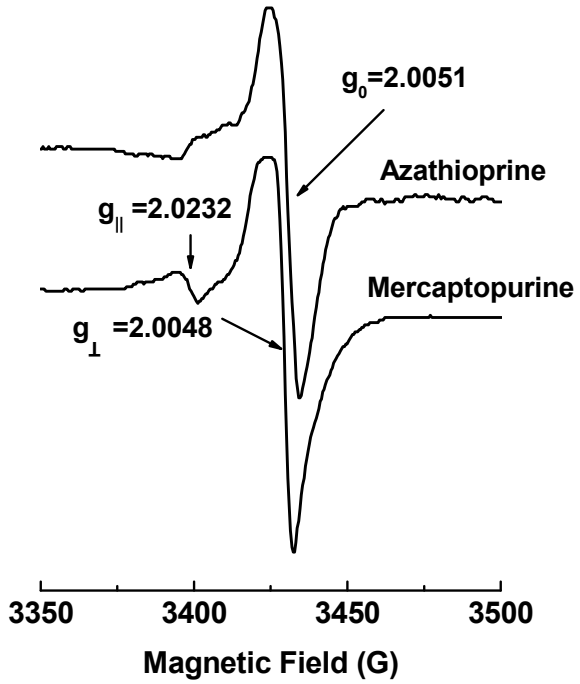

Fig.2. EPR spectra of gamma-irradiated Azathiopine and Mercaptopurine

The value of the isotropic g-factor of g_0=2.0051, is characteristic for carbon or nitrogen-centered radicals. The unresolved spectrum of does not exhibit any resolution similar to that recorded for the non-irradiated drug. Due to lack of resolution of any hyperfine interaction, the character of the paired radicals cannot be concluded. The anisotropic spectrum obtained for Mercaptopurine seems to belong to sulphinyl radical $RSO^{\cdot}$ with $g_{||}$ =2.0232 and $g_{\perp}$ =2.0048 formed on oxidation of the thiyl radical (Damian, 2002). This anisotropy in the EPR spectrum, are due probably,

the localization of radical centers on both aromatic rings giving rise to a local axial arrangement.

Beta-blockers (Pindolol and Atenolol).

The EPR spectra are shown in Figure 3. For Pindolol the magnetic parameters obtained by simulation was one free radical with a_N=25.9G couplated with three protons with a_H=14.6 G (radical centered on nitrogen) and another radical due to a unpaired electron unallocated on the aromatic ring with hyperfine constant a=17G couplated with three protons having a_H=3.8 G.

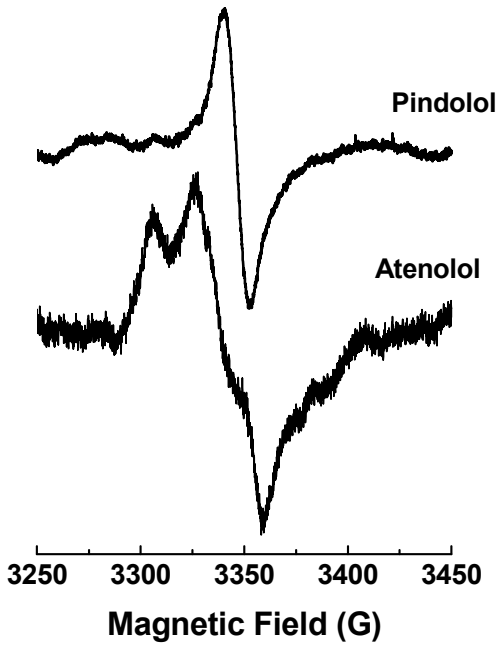

Fig. 3. EPR spectra of irradiated Pindolol and Atenolol.

EPR spectrum of Atenolol has been attributed to the superposition of spectra of two radicals. The first radical gives rise a triplet centered at g=2.0031 with 9.8 G peak-to-peak line width and is due to two equivalent protons with hyperfine coupling a_1(H)=a_2(H)=16.3 G. This radical, in very good agreement with the isotropic coupling generally found for carbon centered π-radicals, is of the form $R-\dot{C}H_2$ and can be produced by removal of hydrogen from methyl group. For the second radical assumed, the unpaired electron can be located on nitrogen atom from imidiazolic group, giving rise a characteristic hyperfine splitting with a(N)=16-18G and g=2.009 (Petrisor et al., 2004).

Species (black and white pepper and sweet and hot pepper).

The EPR spectra of irradiated and mechanical treatment of studied spices are typical for foodstuffs containing high levels of cellulose. The intensity of lines increase after irradiation. As example, for black pepper (Fig.4.) the lines appeared at either side of the g = 2.0050 resonance line of the unirradiated sample (Petrisor et al., 2008).

This indicates the production of an additional new radical beside radical or radicals giving rise to the g =2.0050 resonance line. Our simulation results are in accord with paramagnetic species characteristics for the presence of quinone and carbohydrate type radicals from, cellulose and lignocellulosic material. The similar results were obtained for other studied species.

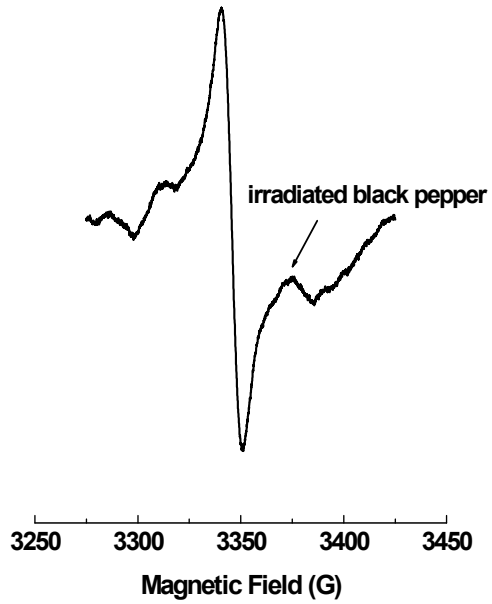

Fig. 4. EPR spectra of noniradiated and irradiated black pepper.

Dried Fruits (banana and pineapple).

Multiplet ESR signals were observed in irradiated dried banana and pineapple while these characteristic signals were not detected in non-irradiated samples.

The EPR spectra are relatively broad (~70 G wide) with asymmetry at the center. In general, powder EPR spectra of radicals show a symmetrical pattern. The asymmetric pattern suggests there are several radical sites in the irradiated dried fruits (Esteves et al., 1999).

The amount of free radicals linearly increased with the applied doses (0.5~5 kGy) and the radicals produced after the irradiation are stable at ambient temperature. It is believed that EPR signals in dehydrated fruits are mostly derived from radicals produced in crystalline saccharides by irradiation. The intensity of the EPR signal and its specific, complex structure is the decisive criterion for the identification of irradiation in dehydrated fruits.

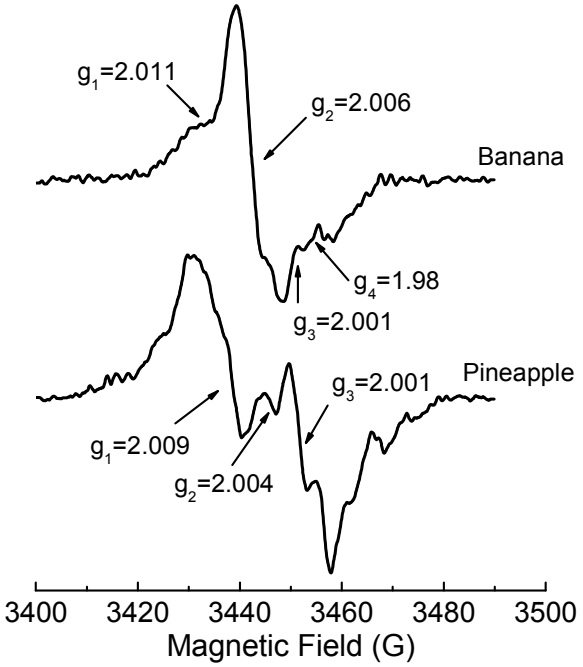

Fig. 5. EPR spectra of noniradiated and irradiated banana and pineapple

CONCLUSIONS

Among many techniques for irradiated drugs and food researches, EPR spectroscopy is the most sensitive method. In particular, EPR techniques for determination of free radicals concentration play an important role in drugs and foods irradiation research and detection method of irradiation dose in irradiated samples. The stability is suitable for the dose accumulated over a period of time. The radical information can be affected by environmental variable as humidity especially but it is not difficult to overcome the moisture.

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A DITHIONITE-INDUCED SIX-COORDINATED SPECIES AT THE HEME IN DEOXY HEMOGLOBIN

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ABSTRACT

Deoxy hemoglobin is well-known to be pentacoordinated, with axial ligation from the so-called proximal histidine. The reducing agent, dithionite, is commonly employed to generate the deoxy from in hemoglobin and other proteins. Here, we report that at room temperature in strongly alkaline medium hemoglobin but not myoglobin can be converted by reduction with dithionite to a hexacoordinated state whose UV-vis absorption spectrum identifies it as a hexacoordinated ferrous heme, where the sixth ligand is most likely SO_2.

Key words: heme, deoxy hemoglobin

INTRODUCTION

The ferrous pentacoordinated deoxy state of the heme iron in hemoglobin and myoglobin are well known (Antonini and Brunori, 1971; Lippard and Berg, 1994; Sigfridsson and Ryde, 1999; Bellelli et al., 2006) The sixth coordination position in these proteins is controlled, via sterical interactions and hydrogen bonding abilities, by the so-called 'distal' histidine, found trans from the iron-ligated 'proximal' histidine (Sigfridsson and Ryde, 1999). Hexacoordination in the ferrous form is known for hemoglobin and myoglobin especially with the physiologically-relevant ligands, dioxygen and carbon monoxide (Sigfridsson and Ryde, 1999; Silaghi-Dumitrescu and Silaghi-Dumitrescu, 2004) On the other hand, the ferric (met) form of hemoglobin is normally found in hexacoordinated states; under physiological conditions the sixth ligand to the iron is a water molecule, which at higher pH becomes a hydroxide (Svistunenko et al., 2000a; Svistunenko et al., 2000b). A small fraction of the met form can, however, engage in a third hexacoordinated state, where the distal histidine is relocated and binds to the iron; a crystal structure of this form is available (Svistunenko et al., 2000a; Svistunenko et al., 2000b; Robinson et al., 2003). Related to this, a more recently-discovered member of the globin class, the neuroglobin, is know to prefer a bis-histidine hexacoordinated state for the heme even for the ferrous state (Dewilde et al., 2001; Hundahl et al., 2006; Dewilde et al., 2008).

Dithionite, $S_2O_4^{2-}$, is commonly employed as a reducing agent in biochemistry, including reduction of metalloproteins. It is generally accepted that neither dithionite nor its monomeric form $SO_2^{-}\cdot$affect protein structure, or the structure of active metal

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centers in metalloproteins in particular. Illustrating this issue, protocols for identifying types of hemoproteins in cell extracts rely precisely on reaction with excess dithionite (Das et al., 2005). Here, we report that under special conditions excess dithionite does in fact change the structure of a metalloprotein: the deoxy form of hemoglobin can be converted to a previously unreported low-spin hexacoordinated state, most likely with an SO_2 ligand at the iron.

MATERIALS AND METHODS

Bovine hemoglobin was purified following the general protocol of Antonini and Brunori (1971). 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. Myoglobin (lyophilized, from horse heart) and bovine serum albumin (fraction V) were purchased from Sigma and used without any further purification. The met forms of hemoglobin and myoglobin were prepared by ferricyanide treatment, while the deoxy forms were produced by reduction with dithionite, as previously described (Dunne et al., 1999; Reeder et al., 2002; Dunne, 2006). Cytochrome *c* (Sigma-Aldrich, Germany) was used without further purification. For pH dependence measurements, the buffers were 50 mM actetate (pH 5), phosphate (pH 6, 7, 8, 12, 13), and borate (pH 9-11).

The UV-vis spectra were recorded on Agilent 8453 (Agilent, Inc.) and Cary 50 (Varian, Inc) instruments. Deoxy forms were obtained either by addition of a few grains of dithionite (large excess) to oxy hemoglobin or myoglobin, or by titration with dithionite of globin solutions previously degassed by purging with argon the headspace of rubber-septum-sealed UV-vis cuvettes. Ferric forms of the globins were obtained with ferricyanide treatment as previously described (Deac et al., 2009).

RESULTS AND DISCUSSION

Figure 1 shows UV-vis spectra for ferric (met) hemoglobin and myoglobin in the pH range 5-13. As previously described, UV-vis spectra indicate two states to be present in these proteins, both of which are hexacoordinated (Svistunenko et al., 2000a; Svistunenko et al., 2000b) The low-pH form features a water molecule ligated to a high-spin (S=5/2) iron, while the high-pH form features hydroxide bound to a low-spin (S=1/2) iron ((Svistunenko et al., 2000a; Svistunenko et al., 2000b). A note may be made of the tendency of the spectra to loose some intensity at pH 13, without changing shape; this is not unexpected at such extreme pH. However, as previously pointed out, globins in ferrous and ferric forms are stable and do not denature at pH values as high as 12 (Makarov et al., 2008).

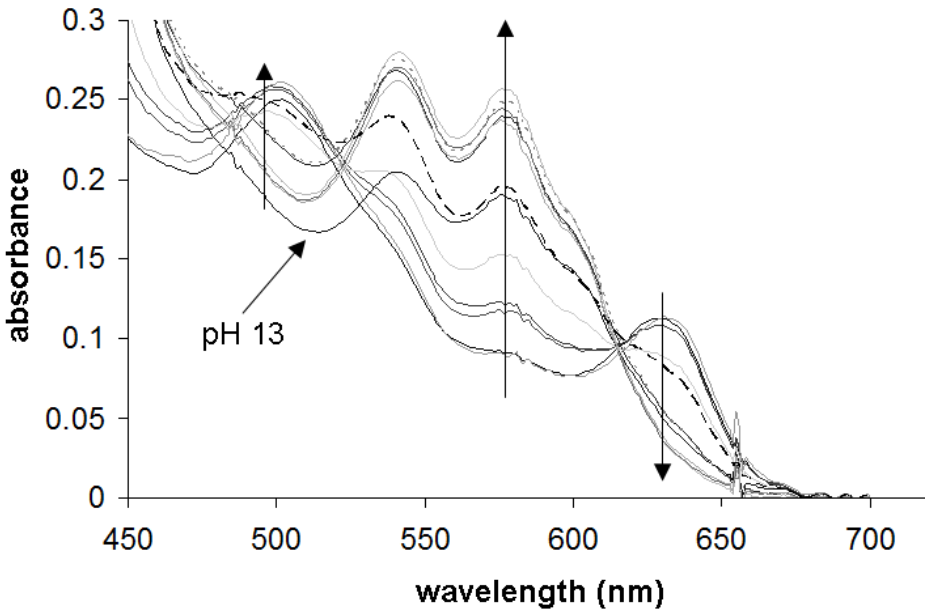

Fig. 1. UV-vis spectra of hemoglobin and myoglobin at pH 5-13.Arrows indicate trends in absorbance with increasing pH. The only exception to these trends is the pH 13 spectrum, indicated separately with an arrow.

Figure 2 shows UV-vis spectra for deoxy hemoglobin and myoglobin at pH 5-13. At pH 5-10 the spectra are clearly in line with well-known data, showing a single maximum in the 500-700 nm region, diagnostic of a high-spin pentacoordinated ferrous heme (Makarov et al., 2008).

However, pH 13 a new species emerges. Figure 2 also shows for comparison UV-vis spectra of hexacoordinated ferrous species – oxy and carbon-monoxy. It is evident that the pH 13 deoxy spectrum, with its two absorption maxima in the 500-700 nm region, is diagnostic of a hexacoordinated low-spin center.Antonin and Brunori, 1971). Figure 2 also shows the UV-vis spectrum of ferrous cytochrome *c*, where the iron is six-coordinated with the axial positions being occupied by two aminoacids.(Silkstone et al., 2005). The similarity in shape between the high-pH deoxy Hb spectrum and the ferrous cytochrome *c* spectrum suggests that at pH 13 a sixth ligand binds to iron in the distal position. This sixth ligand is unlikely to be hydroxide, since at pH 12 there is no evidence for such binding; indeed, by analogy with Figure 1, a well defined titration plot should be apparent if water deprotonation was involved in the deoxy Hb spectral changes between pH 12 and pH 13. Instead, the asymmetric shape of the maxima in the 500-600 nm region of pH 13 deoxy Hb is at odds with the symmetric shapes seen in six-coordinated oxy Hb AND carboxy Hb, but is very much reminiscent of the cytochrome *c* spectrum – where the sixth ligand trans to the proximal histidine is sulfur-based (a methionine) (Silkstone et al., 2005). The most likely explanation is therefore that at pH 13 the hemoglobin iron is bound by a dithionite-derived sulfur-type ligand – which is then proposed to be SO_2. An alternative explanation would be that SO (possibly derived from heme-linked degradation of dithionite) or sulfide is a ligand; however, we are aware of no examples where SO would be bound to a heme protein, and do not expect the highly-charged sulfide to bind to a ferrous heme (nor are large amounts of sulfide expected to be present in fresh dithionite solutions). Also an alternative explanation would be that the distal histidine ligates to iron in the dithionite-reduced forms at high pH. This cannot be excluded directly, especially as we have not yet established a

threshold for the concentration of dithionite at which the six-coordinated species is observed. However, the distinct asymmetry in the two maxima exgibited in the 500-600-nm region, together with the fact that the more intense maximum is centered at 550 nm, offer too strong of a similarity with ferrous cytochrome *c* to afford ignoring the possibility that a sulfur ligand has ligated the iron in ferrous hemoglobin at high pH, too.

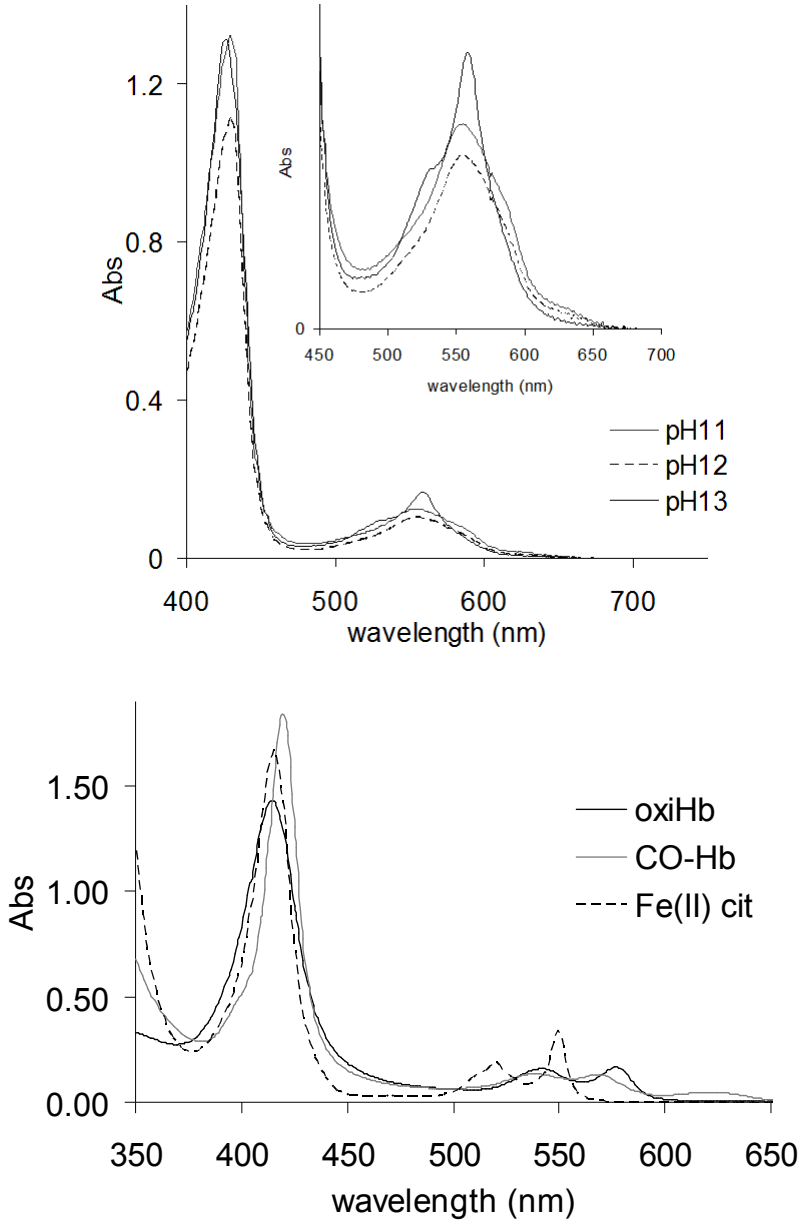

Fig. 2. Top panel- UV-vis spectra of ferrous deoxy hemoglobin at pH 11-13. Bottom banel: spectra of oxy and carbonmonoxy hemoglobin and of ferrous cytochrome *c*.

Figure 3 shows that the pH 13 species induced by dithionite in hemoglobin cannot be observed when a small excess only (~1.5-2 fold) of dithionite is employed for the reaction. These data support the idea that the novel hexacoordinated ferrous Hb shown in Figure 2 is not a mere result of protein denaturation at pH 13, and that the sixth ligand is not an aminoacid within the protein, which may have been relocated closer to the iron due to pH-induced conformational changes. Furthermore,

Figure 3 also shows that the pH 13 dithionite-induced 6-coordinated species is not observable in myoglobin, suggesting that this species is not a general feature of globins or indeed of heme proteins.

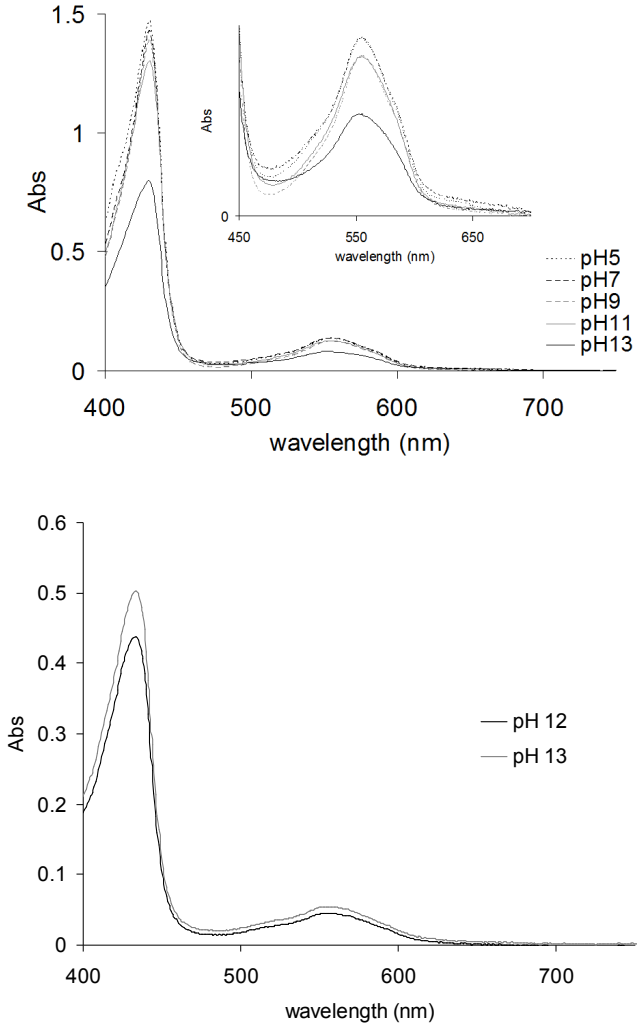

Fig. 3. Top panel: UV-vis spectra of deoxy hemoglobin generated with a slight (~1.5) excess of dithionite from oxy Hb. Bottom panel: UV-vis spectra of deoxy myoglobin at pH 12 and 13, produced with a large excess of dithionite.

In conclusion, the data shown here suggest that a six-coordinated form of ferrous deoxy hemoglobin can be produced with excess dithionite at room temperature and pH 13, where the sixth ligand is most likely the SO_2, in a structure akin to that displayed under physiological conditions by cytochrome *c*. This newly-detected species may hold relevance for catalytic processes in heme enzymes such as sulfite reductase (Crane and Getzoff, 1996; Crane et al., 1997a; Crane et al., 1997b).

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STUDY OF O-H...Π AND N-H...Π INTERACTIONS WITH ACETYLACETONATO RINGS

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ABSTRACT

O-H… π and N-H…π interactions between the coordinated acetylacetonato ligand and phenyl rings were analyzed in the crystal structures from the Cambridge Structural Database and by quantum chemical calculations. Quantum chemical calculations showed that energies in these systems are similar to energies in C-H… π interactions. However, the analysis of crystallographic data showed that there is a small number of N-H… π and O-H…v interactions in crystal structures, compared to number of C-H… π interactions. These results indicate that the O-H and N-H fragments preferably interact with other parts of chelate ring and that these interactions compete with O-H...v and N-H... v interactions.

***Key words*:** N-H…π and O-H…π interactions, acetylacetonato rings, ab initio calculation

INTRODUCTION

The noncovalent interactions involving π-systems have been extensively studied, and it has been documented that they are important for many molecular systems from molecular biology to material science (Steiner, 2002a). Noncovalent interactions in metal complexes between π-system and X-H fragments (X = C,O,N) are of particular interest since it has been shown that they play a role in stability of metalloproteins, crystal engineering and in the mechanism of enzymatic reactions (Zaric et al., 2002). The study of OH/π interactions between water molecule and aromatic groups of amino acids in proteins confirmed the relatively frequent occurrence of aromatic OH/π hydrogen bonding in protein crystal structures (Steiner and Kolelner, 2001; Steiner, 2002b). Besides XH/π aromatic interactions, there are abundant aromatic interactions such as π-π stacking that also play important role for protein structure and protein-ligand recognition. The Protein Data Bank studies revealed that the NH/π interactions are outnumbered by the aromatic-amide stacked structures (Mitchell et al., 1994).

The chelate ring with delocalized π-bonds may engage in two types of interactions: it can be hydrogen atom donor or acceptor. In our previous work we noticed that acetylacetonato ligand, acting as proton acceptor, can be involved in C-H… π interactions (Bogdanovic et al., 2002; Milcic et al., 2006). The calculated

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energy and geometry observed in crystal structures are comparable with C-H…π interactions where proton acceptor is organic aromatic ring (Nishio et al., 1998).

In this work we report on O-H… π and N-H… π interactions with acetylacetonato ligands in square-planar metal complexes. Both O-H… π and N-H… π interactions were studied by searching and analyzing crystal structures in the Cambridge Structural Database (CSD) and by quantum chemical calculations.

MATERIALS AND METHODS

The crystal structures involving M(acac) fragment were screened for intermolecular contacts. A Cambridge Structural Database search was performed using the Quest3D program, to extract all structures of transition metal complexes with coordinated acetylacetonato ligands and looked only for those in which distances between H atom and the center of phenyl ring is shorter than 3.5 Å, α, the angle between the X-H vector and center of aromatic ring, is in range of 110-180°, *β,* the angle between vector H atom center of the ring and vector normal to the ring, is smaller than 30° (Figure 1).

By searching CSD, we found 4 N-H… π and 4 O-H… π interactions which satisfy the following criteria. The number of C-H… π interactions which satisfy the same criteria is 1162.

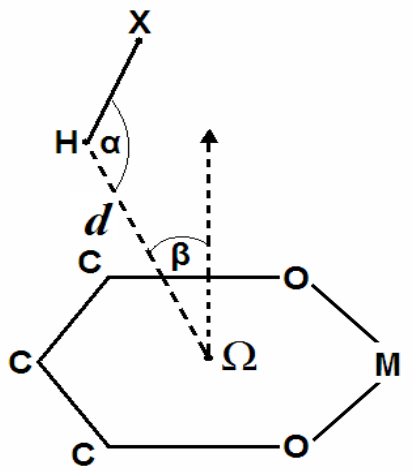

Fig. 1. Geometrical parameters for the X-H/π (X=O,N) interaction with the acetylacetonato chelate ring

High level *ab initio* calculations were carried out on the model system (Figure 2). The systems were made from the crystal structures of acetylacetonato complexes in the way that large groups in complexes were substituted by hydrogen atoms. The energies were calculated for interactions of these systems with water and ammonia molecules.

The calculations of the intermolecular interaction energies were performed at the MP2 level using the lanl2dz basis set for metal atom and 6-31G** basis set for other atoms. All *ab initio* and DFT energy calculations were carried out using the Gaussian 03 program.

RESULTS AND DISCUSSION

The calculations of the intermolecular interaction energies show that the most favorable interactions of Ir(en)(acac) and Rh(en)(acac) complexes with ammonia are at H-Ω distances of 2.9Å. Interactions of $Pd(acac)_2$ and $Pt(acac)_2$ with the same molecule have the lowest energy at H-Ω distances of 3.1 Å, and for $Ni(acac)_2$ at 2.9 Å. The most favorable interactions of Ir(en)(acac) and Rh(en)(acac) complexes with water are at H-Ω distances of 2.6Å, and the interactions of $Pd(acac)_2$, $Pt(acac)_2$ and $Ni(acac)_2$ with the same molecules have the lowest energy at H-Ω distances of 2.7 and 2.9 Å respectively (Figure 2).

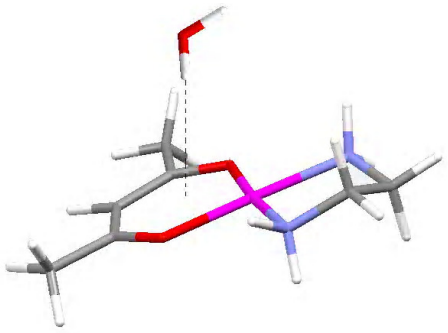

Fig. 2 Model system for calculations of the O-H… π *interaction* of M(en)(acac) complexes

Quantum chemical (MP2) calculations on a few model systems show that O-H… π interactions occur in the range from 2.6 to 2.9 Å(Figure 3.).

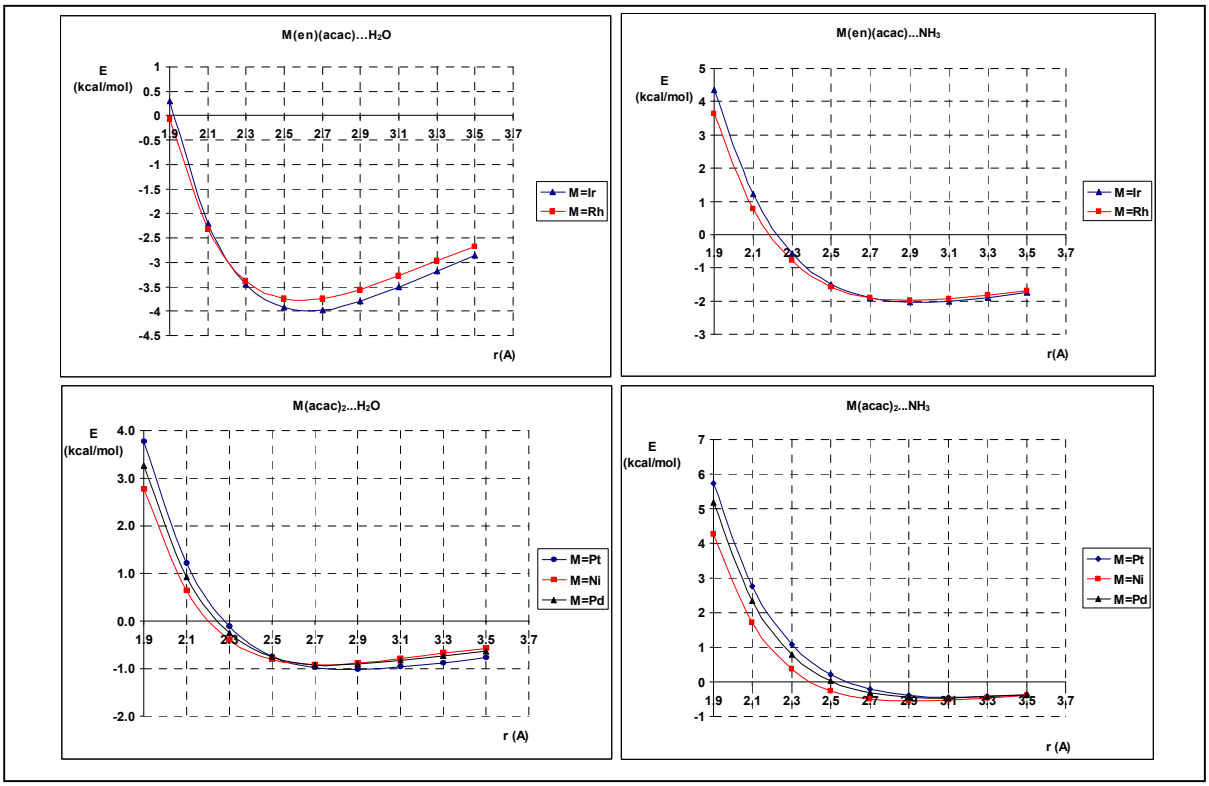

Fig. 3. *Ab initio* calculated energies for interactions of acetylacetonato complexes of Ir, Rh, Pd, Ni and Pt with H_2O and NH_3

These calculations also show that the N-H… π interactions with the chelate ring occur in the range from 2.9 to 3.1 Å.

By searching CSD, we found 4 N-H… π and 4 O-H… π interactions which satisfy the geometrical criteria. The number of C-H… π interactions which satisfy the same criteria is 1162.

CONCLUSIONS

Quantum chemical calculations pointed out that energy for the O-H... π interactions are significantly stronger than N-H... π interactions. It was also shown that the energy of interaction is larger in chelate rings with the soft metals.

By analyzing the data from CSD for structures of acetylacetonato complexes, it was found that there is small number of N-H… π and O-H… π interactions in crystal structures compared to number of C-H… π interactions. These results indicate that the O-H and N-H fragments preferably interact with other parts of chelate ring and that these interactions compete with O-H... π and N-H... π interactions.

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A COMPARISON BETWEEN DIFFERENT EXTRACTION METHODS USED FOR THE DETERMINATION OF IRON MOBILE FORMS

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ABSTRACT

The iron, like other metals, exists in the soils in immobile and mobile forms. The determination of the metals mobile forms is very important for understanding their migration patterns in the soil and their uptake by plants. We compared five methods for extraction immobile ("pseudototal", with aqua regia) and mobile iron forms (using as extractants EDTA, water, $CaCl_2$, CH_3COONa) from soils. The largest amount of mobile iron was extracted with EDTA. The iron extracted with $CaCl_2$, respective with CH_3COONa, is assumed to be the bioavailable iron in soil. The mass ratio between bioavailable : mobile : total iron is around 1 : 19 : 170, which demonstrated the small bioavailability of iron and the low concentration in mobile forms, despite the high concentration of iron in soils.

Key words: iron, bioavailability, soil extraction, metal mobile forms.

INTRODUCTION

The soils are open biogeochemical systems and represent the main source of trace elements for plants, both as micronutrients and pollutants. The soil – plant transfer of elements is a very complex process governed by several factors, of geochemical, climatic and biological origin, both natural and affected by man (Sposito, 2008; Kabata-Pendias, 2004). In the soil, metals exist in immobile (sulfides, phosphates, silicates, etc.) and mobile forms. The metal concentration in the soil solution depends mostly on the pH and organic matter content of the soil, properties which influence metal mobility and availability (Weng et al., 2002). The mobile forms occur with the exchange processes in the soil and with the changing composition, pH, etc. of soil. Determination of the mobile forms of metals, which can be correlated with the metals bioavailability, is important for understanding their migration patterns in the soil and their uptake by plants (Sabienë et al., 2004). Bioavailability of a metal is considered as the fraction of the total element in the interstitial water and soil particles that is available to the receptor organism; the bioavailability is the key to assessment of the potential toxicity of metals (Gheju et al., 2009; Duffus, 2002).

The analysis of metals in the soil depends on the purpose of the analysis. Thus, the determination of the „total" element contents in the soil may be done by the methods that use solid samples (e.g., X-ray fluorescence spectrometry XRF), or by

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various methods which are using solutions prepared by acid dissolution involving hydrofluoric acid or by fusion/dissolution procedures (Sabienë et al., 2004).

For understanding the metals interaction with other soil components (clay minerals, organic matter, soil solution), or to assess their mobility, retention and availability to plants, the usual approach is to use selective chemical extraction. Soil extraction is the method of isolating functionally defined forms of metal. The notion "form of metal" defines the function of matter in the soil, as "plant available form", "exchangeable cations" or "labile form". (Sabienë et al., 2004).

Methods used for the evaluation of the pool of soluble elements in soils are based mainly on extractions by various solutions: (a) mineral acids at various concentrations, (b) chelating agents, e.g., EDTA, DTPA [+ TEA], (c) buffered salts, e.g., NH_4OAc (pH 7 and 4.8), (d) non-buffered neutral salts $NaNO_3$, $CaCl_2$, $MgCl_2$, $Sr(NO_3)_2$, NH_4NO_3, and (e) other extractants, like Coca Cola, proposed for routine soil testing. Some other techniques like electrodialysis, diffusion through membrane, diffusive gradient in thin film (DGT), and bioindicators have been also proposed. Desirable properties of these extractants are relatively weak reactions with soil components and the dissolution of elements related to the amount taken up by most crop plants, and possible independence of soil properties (Kabata-Pendias, 2004).

For iron, the determination of plant available forms is very important, because an issue that plants encounter is the limited bioavailability of iron in many soil types; iron forms insoluble complexes that are not readily accessible at neutral or alkaline pH in aerobic environments (Jeong and Connolly, 2009).

The aim of our study was to compare some „pseudototal" and mobile forms extraction methods for iron in untreated and non-contaminated soil, having in view the estimation of the iron bioavailability.

MATERIALS AND METHODS

1. *The soil samples preparation and characterization.* The unpolluted soil samples used in the research were collected from a private garden (0 – 10 cm depth) of Constanta, Romania. The soil samples were homogenized, air - dried under room temperature and passed through 2.5 mm mesh.

2. Extraction of iron from soil samples. In this study, the mobile iron fraction in soil was considered to be the fraction that is not tightly bound to soil. To extract this fraction were used four procedures (Benton Jones, 2001; Dean, 2007).

2.1. Iron extraction with EDTA. About 0.5 g of the soil samples were mixed with 50 cm^3 of disodium EDTA solution (0.05M at pH 7.0 with NH_3). The mixtures were magnetically stirred for 2 h at room temperature. The solutions were filtered and the filtrates were analyzed for iron (Anyanwu *et al*., 2004).

2.2. Iron extraction with water. The above procedure was carried out, replacing EDTA with distillate water (Anyanwu *et al*., 2004).

2.3. Iron extraction with $CaCl_2$. 5 g dry soil were mixed with 50 mL of 0.01 M $CaCl_2$ solution (1:10 extraction ratio W/V) and stirred magnetically for 2 h at room temperature. The mixture was filtered and the obtained solution was prepared for iron determination (Benton Jones, 2001; Houba *et al*., 2000).

2.4. Iron extraction with Morgan reagent. 25 mL of Morgan reagent were added over a soil sample of 5 cm^3. The mixture was magnetic stirred for 30 min, filtered immediately and the filtrate was collected for iron determination. The Morgan reagent: weigh 100 g of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) into a 1000 mL volumetric flask and add about 900 mL water. Add 30 mL glacial acetic acid (CH_3COOH), just the pH to 4.8 and bring to volume with water (Benton Jones, 2001).

The "pseudototal" iron content of the soil was measured by *aqua regia*.

2.5. Iron extraction with *aqua regia*. 1.5 g air-dried soil were transferred to 20 mL *aqua regia* (concentrated HCl : concentrated HNO_3 = 3:1). The mixture was left to stand for 16 h at room temperature for easy oxidation of organic part of soil, after this time it was boiled till drying. In cooled mixture was added distillate water, was filtered, and in filtrate was added distillate water till 50 mL. Solutions obtained were prepared for iron determination (Butnariu *et al*., 2008).

3. *The determination of iron from extracts*. The iron, as sum of Fe(II) and Fe(III), was determined in the atomic absorption method (FAAS technique) using an aqueous standard calibration curve. Analyses were made triplicate and the mean values are reported. A Shimadzu Atomic Absorption Spectrometer (AAS model AA6200) equipped with air-acetylene flame was used for determination of metal in soil samples extracts. Acetylene of 99.99% purity at a flow rate 1.8 - 2.0 L/min was utilised as a fuel gas. Concentrations of metal were measured using monoelement hollow cathode lamp. The characteristics of metal calibration curve are: wavelength 228 nm, concentration range 0.040 - 8.000 ppm and correlation coefficient 0.9932 (Skoog *et al*., 1998).

RESULTS AND DISCUSSIONS

The results of the determinations are presented in Table 1. The reported values are the averages for three determinations.

Table 1. The concentration of extracted iron in soil (mg/kg, d.w.).

Extraction reagent	pH of extractant	pH of extract	Concentration
1. *Aqua regia*	-	0.80	680.01 $\pm$ 3.28
2. NH_4 – EDTA	7.00	8.40	75.81 $\pm$ 1.13
3. H_2O	7.00	6.95	56.13 $\pm$ 0.94
4. $CaCl_2$	7.00	6.50	3.69 $\pm$ 0.32
5. CH_3COONa	4.80	6.20	4.62 $\pm$ 0.51

Aqua regia, used for the extraction of "pseudototal" iron, is not totally dissolvent for the most of soils; the efficiency of extraction is different from a metal to another and from a soil to another, being influenced by matrix compound. Metals extracted in *aqua regia* can't be considered total fractions, but also can't be consider bioaccessible fractions, because the extraction process is to power for representing a biological process (Butnariu *et al*., 2008).

Using *aqua regia* as extractant, the extracted iron concentration was more than 10 times higher (12 times higher) than those extracted with water, meaning that iron is present in soil especially in insoluble and immobile form. The insoluble iron is also not bioaccessible for plants.

The largest amount of iron was extracted with EDTA, a chelating agent which has the capacity to extract the metals from inorganic and coordinative compounds. Some authors proposed EDTA extraction on soil analysis as an ideal method for quantifying the empirical relationships between plant uptake and soil metal contents, because the good agreement between the concentrations of metals extracted with EDTA and the concentrations of metals in plants (Anyanwu *et al*., 2004). In our study, the difference between the concentration of iron in EDTA solution and in water solution was lower than we expected. The concentrations of iron in EDTA solutions were also lower than in our previous studies. It could mean that in studied soil

(untreated) the insoluble forms of iron are very stable and the metal is hardly replaced, even with a chelating agent, and mostly of mobile iron is water soluble.

The procedure of extraction with 0.01 M $CaCl_2$ solution is simple, easy to perform, and cheap. The method receives internationally more attention as an alternative for the many extraction procedures for a single nutrient or pollutant that are still in use. The soil is extracted with a solution what has more or less the same ionic strength as the average salt concentration in many soil solutions. The extracted iron may be assumed to be the bioavailable iron in soil (Houba *et al*., 2000) In our study, the fraction of bioavailable iron was very low, being only about 1/170 of "pseudototal" iron, respective 1/19 from mobile iron forms.

The concentration of iron extracted with $CaCl_2$ solution is comparable with those obtained by extraction with sodium acetate solution. Despite the different pH values for extractants solutions, the pH values of both extracts are similar.

CONCLUSIONS

1. All tested extraction methods are suitable for iron determination in soil samples.
2. We assumed that the iron extracted with $CaCl_2$ and CH_3COONa is the bioavailable iron and the iron extracted with EDTA represents the mobile iron in soil.
3. Our results indicate that, in an unpolluted and untreated soil, iron has a limited bioavailability, probably because of iron insoluble complexes which are not readily accessible at neutral or alkaline pH in aerobic environments. Among the iron mobile forms, the bioavailable iron represents a small part. The increasing of iron bioavailability is an important issue, which could lead to better crops biofortification.

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CHELATING AND BRIDGING ARSINOARYLTHIOLATO GALLIUM COMPLEXES WITH POTENTIAL BIOLOGIC ACTIVITY

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ABSTRACT

*Due to the biologic activity of the gallium complexes as antitumoral and antiproliferative agents, our interest was pointed to the synthesis and characterized of the new arsinoarythiolato gallium complexes, through to reaction of arsino-ligands of type 2-$AsPh_2$-C_6H_4SH (**AsSH**), 2-AsPh-$(C_6H_4SH)_2$ (**AsS_2H_2**) and 2-As-$(C_6H_4SH)_3$ (**AsS_3H_3**) with $GaCl_3$ and/or organogallium derivatives. The reaction of **AsSH** with $GaMe_3$ in 2:1 molar ratio led to a mixture of bridging $[Ga(Me)_2\{(\mu_2\text{-}SC_6H_4\text{-}2\text{-}AsPh_2)\text{-}\kappa S\}]_2$ and chelating $[Ga(Me)\{(SC_6H_4\text{-}2\text{-}AsPh_2)\text{-}\kappa S\}_3][(CH_3)_2NHCH_2CH_2N(CH_3)_2]$ compounds. The molecular structure of $[Ga(Me)\{(SC_6H_4\text{-}2\text{-}AsPh_2)\text{-}\kappa S\}_3][(CH_3)_2NHCH_2CH_2N(CH_3)_2]$ is also reported.*

Key words: gallium complexes, biologic activity

INTRODUCTION

The essential role of metal ions, especially transition metal ions in biological systems is well known (Sigel and Sigel, 1979). In medicinal chemistry, which has traditionally been dominated by organic chemistry, metal complexes have gained considerable attention as pharmaceuticals (Guo and Sadler, 1999) for the use as diagnostic tools or as chemotherapeutic drugs mainly against cancer. Research in this field has been stimulated by the worldwide success of cisplatin, *cis*-diamminedichloroplatinum(II) (Lippert, 1999). After this landmark discovery, thousands of platinum(II) and platinum(IV) complexes have been synthesized and tested with respect to their tumor-inhibiting properties. Of the non-platinum metals attention is focused in particular on ruthenium and gallium compounds. Generally, coordinated gallium may endow tumor-inhibiting organic ligands with pharmacologically advantageous properties in a dual manner: first, because of the changed pharmacokinetics and the affinity of gallium to tumour cells and, second, because of its antiproliferative effects (Bernstein, 1998; Collery et al., 2002; Jakupec and Keppler, 2004).

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The combination of a central metal and a ligand, which are both supposed to be directed at the same molecular target, seems a worthwhile task. In many cases reported by literature, gallium is of special interest because it may contribute to the biological activity in a cooperative manner (Arion et al., 2002; Dobrov et al., 2009).

Taking into consideration the interest of our group in the study of coordination chemistry of arsinoarylthiol ligands (2-AsPh$_2$-C$_6$H$_4$SH (**AsSH**), 2-AsPh-(C$_6$H$_4$SH)$_2$ (**AsS$_2$H$_2$**) and 2-As-(C$_6$H$_4$SH)$_3$ (**AsS$_3$H$_3$**) (Hildebrand, 2006; Hildebrand et al., 2008) toward main group metals (Vălean, 2008; Vălean et al., 2008; Vălean et al., 2009), especially gallium (III), and also the potential biologic activity of the new gallium complexes, we report the synthesis and the molecular structure of the new gallium arsinothiolato complex: [Ga(Me){(SC$_6$H$_4$-2-AsPh$_2$)-κ*S*}$_3$][(CH$_3$)$_2$NHCH$_2$CH$_2$N(CH$_3$)$_2$].

MATERIALS AND METHODS

General procedure. All reactions were carried out using standard Schlenk and vacuum line techniques under an atmosphere of dry nitrogen, using dry oxygen-free solvents. Cyclohexane, *n*-hexane, toluene, diethyl ether and THF were dried over sodium wire/benzophenone, distilled under an atmosphere of dry argon and stored over molecular sieves. TMEDA were refluxed over CaH_2, distilled and kept under argon. $CDCl_3$ was dried over $LiAlH_4$, distilled and kept over molecular sieves. Thiophenol, ^{n}BuLi, TMEDA, NEt_3, $GaMe_3$ were obtained from commercial suppliers. Ph_2AsCl was prepared according to the literature (Blicke and Smith, 1929). The NMR spectra were recorded using a Bruker Avance 300MHz instrument. ^{1}H and ^{13}C chemical shifts are quoted in parts per million (ppm) relative to tetramethysilane (TMS). The infrared spectra were recorded on a Perkin-Elmer System 2000 FT-IR spectrometer scanning between 4000 - 400 cm^{-1} using KBr. The mass spectra were recorded on a VG12-250 mass spectrometer (EI-MS, 70 eV, 200 °C). The crystallographic data were collected on a Siemens CCD diffractometer (SMART), *ω* scan rotation, data reduction with SAINT (1999), empirical absorption correction with SADABS (Sheldrick, 1997a) Structure refinement was carried out with SHELXL-97 (Sheldrich, 1997b). Non-hydrogen atoms, except poorly defined disordered regions, were refined anisotropically, and H atoms were calculated on idealized positions. Structure figures were generated with ORTEP (Johnson, 1976; Farrugia, 1997). The relevant crystallographic data and refinement details are shown in Table 1.

Synthesis of the AsSH (Hildebrand, 2006). TMEDA (11ml, 74 mmol) was diluted in cyclohexane (50ml). The reaction mixture was cooled at 0º C in a cooling bath and ^{n}BuLi (72 ml, 82.8 mmol) was added dropwise. After adding thiophenol (3.3 ml, 3.75 g, 32.3 mmol) in the system and after long time of stirring (22 h), at room temperature, yellow solution and white precipitate was obtained. The precipitate was washed with hexane (50 ml) and dried under vaccum. The ^{1}H NMR spectrum of the white precipitate show the formation of the lithium derivative: $Li_2(S\text{-}C_6H_4)(TMEDA)_{1.3}$ (lithium 2-lithiobenzenethiolate). The stirred solution of $Li_2(2\text{-}S\text{-}C_6H_4)(TMEDA)_{1.3}$ in THF (60 ml) was treated dropwise with a solution of chlorodiphenylarsine (11.75 g, 23 mmol) in 20 ml THF at –78 °C. The reaction mixture was warmed to room temperature overnight. The deep red solution was acidified at 0 °C with degassed dilute sulfuric acid (7%) to ca. pH 2–3, concentrated in vacuo to 1/3 volume, and the residue was taken up in diethyl ether (100 ml). The ether phase was washed with degassed water (3 x 50 ml), dried over $CaCl_2$ and concentrated to afford crude **AsSH**

Table 1. Summary of data collection, structure solution and refinement details for compound $[Ga(Me)\{(SC_6H_4\text{-}2\text{-}AsPh_2)\text{-}\kappa S\}_3][(CH_3)_2NHCH_2CH_2N(CH_3)_2]\cdot 2Et_2O$

empirical formula	$C_{66}H_{74}As_3GaNO_2S_3$
Fw	1303.92
T, K	180(2)
cryst syst	Triclinic
space group	$P\bar{1}$
unit cell dimens	
a, Å	1510.8(5)
b, Å	1521.1(5)
c, Å	1564.0(5)
β, deg.	80.628(5)
vol, $Å^3$	3.1620(18)
Z	2
$D_{calc,}$ mg/m^3	1.370
μ (Mo Kα), mm^{-1}	2.134
F(000)	1342
crystal size, mm^3	0.1 x 0.02 x 0.02
$\theta_{Min}/\theta_{Max}$, deg.	2.63 to 25.68
no of reflns. Collected	32508
no of indep. reflns.	11930 [R(int) = 0.0626]
Completeness to θ_{Max}, %	99.3
final R indices [I>2σ(I)]	R_1 = 0.0491, wR_2 = 0.1020
R indices (all data)	R_1 = 0.1231, wR_2 = 0.1238
goodness-of-fit on F^2	0.909
largest diff. peak, $e\cdot Å^{-3}$	0.825 and -0.535

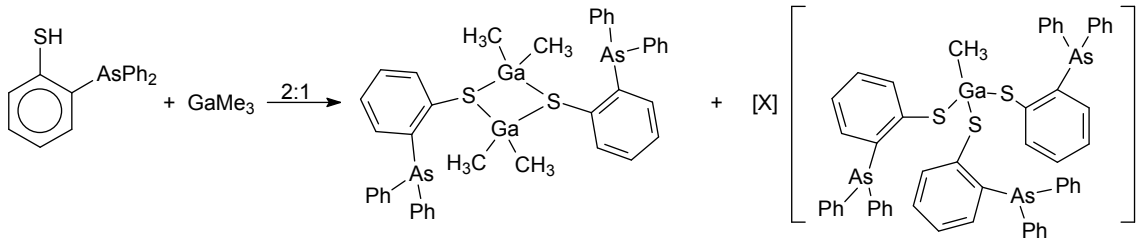
(yield 45% based on Ph_2AsCl). M.p.: 91–95 ºC. ^1H NMR (δ, $CDCl_3$): 7.34 (m, 10H, aryl-H), 7.19 (m, 2H, aryl-H), 7.05 (t, $^3J_{HH}$ = 8 Hz, 1H, aryl-H), 6.88 (d, $^3J_{HH}$ = 4 Hz, 1H, aryl-H), 3.67 (s, 1H, SH). $^{13}C\{^1H\}$ NMR (δ, $CDCl_3$): 140.5 (C^2), 138.3 (C^1), 136.4 (C^7), 134.0 (C^3), 133.8 (C^8), 131.5 (C^6), 129.1 (C^4), 128.8 (C^{10}), 128.7 (C^9), 126.6 (C^5). IR (KBr, cm^{-1}): 3112 (w), 3051 (m), 3006 (m), 2962 (m), 2864 (w), 2552 (m, ν_{S-H}), 2073 (w), 1955 (w), 1917 (w), 1884 (w), 1805 (w), 1570 (m), 1478 (m), 1430 (s), 1305 (w), 1260 (m), 1183 (w), 1154 (w), 1120 (w), 1101 (m), 1071 (m), 1020 (m), 997 (m), 911 (m), 801 (w), 743 (s), 694 (s), 503 (w), 470 (s), 432 (m). Mass spectrum (EI–MS), *m/z*: 338.4 (25%, ($[M]^+$), 306.4 (14%, $[Ph_3As]^+$), 260.3 (12.5%, $[M–H–Ph]^+$), 229.0 (17%, $[Ph_2As]^+ \equiv [C_{12}H_{10}As]^+$), 227.2 (56%, $[C_{12}H_8As]^+$), 184.2 (24%, $[M–2Ph]^+$), 154.3 (100%, $[C_{12}H_{10}]^+$), 152.0 (55%, $[PhAs]^+$), 110.2 (24%, $[PhSH]^+$), 77.1 (28%, $[C_6H_5]^+$), 51.0 (26%, $[C_4H_3]^+$).

Synthesis of [Ga(Me){(SC_6H_4-2-$AsPh_2$)-κ*S*}$_3$][$(CH_3)_2NHCH_2CH_2N(CH_3)_2$]. Only very small amounts of the complex [Ga(Me){(SC_6H_4-2-$AsPh_2$)-κ*S*}$_3$][$(CH_3)_2NHCH_2CH_2N(CH_3)_2$] were obtained from the 2:1 reaction of **AsSH**:$GaMe_3$ at room temperature, long time of stirring (3 days). To a stirred solution of the **AsSH** (0.3 g, 0.8875 mmol) in toluene (12 ml) at –78 °C, trimethylgallium (0.4437 mmol, 0.292 ml 1.52M in *n*-hexane) was added. During the addition of trimethylgallium, a vigorous evolution of gas was observed. The volatiles were removed *in vacuo* to reveal a white powder. A mixture of [Ga(Me)$_2${(μ_2-SC_6H_4-2-$AsPh_2$)-κ*S*}]$_2$ (Vălean et al., 2008) and [Ga(Me){(SC_6H_4-2-$AsPh_2$)-κ*S*}$_3$][$(CH_3)_2NHCH_2CH_2N(CH_3)_2$] were obtained. The complex [Ga(Me){(SC_6H_4-2-$AsPh_2$)-κ*S*}$_3$][$(CH_3)_2NHCH_2CH_2N(CH_3)_2$] was isolated as few low quality crystals from an Et_2O solution and the molecular structure was determined by X-ray diffraction. Taking into consideration the less amount obtained no other spectroscopic measurements were performed. The 3:1 molar ratio did not occurred with the obtaining of [Ga(Me){(SC_6H_4-2-$AsPh_2$)-κ*S*}$_3$][$(CH_3)_2NHCH_2CH_2N(CH_3)_2$] as expected.

RESULTS AND DISCUSSION

The reactivity and the coordination chemistry of the phosphino- and arsinoarythiol ligands (2-EPh_2-C_6H_4SH (**ESH**), 2-EPh-$(C_6H_4SH)_2$ (**ES_2H_2**) and 2-E-$(C_6H_4SH)_3$ (**ES_3H_3**), E=P, As) toward gallium(III) was the subject of our research for more then 4 years. Our previous work shown that although, similar phosphorus and arsenic ligands usually exhibit the same coordination behavior towards the same metal complex fragment, different structures were obtained through the reaction of **ESH** with $GaMe_3$ in 1:1 molar ratio: a monomeric structure with a chelating phosphinoarylthiolato ligand in $GaMe_2${(SC_6H_4-2-PPh_2)-κ^2*S,P*}, and a dimeric arsinoarylthiolato-bridged complex [$GaMe_2${(μ_2-SC_6H_4-2-$AsPh_2$)-κ*S*}]$_2$ (Vălean et al., 2008). Attempts to replace more than one alkyl group in GaR_3 by phosphinoarylthiol were unsuccessful in case of $GaMe_3$. Compound $GaMe_2${(SC_6H_4-2-PPh_2)-κ^2*S,P*} was the only product even when a 2:1 or 3:1 **PSH**:$GaMe_3$ molar ratio was used.

The 2:1 reaction of **AsSH** and $GaMe_3$ led to a mixture of [Ga(Me)$_2${(μ$_2$-SC_6H_4-2-$AsPh_2$)-κ*S*}]$_2$ (**1**) and the new complex [Ga(Me){(SC_6H_4-2-$AsPh_2$)-κ*S*}$_3$][$(CH_3)_2NHCH_2CH_2N(CH_3)_2$] (**2**) (very low yield) (Scheme 1).



1 **2**

Scheme 1.

Where: X = protonated TMEDA

The 3:1 molar ratio did not occurred with the obtaining of **2** as expected. Moreover, different times of refluxing were used but in any of these cases the compound **2** was obtained. These results reveal that the synthesis of **2** is not yet controllable and reproducible. The formation of the anionic compound **2** could be determined by the presence of some left TMEDA in the ligand molecule. A few low quality colourless crystals of **2** were obtained from Et_2O at room temperature. This salt contains cationic unit of $[(CH_3)_2NHCH_2CH_2N(CH_3)_2]^+$ and the $[Ga(Me)\{(SC_6H_4\text{-}2\text{-}AsPh_2)\text{-}\kappa S\}_3]^-$ anion. A view of the cationic-anionic complex **2** is shown below (Figure 1) and selected bonds distances and angles are given in Table 2. The complex crystallises in the triclinic P $\bar{1}$ space group with 2 molecules in the unit cell. The unit cell also contains 2 molecules of Et_2O as solvate molecules.

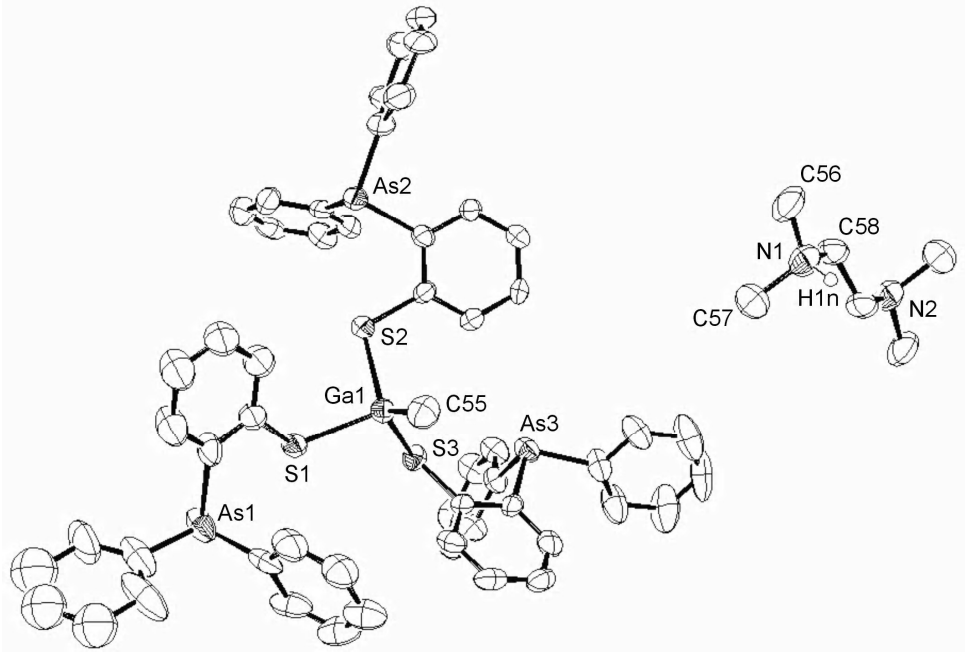


Fig. 1. Molecular structure of $[Ga(Me)\{(SC_6H_4\text{-}2\text{-}AsPh_2)\text{-}\kappa S\}_3][(CH_3)_2NHCH_2CH_2N(CH_3)_2]$

The gallium atom in the anionic unit is four-coordinated by one methyl group and three sulphur atoms of the **AsS**$^-$ ligand in a distorted tetrahedral geometry. The S–Ga–S bond angles range from 97.85(6)° to 104.02(6)° since the C(55)–Ga(1)–S angles are slightly longer, in the range 116.53(19)–119.6(2)°. Similar with other similar complexes, previously reported (Vălean et al., 2008), the arsenic atoms are not coordinated to gallium, the **AsS**$^-$ acts as a monodentate ligand in contrast with **PS**$^-$ which preffers a chelate coordination to the gallium.

The three Ga–S bond distances fall in the range 2.3011(17)–2.3309(16)Å close to those found in gallium complexes $GaCl[PhC(S)CHC(O)Ph]_2$ 2.273Å), (Bhattacharya et al.,1996) $[HNEt_3][Ga(SC\{O\}Ph]_4]\cdot H_2O$,(Deivaraj, et al., 2003) $Ga(^t Bu)_2\{(SC_6H_4\text{-}2\text{-}PPh_2)\text{-}\kappa^2 S,P\}$ and $[PPh_4][Ga\{[(SC_6H_4)_2\text{-}2\text{-}PPh]\text{-}\kappa^3 S,S',P\}\{[(SC_6H_4)_2\text{-}2\text{-}PPh]\text{-}\kappa^2 S,S'\}]$ (Vălean et al., 2008). The S(1)–C(1) (*av.* 1.773Å) and Ga–C (1.956(6)Å) bong lengths are within the accepted range for such compounds and comparable with those previous described for related compounds. The Ga–C bond length in **2** fits in the same range with those found in the literature for similar complexes (Hoffman and Burschka, 1984; Boardman et al., 1985; Hendershot et al., 1991; Keys et al., 1998; Coward et al., 2000).

Table 1. Selected bond lengths (Å) and bond angles (°) in compound **2**

N(1)–H(1n)	0.86(6)	C(55)–Ga(1)–S(1)	116.53(19)
S(1)–C(1)	1.767(6)	C(55)–Ga(1)–S(2)	117.18(19)
S(2)–C(19)	1.781(5)	C(55)–Ga(1)–S(3)	119.6(2)
S(3)–C(37)	1.773(5)	S(2)–Ga(1)–S(1)	97.88(5)
Ga(1)–C(55)	1.956(6)	S(2)–Ga(1)–S(3)	104.02(6)
Ga(1)–S(1)	2.3309(16)	S(3)–Ga(1)–S(1)	97.85(6)
Ga(1)–S(2)	2.3011(17)	C(1)–S(1)–Ga(1)	110.9(2)
Ga(1)–S(3)	2.3013(18)	C(19)–S(2)–Ga(1)	107.79(18)
		C(37)–S(3)–Ga(1)	106.42(19)
		C(56)–N(1)–C(57)	110.7(5)
		C(58)–N(1)–C(56)	111.3(5)
		C(58)–N(1)–C(56)	113.9(5)
		C(58)–N(1)–H(1n)	104.0(4)
		C(56)–N(1)–H(1n)	112.0(4)
		C(301)–N(1)–H(1n)	105.0(4)

The intramolecular S(1)···S(2) (3.493Å) and S(1)···S(3) (3.492Å) distances are slightly below the sum of the sum of van der Waals radii of the atoms involved, which could be indicative of some degree of S···S interactions (Σ v.d. Waals radii (S···S) = 3.6 Å (Bondi, 1964). No intramolecular Ga···As or S(2)···S(3) interactions were observed, the distances between these atoms are longer than the Σ v.d. Waals radii. On the other hand, N···S(1) interactions between the each two vicinity molecules were observed. The N···S(1) distances of 3.198Å, are within the range suitable for hydrogen bonding interactions. These interactions establish the arrangement of the two molecules so that π–π stacking interactions between the phenyl groups are formed.

The $[(CH_3)_2NHCH_2CH_2N(CH_3)_2]^+$ cation is hydrogen bonded to the thiolate S(2) and S(3) sulfur atoms through **H**–(CH)$N(CH_3)_2$ with the S···H distances of 2.728Å and 2.878Å respectively and C–H···S angles of 127.45° (C–H···S(2)) and 157.3° (C–H···S(3)) respectively. Besides of these interactions, no significant contacts between molecules of **2** in the lattice were observed.

CONCLUSIONS

Bidentate arsinoarylthiol ligand **AsSH** was synthesized and the reaction of this with GaMe3 in different molar ratio and reaction conditions were performed. In order to replace more methyl groups in the comlpex $[Ga(Me)_2\{(\mu_2\text{-}SC_6H_4\text{-}2\text{-}AsPh_2)\text{-}\kappa S\}]_2$ presented in Sheme 2 (complex **1**), which is obtained from 1:1 reaction between **AsSH** and $GaMe_3$, the complex $[Ga(Me)\{(SC_6H_4\text{-}2\text{-}AsPh_2)\text{-}$

$\kappa S\}_3][(CH_3)_2NHCH_2CH_2N(CH_3)_2]$ was obtained from the above presented 2:1 reaction.

Different attempts to obtain it as a pure product failed, different molar ratio and reaction conditions were used and in all the cases a mixture of products was obtained. A few crystals of $[Ga(Me)\{(SC_6H_4\text{-}2\text{-}AsPh_2)\text{-}\kappa S\}_3][(CH_3)_2NHCH_2CH_2N(CH_3)_2]$ were isolated and characterised by X-ray diffraction. The X-ray structure analysis shows that the formation of the compound **2** was determined by the presence of some left TMEDA in the ligand molecule.

The gallium atom in the anionic unit is four-coordinated by one methyl group and three sulphur atoms of the **AsS**$^-$ ligand in a distorted tetrahedral geometry. The Ga–S and Ga–C bond lengths are within the accepted range for such compounds and comparable with those previous described for related compounds. The intramolecular S···S distances are below the sum of the van der Waals radii of the atoms involved, which could be indicative of some degree of S···S interactions.

Outlook

The potential application of the obtained gallium complexes in medicine, as antitumor agents, or in material science, as semiconductors (GaP, GaAs, etc.), will make the subject of our future research work.

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BIOGENESIS OF DNA ADDUCTS IN THE DNA/M^{n+} SYSTEMS WHIT IMPLICATIONS IN NUTRITION, PATHOBIOCHEMISTRY AND CYTOSTATIC PHARMACOTHERAPY

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ABSTRACT

The approach of the problem regarding deoxyribonucleic acid (DNA) adducts is important both for biochemistry and xenobiochemistry. In the case of biochemistry is important the study of DNA adducts with various organic compounds and/or organometallic compounds which are present alongside nutrients from food. These types of compounds may be bound predilectly to protein, lipid or carbohydrate macronutrients. The study of adducts is also of great importance for xenobiochemistry due to the fact that chemical xenobiotics are found in the environment being present into water, air and soil. The problem of xenobiochemistry is correlated with the domain of pathobiochemistry due to the fact that numerous xenobiotics, e.g.: metallic ions (M^{n+}), polycyclic aromatic hydrocarbons (HPA), mycotoxins, nitrosamines etc. may interfere with various metabolites during the process of catabolism and anabolism. The interactions of various chemical substances obtained synthetic or by extraction are also in pharmacology and pharmacotherapy and especially in the cytostatic pharmacotherapy. This type of approach regards especially the chemical xenobiotics of pharmaceutical interest.

Key words: Adducts DNA-M^{2+} in nutrition, patobiochemistry and pharmacology

INTRODUCTION

Nutrients which accede in the organism undergo specific metabolic processes. In the first phase nutrients are degraded into smaller units or metabolites (amino acids, fatty acids, monosaccharides etc) through biodegradations processes; these

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processes are known as the catabolism. In the second phase the metabolites are used at the cellular and tissular level to synthesize essential molecules which are indispensable to the organism; this process of biosynthesis is called anabolism.

From water and air, the xenobiotics accede directly into the organisms, while from soil they can be taken indirectly, through the consumption of plants (xenobiotics are taken up by plants from soil through the rhizome). The xenobiotics that accede in the organism undergoes biotransformation processes in two distinct phases: xenobiodegradation and xenobiosynthesis. The xenobiodegradation phase involves oxidoreduction and hydrolyses reactions while the xenobiosynthesis phase involves conjugation and adductation reactions.

From the oncology point of view it is known the fact that numerous xenobiotics are considered to be carcinogens (Dipple, 1995; Garban et al., 2007).

One of the most important classes of compounds that may interact with DNA is the metallic compounds. In this case one must distinguished between biometals which in certain quantity are important for the good functioning of the organism, and metals with toxicological potential which affect the function of the organism. Metallic compounds and their potential adducts with DNA are important in nutrition (various biometals are considered to be micronutrients and some of them led to formation of DNA adducts which are important in the biological function of DNA) but also in pathobiochemistry (toxic metals which may lead to DNA adducts that affects the normal function of DNA and in some cases are incriminated for carcinogenic and mutagenic effects) and cytostatic pharmacotheraphy (most of the alkylating agents used in oncotherapy are organometallic compound which bind to DNA and interfere whit the cell metabolism, killing the cancerous cells).

1. DNA-M^{n+} ADDUCTS IN NUTRITION

In the case of DNA-M^{n+} adducts with importance in the field of nutrition, the most important are the adducts of divalent biometals cations (M^{2+}) – see Ames and Gold (1990); Garban and Garban (2003). The complexes resulted from the interaction between DNA and divalent metallic ions (both in the case of biometals and also in the case of potentially toxicological metals) may present various type of binding: I) binding to the phosphodiesteric group; II) binding between a phosphodiesteric group and nucleobases; III) binding between two intecatenary and complementary nucleobases; IV) binding between two vicinal nucleobases; V) binding in different positions of the same purinic nucleobase.

Native DNA is known to bind relatively strong the divalent biometals like Mg^{2+} because they stabilize the macromolecule (Sigel and Sigel, 1996). The role of magnesium in DNA stabilization is concentration dependent. At high concentrations there is an accumulation of Mg binding, which induce conformational changes leading to Z-DNA, while at low concentration there is deficiency and destabilization of DNA (Anastassopoulou and Theophanides, 2002). These type of adducts are specific usually for alkaline-earth biometals. The bond that is formed depends on the absence or presence of a water molecule – as is shown in fig. 1, there can be three different structures. There is a direct binding (fig. 1a) or one intermediated by the water molecule (fig. 1b and 1c). Such bindings are achieved in the DNA samples with Mg^{2+} or Ca^{2+} (Garban et al., 2007).

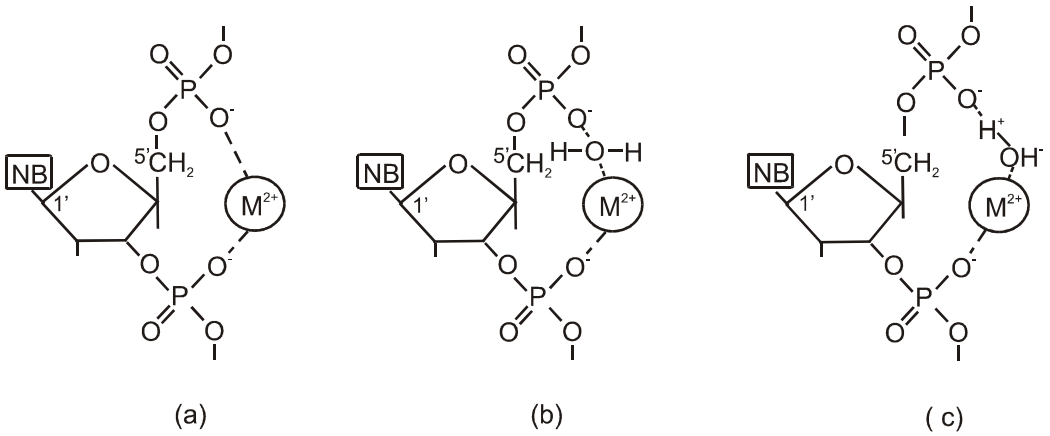


Fig.1. Binding of M^{2+} to the phosphodiesteric groups of DNA

Another type of bound that is more often found in the case of biometals is the binding between phosphodiesteric groups and nucleobases. This can be done a chelation of the phosphorus group with N_7 of the purine nucleobase from GMP (Fig. 2). Such structure characterizes the DNA complexes with Mn^{2+} and Zn^{2+}, which have a strong affinity for the phosphodiesteric groups and a low affinity for the coordination with purine nucleobases.

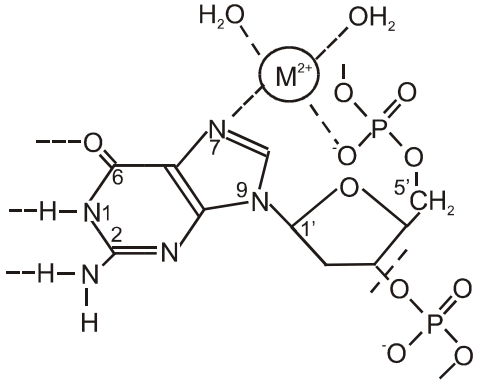


Fig.2. Binding of M^{2+} to guanine and to phosphodiester group of DNA

The other three types of binding are more often found in the case of metals with toxicological potential and will be presented in the next chapter.

In the case of transition biometals, covalent bonds between these metals and various DNA nucleobases are formed. The interaction takes place especially between the metallic ion and a nitrogen atom from the nucleobase structure. An overview on this type of interactions shows that usually there is a destabilizing effect due to the bond that is formed between the divalent transition metal and DNA nucleobases. Such effects were observed in the case of Cu^{2+}, Zn^{2+}, Mn^{2+}.

The destabilization of the DNA macromolecule by Cu^{2+} depends on the molar ratio M^{2+}/DNA-P, the G-C content of the studied DNA and the ionic force of the environment. Copper is a natural constituent of cell nuclei which it seems to play a key role in the structural organization and function of chromosomes. Copper may

also be toxic in biological systems, especially in the presence of hydrogen peroxide and when activated by cellular reductants, including thiols. For example, ligands such as 1,10-phenanthroline are known to lead to the degradation of DNA in the presence of copper(I) and hydrogen peroxide (Gilbert et al., 1999).

The DNA macromolecule interaction with Zn^{2+} gives rise to a DNA-Zn^{2+} adduct which by repeated heating and cooling prove to be a reversible denaturation process, unlike in the case of Mg^{2+}. In the case of a DNA rich in G-C (guanine-cytosine) pairs, the Zn^{2+} ions binds preferentially to these nucleobases. In the case of Mg^{2+}, wich usually binds to the phosphodiestheric groups, there is also the possibility of binding to the G-C bases (Garban, 2004).

It can be affirmed that in the case of DNA-M^{n+} interactions, the adduct formation, the types of bonds and the impact on DNA biological activity is influenced by the cation nature, the concentration of components, the ionic strength, pH and temperature.

2. DNA-M^{n+} ADDUCTS IN PATHOBIOCHEMISTRY

In order to better understand the mechanism of DNA adducts biogenesis one must briefly discuss some notions about xenobiotics kinetic in the organism and especially about the biotransformation process which is specific to xenobiotics (Ames et al., 1993; Dipple, 1995). The kinetic or the transit of xenobiotics through the organism involves four important phases: absorption, biotransformation, distribution and elimination.

The first phase (absorbtion) depends on the nature (lipophylic or hydrophilic) of the xenobiotic (Beyersmann, 1994; Faber, 1999).

The second, and the most important phase regarding both pathobiochemistry and pharmacology, is biotransformation and involve xenobiodegradation and xenobiosynthesis reactions (Kazantzis and Lorna, 1979; Garban, 2007).

During the xenobiodegradation, smaller and more reactive compounds are usually exerted. The xenobiodegradation process is divided in oxidoreduction and hydrolyses reactions. Oxidoreduction reactions include oxidation and reduction reactions. The oxidation reactions are usually: a) hydroxylation reactions - hydroxylation of aliphatic compounds (involved in the biotransformation of aliphatic hydrocarbons, quinidine, phenylbutazone etc.) and hydroxylation of aromatic compounds (involved in biotransformation of polycyclic aromatic hydrocarbons, aniline, phenols, salicylic acid etc); b) oxidative dealkylation reactions - O-dealkylation reactions (involved in biotransformation of anisol, mescaline, codein etc) and N-dealkylation reactions (involved in the biotransformation of caffeine, nicotine, codeine, morphine etc); c) oxidative deamination reactions (involved in the biotransformation of methylamine, amphetamine etc); d) oxidation reactions - N-oxidation reactions (involved in biotransformation of acetaminophluoren, chlorpromazine etc), S-oxidation reactions (involved in biotransformation of sulfoxides, spironoloactone etc); e) the reaction of alcohols and aldehydes oxidation (involved in the biotransformation of ethanol, formaldehyde etc). The reduction reactions are: a) the reaction of carbonylic compounds reduction (reaction involved in biotransformation of acetone, cortisone, prednisone etc.); b) the reaction of nitro- and azoderivates reduction (involved in the biotransformation of nitrophenols, nitrobenzene, diazoderivates, cloramphenicol etc); c) the reaction of disulfidic compounds reduction

(involved in the biotransformation of sulfoxides, disulfiram etc). The hydrolyses reactions include: a) hydrolyses reaction of esters (organophosphoric pesticides, atropine etc); b) hydrolyses reaction of glycosides (involved in the biotransformation of salicin, amigdalin, digitaloid drugs etc); c) hydrolyses reaction of amides (involved in the biotransformation of biogenic amines from meat products).

Xenobiosynthesis usually exerts inert (inactive) compounds from the biological point of view, therefore is usually considered a phase that reduces the toxicity of the xenobiotics. The xenobiosynthesis involves conjugation and adductation reactions. Conjugation reactions are: a) glucurono-conjugation reaction (involved in the biotransformation of carboxylic acids, phenols, nicotinic acid, acetaminophen etc); b) sulfono-conjugation reactions (involved in the biotransformation of aromatic acids, methyldopa etc); c) amino acid-conjugation reaction – conjugation with glycocol (biotransformation of benzoic acid, izonicotinic acid etc), conjugation with glutamine (biotransformation of epoxydic compound resulted from xenobiodegradation of polycyclic aromatic hydrocarbons, phenylacetic acid etc), conjugation with cystein (biotransformation of polycyclic aromatic hydrocarbons etc); d acetyl-conjugation reactions (involved in the biotransformation of sulfonamidic compounds, hydrazine etc); e) methyl-conjugation reactions (involvd in the biotransformation of contraceptive estrogens, methadone etc); f) tiocian-conjugation reaction (involved in the biotransformation of cianhydric acid derivates etc.). Adductation reactions involve reactions which generates complexe compound called adducts (e.g.: protein adducts, DNA adducts). The term of „adductation" is used relatively rare although the reaction presents similarities with the conjugation reaction. Therefore it may be considered opportune the use of the terms "conjugation reactions" and "adductation reactions" for the types of reactions specific to xenobiosynthesis. If in the case of conjugation metals the end product is usually a stable compound with low toxicity, the adductation reaction may lead to the formation of macromolecular complexes with DNA and in the end may generate carcinogenic and mutagenic effects.

The third phase (distribution) the transit of xenobiotics and/or their xenobioderivatives through the extracellular fluids

The fourth and last phase represents the process of elimination. The elimination is performed through the digestive system (bile excretion), through the renal system (urinary excretion), through the respiratory system (exhalation of the volatile compounds) or through the skin (sweat and sudoripary excretion).

The metals with toxicological potential may present all the five types of binding previously described in chapter 1, but they usually present the last three types namely: binding between two intecatenary and complementary nucleobases; binding between two vicinal nucleobases; binding in different positions of the same purinic nucleobase.

One of the types of binding that is specific to metals with toxicological potential is the intercalation of the metallic ion between complementary intrastrand nucleobases. The structure of the resulted adducts shows an intrahelical disposition of the divalent cation M^{2+}: (a) in the case of A-T chelation, bindings appear at N_1 adenine and N_3 thymine; (b) and (c) in the case of the G-C chelation the bindings appear at N_1 guanine and N_3 cytosine (Fig. 3.). The first way is characteristic for Hg^{2+} and the second for Cu^{2+} and Cd^{2+}. The M^{2+} binding is also possible to N_7 guanine, N_3 cytosine and to O from C_6, respectively C_2.

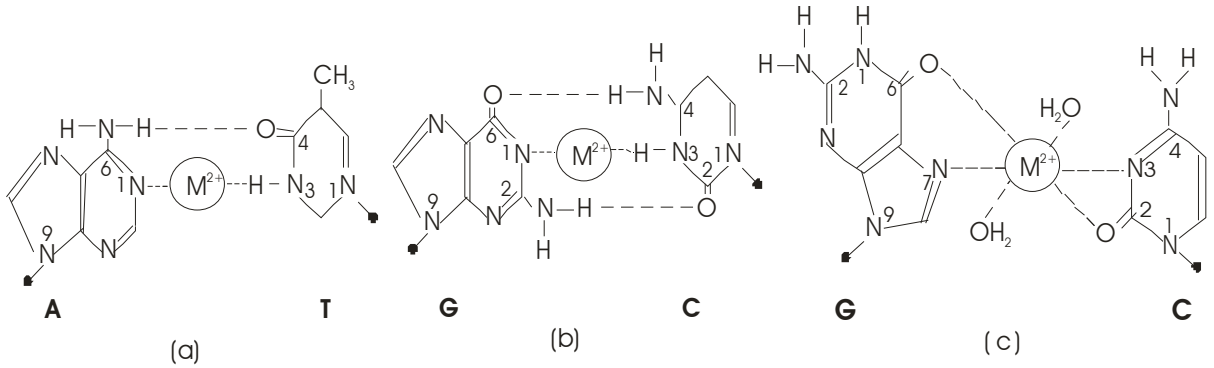


Fig. 3. Internal chelatization binding of M^{2+} to adjacent nucleobase pairs of DNA strands: a) M^{2+} - A-T pair; b) M^{2+}- G-C pair; c) M^{2+} - G-C pair (other position) (o---- binding to DNA strand)

Another type of binding that is more often found in the case of metals with toxicological potential is the chelation made at the level of two nucleobases situated on the same strand (i.e. adjacent nucleobases) like the case of the GpG` sequence. The binding can occur at N_7 and O from C_6 of nucleobases (Fig. 4). Such "sandwich" type bindings, appear in case of Cu^{2+} and Hg^{2+}.

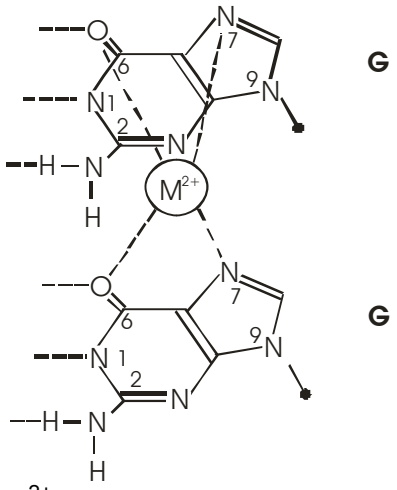
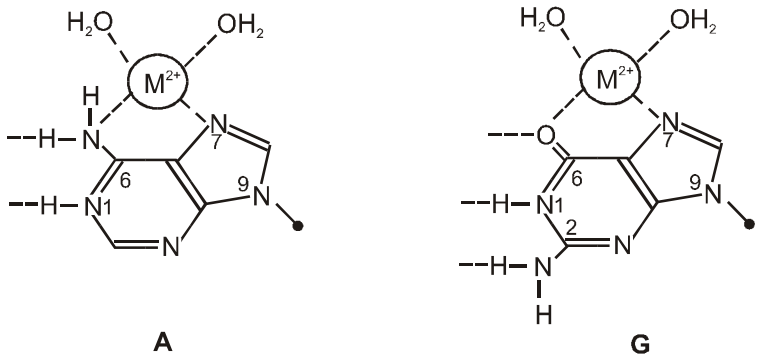


Fig. 4. Binding of M^{2+} to adjacent guanine nucleobases of DNA

The list type of binding specific to metals that have toxicological potential is the bond formed between M^{2+} and a purine nucleobase. This type of bond occurs at N_7 and O from C_6 of adenine or N_7 and O from C_6 of guanine (Fig. 5). In these cases water molecules can bind at the chelate. This type is usually met at transition metals and affects the conformation of helix causing local denaturations in the macromolecular structure (Garban, 2008).



(a) (b)

Fig. 5. Binding of M^{2+} to purine nucleobases of DNA
a) M^{2+} - adenine; b) M^{2+} - guanine

The ability of these metallic compounds to form DNA adduct is resposable for the various carcinogenic effects reported in the case of certain compounds with metals that have toxicological potential. An example is cadmium which induce carcinogenicity based on a complex mechanism that involves not only the direct DNA damage (due to DNA adducts formation) but also the interaction with the DNA repair process and the increase of the oxidative stress (Hartwig, 2010). Therefore, in addition to the direct interaction with DNA, cadmium also disturb the DNA repair process due to its interaction with zinc-containing transcription factor, presumably through the displacement of zinc by cadmium (Kothinti et al, 2010).

Even biometals can, in certain condition, become carcinogens. An example would be chromium. Some hexavalent chromium compounds are known to be environmental contaminants and respiratory carcinogens. These compounds are mainly generated from industrial processes. The primary route of exposure is inhalation. Additional potential routes may be oral ingestion of contaminated water or by direct dermal contact with products manufactured using chromium such as pressure treated wood. Chromium carcinogenesis is initiated and promoted through the process of xenobiodegradation in which chromium compounds undergoes reduction reactions and produces chromium species that are able to interact with DNA and to yield genotoxic and mutagen effects (Nickens et al, 2010).

3. DNA-M^{n+} ADDUCTS IN PHARMACOLOGY

The use of metal-based compounds in pharmacology is a domain that is increasing in importance especially due to the tendency of finding and studying new organometallic compounds with applications in cytostatic chemotherapy (Haiduc and SIlvestru, 1989). Anyway, compounds containing metals are used nowadays not only as cytostatics for cancer therapy but also in other fields of pharmacology. It is worth mentioning some derivatives of the group Va containing arsenic (e.g.: melarsapol – a drug containing As which is used in the treatment of trypanosomiasis), stibium (e.g.: sodium stibogluconate – a compound used in the treatment of leishmaniasis) and bismuth (e.g.: bismuth citrate, bismuth subsalicylate – drugs used in the eradication of Helicobacter pylori). Also, there are worth mentioning compounds containing metals from the Ib group, like silver (e.g.: silver sulfadiazine – a drug used in the prevention of burn infection) and gold (e.g.: auranofin – a drug used in the treatment

of rheumatoid arthritis). Finally one must mention the lithium containing compounds (e.g.: lithium carbonate, lithium citrate) which are used for the treatment of mania and for the prophylaxis of bipolar disorders.

In the field of oncology the organometallic compounds have a long history due to cisplatin which is used in cancer therapy since 1978. Cisplatin (cis-dichlorodiaminoplatin or cDDP) it is often considered an alkylating agent, although it contains no alkyls groups and does not instigate alkylating reactions, so it is properly designated as an alkylating-like drug. The mechanism of action in the case of cisplatin involves the formation of DNA adducts which not only inhibits replication and transcription of DNA, but also leads to programmed cell death (Bertino, 1997).

The binding of cisplatin to DNA nucleobases can be made at one chain or between the two chains of DNA macromolecule; in the end it will result DNA-cDDP complexes (Garban 2009). The binding to nucleobases is done preferentially in the next order: guanine > adenine > cytosine, without the involvement of thymine. The formation of DNA-cDDP complexes is illustrated in figure 6.

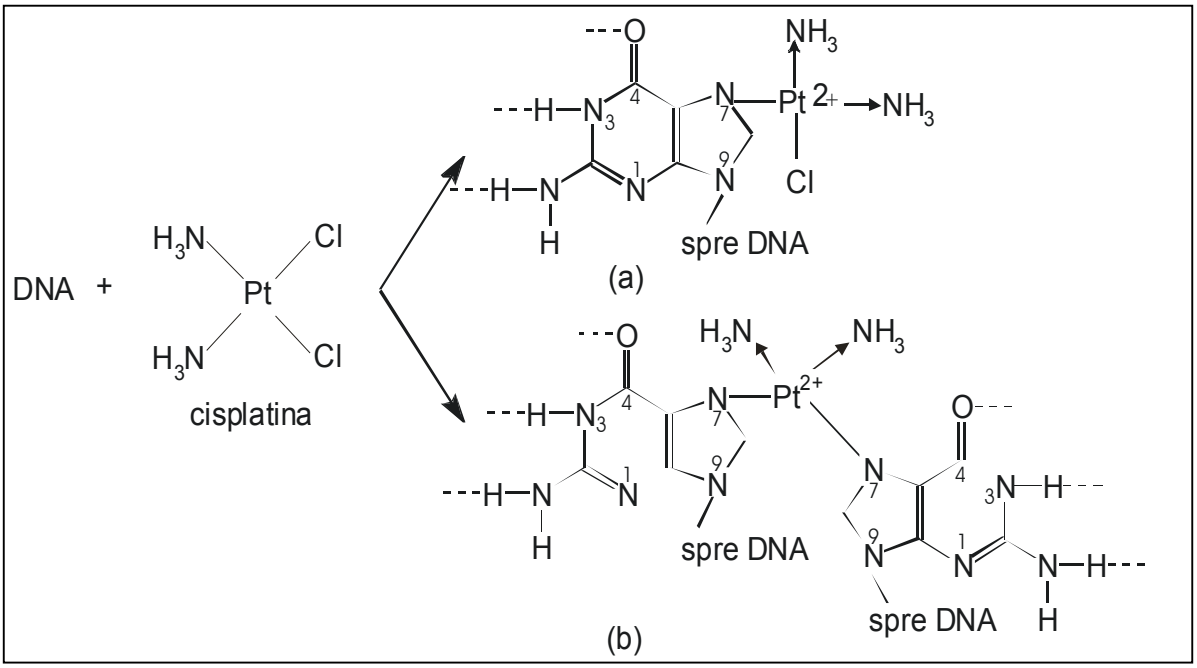


Fig.6. Biogenesis of DNA-cDDP adducts: (a) binding of cDDP to guanine from one chain of DNA; (b) binding of cDDP to two guanine nucleobases from both chains of DNA

Metal based compounds have an increased potential for building up molecules that are better suited for binding to specific biological targets. Metal ions have a wide range of coordination numbers and exhibit a great number of possible geometries which allow organizing a great variety of anions and organic ligands in the desired spatial distribution, affording better ways of attacking the target molecules (Alama et al, 2009).

As we already shown, cisplatin is one of the most important metallic compounds used in cancer therapy together with others platinum complexes like: carboplatin, oxaliplatin, thioplatin etc. The platinum compounds are the well known in the field of oncology and nowadays the scientists focuses more and more to the research of non-platinum metal compounds that can be used as antitumor drugs.

Whit the exception of platinum, the metal elements which have organometallic compounds more promising in cancer therapy are ruthenium, iron, cobalt and gallium. Chemical structures for some of the compounds containing these metals are shown in figure 7.

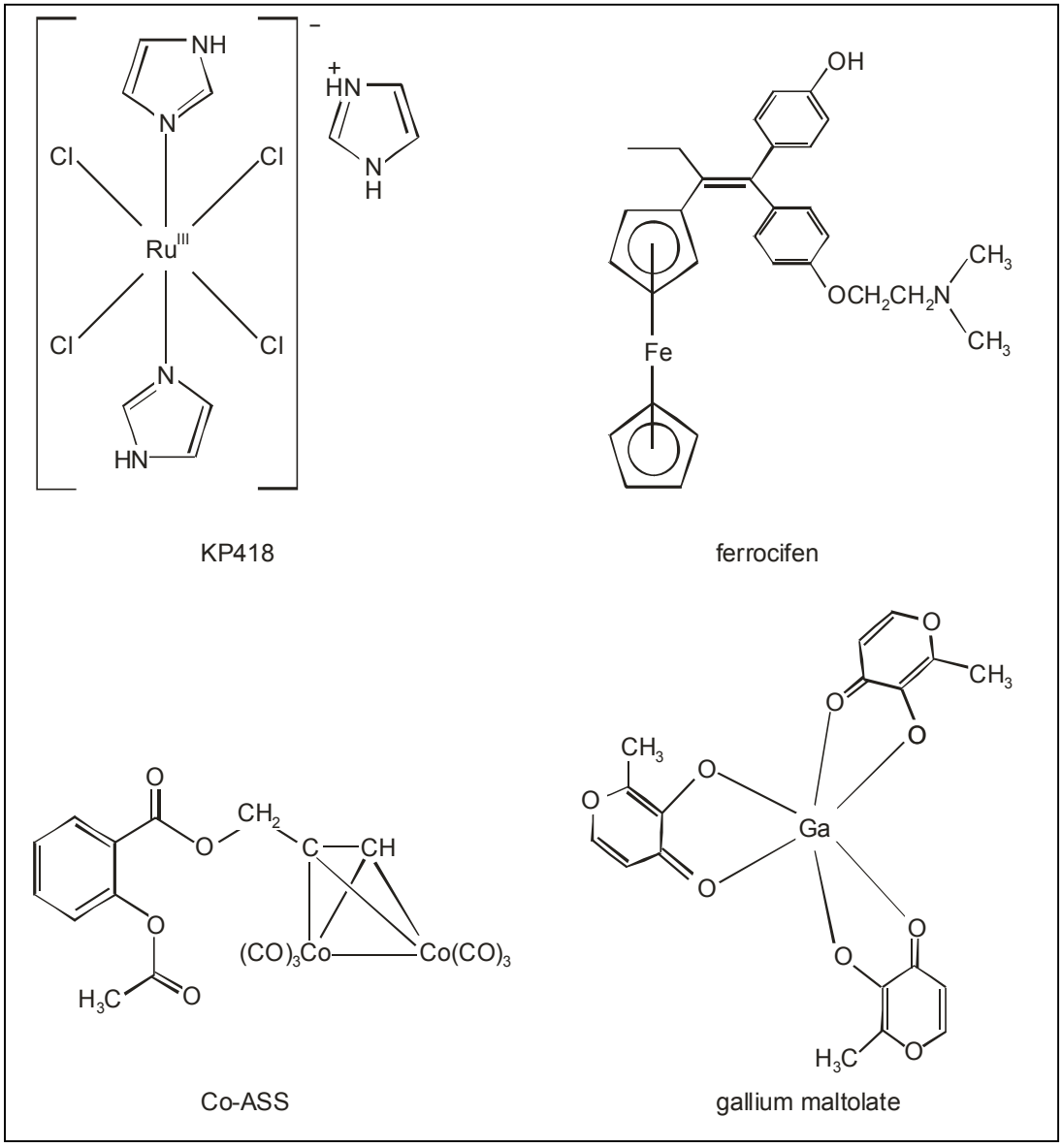


Fig.7. Chemical structures of some non-platinum metal compounds

Ruthenium complexes have lower cytotoxicity compared with cisplatin but they are better tolerated in vivo (Levina et al, 2009). The Ru^{III} complexes maintain the metal oxidation state until they reach the tumor cells where the low oxygen level allows their activation through reduction to Ru^{II} compounds (Kostova, 2006). The studies have shown that ruthenium-derived compounds interact with DNA and this interaction that generates DNA adducts is the mechanism of their cytotoxic effect (Brabec 2002; Zeglis, 2007; Meng et al., 2009). One of the most promising ruthenium compound is KP418 whose chemical structure is shown in figure 7.

The fericenium salts were the first iron complexes found to have some antitumoral activity (Köpf -Maier et al, 1984). Recently, ferrocene derivatives of

tamoxifen like ferrocifen (fig. 7) have been shown to have antiproliferative effect (Vessières et al., 2005; Hillard et al., 2005; Zanellatoa et al., 2009).

In the case of cobalt compounds is worth mentioning hexacarbonyl dicobalt which can form with alkynes organic complexes that exhibit antiproliferative activity in the case of several types of cancer (especially breast cancer). When the alkyne is the propargylic ester of aspirin the resulted compound is Co-ASS (fig. 7.) which is one of the cobalt compounds with the most remarkable antitumoral effect (Ott et al., 2005). The antiproliferative activity of CO-ASS may be exerted through a dual mechanism because studies have shown that CO-ASS has also a strong inhibitory effect on cyclooxygenase and therefore it slows the tumoral growth and it increases the tumor response to therapy (Jeon and Song, 2006).

The last metal that is discussed in this paper for the cytostatic potential of some of its complexes is gallium. Gallium (III) has coordination characteristics similar to iron (III), but unlike iron, gallium is redox-inactive in cellular environments. Among gallium compounds one must remind gallium maltolate (fig. 7) which is a promising chemotherapeutic agent for the treatment of hepatocellular carcinoma (Chua et al., 2006) and it also proves to have antimicrobial activity (Coleman et al., 2010).

4. EXPERIMENTS ON ANIMALS

Research on animals has a great importance in nutrition toxicology and medicine. In the case of nutrition experiments on animals are used especially in investigation on the importance of various nutrients and in order to establish the recommended daily allowance for various micronutrients. In toxicology, animal test are used especially in order to determine the toxic effects of various chemical xenobiotics and to establish various doses and limits for each toxic substance (e.g.: lethal or semilethal doses, no observable adverse effect level – NOAEL etc). In the case of medicine this type of studies are used in understanding diseases and in developing ways to prevent and treat them. Animal studies have a dramatic impact on the progress of medicine as scientists need these kinds of studies to test medical treatments for effectiveness and test new drugs for safety before beginning human testing. Small rodents (rats, rabbits, mice) are usually used to determine the possible side effects of new drugs. One must note that there is no real alternative to animal research as biological systems are complex and cannot be fully replaced by cell or tissues cultures. The nervous system, blood and brain chemistry, the gland secretions are all interrelated and it is not possible to predict the result of a treatment without observing and monitoring the entire living system.

The experiments on animals regarding the effect of various organometallic compounds are usually of toxicological interest or of pharmacological interest. The toxicological studies aim mainly to find the health effects produced by metallic compounds while the pharmacological studies try to develop new organometallic based drugs or to identify the potential side effects of the existing or of the new developing drugs. In order to understand the way in witch the various xenobiotics exerts their effects, it is considered opportune to remind the specific phases of xenobiotic kinetic that were discussed in chapter 2. Thus the four specific phases, as illustrated in fig. 8, are the absorption of chemical xenobiotics, biotransformation (xenobiodegradation and xenobiosynthesis), distribution of xenobiotics and/or their xenobioderivatives in extracellular fluid and elimination of xenobiotics and/or their xenobioderivatives.

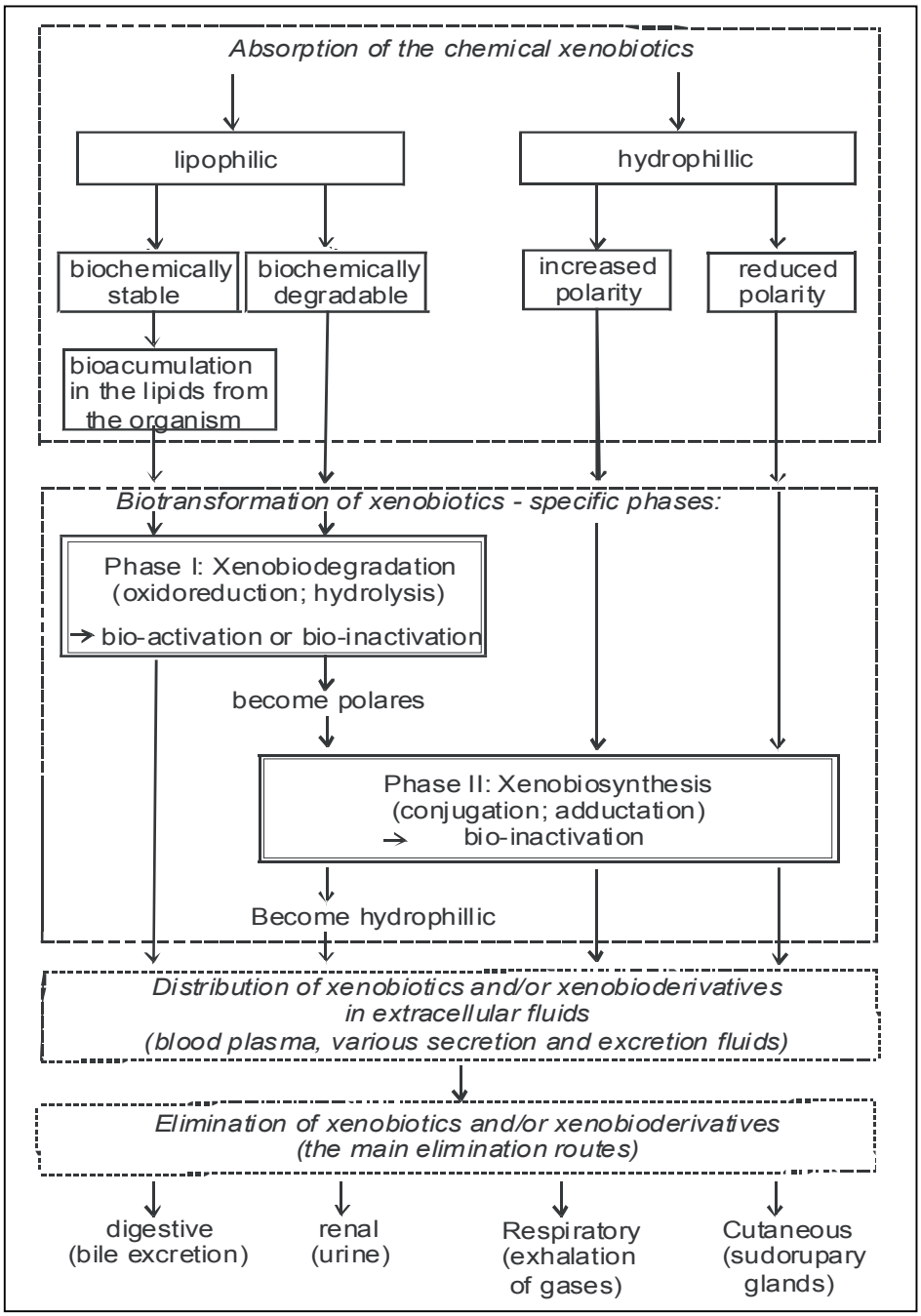


Fig. 8. Specific phases of the xenobiotic kinetics (Garban, 2007)

When discussing about metlas and their compounds one must take into account that there are cases of metal elements (e.g. Ni, Cr, Zn) which have a great importance in nutrition but also are found incriminated for carcinogenic effects or studied for their potential in cancer therapy. It is a known fact that in the case of metals their effects are strongly influenced various factors like dose or chemical form. An example that clarifies the situation presented above is the case of nickel, an element which plays an important role in the function of some metallo-enzymes (thus it can be considered a biometal) but simultaneous it is known as a environment carcinogen causing DNA damage and protein–DNA crosslinks. At the same time certain nickel complexes are studied for their potential in antitumoral drugs development. Nickel compounds have two characteristics in common with leading

antitumour drugs: direct metal binding to N_7 of guanine is possible and nickel complexes are able to catalyze oxidative damage to nucleic acids (Surendra Babu et al., 2010).

The importance of animal studies with metal compounds will be discussed separately for nutrition, pathobiochemistry and pharmacology.

4.1. Experiments on animals of nutritional importance

Regarding the field of nutrition there are few studies made on the impotance of biometals DNA adducts. Most of the current experiments on animals regarding this domain are aimed in establishing the effects and biological role of biometals and the outcome of their excess or deficiency from the diet (Knudsen, 1989). Biometals are found usually in proteic compounds (metalloproteins). An example is the investigation of magnesium deficiency on Sprague-Dawley female rats (Stendig-Lindberg et al, 2004) which underlined the fact that prolongued magnesium deficiency causes osteoporosis. Another animal study which is at threshold of nutrition and medicine investigates using twenty-two Sprague Dawley rats with a induced unilateral cervical hemicontusion that received 2 hours later magnesium chloride ($MgCl_2$) in polyethylene glycol formulation. The results showed an amelioration of the secondary damage and improved behavioral recovery (Lee et al, 2010). Other animal studies underline the effects of supplementation or deficit of Zn on bone metabolism and related gene expression in rats (Sun et al., 2010) or on healing of colon anastomosis in rats (Grommes et al, 2010).

4.2. Experiments on animals of pathobiochemical importance

A study focused on the DNA damage induced by lead (Pb), cadmium (Cd), and arsenic (As) in rat germinal cells and which tried to determine the relationship between DNA damage and blood Pb, blood Cd, and urine As levels is the study made by Nava-Hernández team. In this study blood from rats was collected by cardiac puncture (for lead acetate and cadmium chloride), while for arsenic trioxide a 24-h urine sample was collected. After the animals were sacrificed, pachytene spermatocytes from rat testes were extracted and purified. After reaching the desired cell purity and viability, DNA damage (tail length) was measured using single cell gel/comet assay. Significant DNA damage was found in primary spermatocytes from rats with chronic exposure (13 weeks) to toxic metals, suggesting that exposure to toxic metals may affect primary spermatocyte DNA and is responsible for direct testicular toxicity (Nava-Hernández et al., 2008). Experiments made on laboratory rats have shown that cadmium has also the ability of inducing apoptosis in rat thymus and testicle (Krichah et al., 2003).

Another important metallic element from the toxicological point of view is mercury. One of the most discussed mercury based organometallic compounds is methylmercury which is a potent carcinogen and also is known to induce neurotoxicity and apoptosis (Ceccatelli et al., 2010). A study on male albino rats exposed to 1mg/kg body wt of methylmercury chloride for seven days shown after performing the biochemical investigations for rate of lipid peroxidation, nucleic acids, proteins in cerebrum, cerebellum and brain stem that there was an increase in the rate of lipid peroxidation (showing methyl mercury induced free radical stress) and a lowering in the levels of nucleic acids and proteins (showing that methymercury inhibits DNA and protein synthesis) as compared to controls. The

motor and memory functions of the animals were also assessed and shown a clear decline indicating neurotoxic and neurodegenerative effects (Zahir et al., 2006).

Experiments made on laboratory mice with nickel chloride have that nickel exposure produced moderate oxidative stress in testis of mice, which was apparently associated with apoptotic cell death and DNA damage in testis. The genotoxic effects can be interpreted as a specific effect on spermatozoa and spermatids, which can play a significant role in the development of male infertility. These results suggest that nickel-induced testicular dysfunction at lower sublethal doses is wholly or partly mediated through oxidative damage to macromolecules, including damage to DNA (Doreswamy et al., 2004).

4.2. Experiments on animals of pharmacological importance

In the case of platinum compounds with antitumoral effects various studies were made on laboratory animals in order to asses the efficiency and the potential side effects. An example is the experiment carried out by Esteban-Fernández team (Esteban-Fernández et al., 2008) with Wistar rats treated with cisplatin, carboplatin, and oxaliplatin. The experiment aimed to study Pt-drugs accumulation and elimination, and Pt-biomolecule distribution in the cells and cytosols of ear, kidney, and liver. The bioaccumulation of platinum was determined using inductively coupled plasma-mass spectrometry (ICP-MS) and the results shows that cisplatin bioaccumulation capability is situated between oxaliplatin (the highest) and carboplatin (the lowest). Animal models were also used in order to determine the link between the repair capacity of DNA as result of cisplatin administration and the incidence of neurotoxicity induced by cisplatin (Dzagnidze et al., 2007). The results of Dzagnidze experiment on mice underlined the fact that suboptimal DNA repair in critical cells of the nervous system accelerates the accumulation of DNA cross-links during chronic application of cisplatin and may thus represent an important risk factor for drug induced neurotoxicity. Also cisplatin-DNA adducts formation and the potential of other drugs to interfere in this process was studied on animals. Such an experiment was performed on rats and was focused on the effects of amifostine on cisplatin induced DNA adduct formation (Bergström et al., 1999).

Nowadays most of the animal studies in the field of oncology are focused on new organometallic compounds containing metals like ruthenium, iron, cobalt, gallium, titanium, iridium, gold, tin etc. An example is the novel ruthenium-gamma-linolenic complex [Ru(2)(aGLA)(4)Cl] which was found to inhibits C6 rat glioma cell proliferation and to induce changes in mitochondrial membrane potential (Ribeiro et al., 2010).

Another interesting study is a comparison of the antiproliferative activity of mefenamic acid and its metal complexes with manganese (II), cobalt (II), nickel (II), copper (II) and zinc (II). Their inhibitory effects on rat paw edema induced by Carrageenan was studied and compared. The complex [Zn(mef)2] exhibited a strong inhibitory effect, superior to the inhibition induced by mefenamic acid at the same dose (Kovala-Demertzi et al., 2009).

Titanocene compounds are a novel series of organometallic compounds containing titanium that exhibit cytotoxic effects. Mice treated through intraperitoneal injection with Titanocene Y and cisplatin. The results shown that Titanocene Y induced a significant inhibition of tumor growth, with 40% inhibition in mean tumor volume following drug treatment in comparison to control animals.

From this study, it is apparent that Titanocene Y efficiency is comparable with cisplatin (Bannon et al., 2007).

CONCLUDING REMARKS

As this paper has underlined, a large number of metallic ions may interact with the DNA macromolecule. In the case of biometals, the interaction usually plays an important role in the stability and in the biological function of the DNA. The problems arise when instead of a biometal, the metallic ion that interacts with DNA belongs to a metal with toxicological potential (e.g.: Cd, Hg etc). In this case the new complex that is generated (the DNA adduct) usually disturb the function of the DNA, especially the protein synthesis, the replication and transcription of DNA. Once the adduct is formed, the DNA repair enzymes will try to correct the error by eliminating the new formed DNA adduct. If the enzymes do not succeed to repair the DNA, or if the repairing is not done properly then the cell is compromised and the process of apoptosis (programmed cell death) will be triggered. In some cases the failure of DNA repair process may lead to mutagenic and carcinogenic effects. So the metals with toxicological potential often have the ability to generate DNA adducts and are often considered potent carcinogens. Anyhow, the same ability of generating DNA adducts and of inhibiting the DNA transcription and replication is what makes some of the organometallic compounds such effective cytostatic or antitumoral drugs. In addition to this, one must consider the fact that metal ions have a wide range of coordination numbers and of possible geometries which leads to an increased potential for building up molecules that are better suited for binding to specific biological targets. Therefore organometallic compounds remain a field with great potential for developing new drugs with applications in oncology.

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SERUM CREATININE LEVEL AND MUSCLE METALLOGRAMS IN RABBITS AFTER EXCESS OF SODIUM NITRATE ADMINISTRATION IN DRINKING WATER

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ABSTRACT

In order to evaluate the effects of sodium nitrate administration on serum creatinine a spectophotometrical method was used while for the determination of muscle tissue metallograms in rabbits it was used the atomic absorption spectrophotometry technique. The experimental model was created with the purpose of evaluating the effects of nitrates on the biochemical homeostasis, regarding especially the variations of serum creatinine and of some macro- and trace elements in muscle. Sodium nitrate was administered in drinking water of rabbits in high concentrations compared with the reference value equal to the maximum contaminant level (MCL) in drinking water set by Environment Protection Agency. The biochemical investigation revealed the effects of sodium nitrate - $NaNO_3$ administered to two groups of rabbits in concentrations of 20 x MCL and 40 x MCL. The analytical determinations underline the dyshomeostatic effects of nitrates on serum cfreatinine levels and on macro elements (Na, K, Ca, Mg) and trace elements (Fe, Zn, Cu, Mn, Ni) in the muscle of rabbits.

Key words: sodium nitrate, muscle, serum creatinine, metalograms

INTRODUCTION

Nitrates are natural components that are part of the nitrogen cycle. Nitrates can be generated in nature through the nitrification on ammonia ions (NH_4^+), which is oxidized to nitrite by the *Nitrosomonas bacteria* and than further oxidized to nitrate by *Nitrobacter bacteria* (Bhaskar and Charyulu, 2005). This conversion takes place in the environment and also in the digestive tract of animals and humans. So in the case of animals and human there is an exogenous intake and an endogenous source (Terblanche, 1991).

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Nitrates distribution in soil, water and air is made accordingly to an equilibrium imposed by the peculiarities of the nitrogen cycle. The extensive agriculture with the use of fertilizers based on nitrates and also the industrial pollution often leads to the accumulation of high levels of nitrates in the environment affecting the cycle of nitrogen and the natural distribution of nitrates (Prakasa and Puttanna, 2002). From the environment the excess of nitrates accedes in plants and finally they accumulate in the organism of animals and humans thus exerting their toxicogenic effects by generating nitrites with methemoglobinizing effects (Speijers et al, 1989) or if certain conditions are met (i.e.: the presence of nitrites and amines) nitrates consumption may lead to carcinogenic nitrosamines (Vermeer et al., 1998; Rostkowska et al., 1998; Gârban, 2005).

Nitrate excess resulted accidentally or induced experimentally known to cause disturbances of kidney and liver function and also to alter the biochemical parameters of blood serum (urea, cretinine, uric acid), but the major manifestation of their toxic effects remains the generation of nitrites which react with hemoglobin and cause methemoglobinemia (Zabulyte et al., 2007; Ghibu et al, 2008a).

Regarding the rabbit muscles, the investigation of nitrate action is important not only in medicine but also in nutrition and in food sciences because the rabbit muscles have also alimentary importance. An aspect which is often neglected in the scientific literature is the fact that nitrates have in their chemical structure metallic ions which are released inside the organism and may interact with other bioconstituents and interfere in the absorption and biodisponibility of other biometalic ions (Ghibu et al, 2008b). In the case of muscles, where the balance of water and electrolytes is very important for the normal functioning, sodium nitrate may disturb especially the electrolytes balance with physiopathological implications.

The content of macro and trace elements in rabbit muscles is important from the nutritional point of view because the low content of sodium and the high content of magnesium as compared with other animal species used in alimentation make the rabbit suitable for diets for cardiovascular diseases (Hermida et al, 2006). Therefore a study on the influence of sodium nitrate on the mineral composition of rabbit muscle can offer useful information in the field of nutrition and food science.

MATERIALS AND METHODS

Experimental model. The effect of sodium nitrate excess in drinking water was studied using rabbits as laboratory animals. The nitrate solutions were prepared by dissolving sodium nitrate - $NaNO_3$ in the tap water of rabbits. The water was administrated “ad libitum” and the effects of nitrates consumption on serum creatinine concentration and the mineral metabolism of rabbits were studied. The reference value chosen in the preparation of nitrates solutions was the maximum contaminant level (MCL) admitted in drinking water, a value 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 $N\text{-}NO_2$).

The animal subjects used were rabbits (Oryctolagus cuniculus) with the age of 30 days and the average weight of 700 ± 25 g which were included in two experimental groups: $E_{A(1)}$, $E_{A(2)}$ and a control group. All groups were comprised of 10 animals (5 males and 5 females). The rabbits were fed with VivaBio - a granulated fodder 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 tap water from the same source as the water used to prepare the nitrate solutions. The animals from $E_{A(1)}$ group received $NaNO_3$ solution with a concentration equivalent with 20 x MCL and $E_{A(2)}$ group received $NaNO_3$ solution with a concentration equivalent with 40 x MCL established for nitrite in drinking water.

In order to allow the rabbits to accommodate with the laboratory environment and the new diet, a quarantine period of 10 days was kept previous to the beginning of the experiment. Also in the quarantine period the health status of rabbits was observed. After the quarantine, the experiment has started and lasted 20 days.

Blood samples necessary for the biochemical investigations regarding serum creatinine levels were collected in three different periods. First prelevation was made before the beginning of sodium nitrate solutions administration and the collected blood was used as reference value. The second blood prelevation was made at the end of the firs decade of the experiment (10^{th} day) and the last prelevation was performed at the end of the experiment (20^{th} day). The blood was collected only from the experimental groups $E_{A(1)}$ and $E_{A(2)}$ as the prelevation made before the start of the experiment was considered to be the control value.

Venipuncture was made after a preliminary narcosis with acepromazine maleate (1 mg/kg), a tranquilizer which also a have a vasodilatatory effect that makes the blood collecting procedure easier. The election place for the bleeding procedure was the auricular vein. The blood had been collected in 7 ml gradated vacuumtainers, the collected blood quantity being 3 ml. After prelevation, the blood samples were left at the room temperature in order to obtain the serum.

In order to determine the muscular metallograms, the subjects were killed and muscle samples were prelevated. On this purpose ketamin was administered intravenously. Muscle samples were taken according to the techniques of laboratory animal's necropsy and stored in 25 ml glass bottles which were placed in a refrigerator until the analytical determinations were performed.

Biochemical investigations. Serum creatinine concentration was determined in each serum samples obtained from experimental groups $E_{A(1)}$ and $E_{A(2)}$ using a biochemical analyzer. In all the situations, the analytical determinations were preceded by the calibration of the apparatus. In order to determine serum creatinine the biochemical analyzer uses a colorimetric method with sodium picrate (Jaffé method). Creatinine forms with sodium picrate a colored complex containing ionic bonds. The rate of formation of the colored complex is proportional to the creatinine concentration. The colored complex is determined at the wavelength of 492 nm.

Muscle mettalograms was determined in the samples prelevated from each animal of the control and experimental groups. The determinations of macro elements (Na, K, Ca, Mg) and trace elements (Fe, Zn, Cu, Mn, Ni) were performed in the "Laboratory of molecular and atomic spectroscopy" of the Faculty of Food Products Technology from Timişoara.

The muscle tissue samples were weighted and then calcinated at a 700 ºC temperature for a 3 hours time. The obtained ash was mineralized with nitric acid (0,5 N) and brought in gradated flasks of 50 ml. The obtained solutions were analyzed using a spectrophotometer with continuous atomic absorption. The model of the spectrophotometer was Analytik Jena ContrAA 300. Analytical data were expressed in μg/g wet weight.

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

RESULTS AND DISCUSSIONS

The obtained results will be discussed focusing first on the influence of sodium nitrate on serum creatinine levels and then regarding the effects of sodium nitrate on muscle mettalograms.

Sodium nitrate effect on serum creatinine level

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).

Regarding the modification of serum creatinine quantum after sodium nitrate administration, data are given in table 1.

Table 1. Homeostatic variations of serum creatinine after $NaNO_3$ administration in different concentrations.

Specification	UM	$E_{A(1)}$		$E_{A(2)}$	
		n	$\overline{X} \pm DS$	n	$\overline{X} \pm DS$
Preliminary	mg/dL	10	0,89 ± 0,08	10	0,83 ± 0,05
Decade I	mg/dL	10	0,99 ± 0,09	10	1,04 ± 0,05*
ΔX_I			+ 0,10		+ 0,21
Decade II	mg/dL	10	1,11 ± 0,14**	10	1,12 ± 0,15*
ΔX_{II}			+ 0,22		+ 0,29

* $p < 0,01$; ** $p < 0,05$

From the obtained values an increase of serum creatinine levels consecutive to nitrate solutions administration is revealed. The variation of creatinine concentration is directly proportional with the concentration of the sodium nitrate solution. The Student test shows significant variations in the case of $NaNO_3$ administration in all cases except for the first decade blood prelevation from $E_{A(1)}$ group.

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 hiperkinesy (explained through the biochemical interactions regarding the interconversion between creatin and creatin-phosphate)

Similar data on biochemical homeostasis after to sodium nitrate administration have been found in rats by Zabulyte et al. (2007). In another study that approached the problem of $NaNO_3$ administration on experimental groups of 5 rabbits there was revealed that serum cretinin values increase together with those of uric acid and urea from serum (Shour et al, 1999).

Sodium nitrate effect on muscle metallograms

The study of the sodium nitrate influence on muscle metallograms regarded the variations of some essential macro and trace elements from the muscle tissue.

The macro elements investigated in this paper were: Na, K, Ca and Mg. The homeostatic modifications produced by sodium nitrate solutions administrations in different concentrations (20xMCL and 40xMCL) are presented in table 2.

Table 2. The quantum of muscle macro elements in rabbits after administration of $NaNO_3$ solutions in different concentrations

Group	**UM**	**n**	**Na** $\overline{X} \pm DS$	**K** $\overline{X} \pm DS$	**Ca** $\overline{X} \pm DS$	**Mg** $\overline{X} \pm DS$
C	μg/g	10	589,05 ±39,73	3108,80 ±194,53	91,49 ±8,03	241,21 ±30,37
$E_{A(1)}$	μg/g	10	613,23 ±74,81	3016,59 ±194,07	103,84 ±11,45**	237,09 ±12,43
$\Delta X_{A(1)}$			+ 24,18	- 92,21	+ 12,35	- 4,12
$E_{A(2)}$	μg/g	10	647,25 ±79,24	2936,28 ±291,00	108,15 ±11,12*	233,40 ±16,87
$\Delta X_{A(2)}$			+ 58,20	- 172,52	+ 16,66	- 7,81

* $p<0,01$; ** $p<0,05$

According to the obtained values, increase of Na and Ca (elements with extracellular distribution) and a depression of K and Mg (elements with intracellular distribution) were revealed. The variations are direct proportional with the concentrations of the sodium nitrate solutions. Significant variations were found only in the case of Ca

This variations evidentiate a disequilibrium with implications in the functioning of “N-K pumps”, “Ca-Mg pumps” and in the muscular contraction and distension mechanism. These effects correlate with the homeostatic variations of creatinine in explaining the behavioral manifestations of rabbits during the experiment (e.g.: hyperkinesy).

In muscle tissue the antagonism between extracellular (Na, Ca) and intracellular (K, Mg) macro elements and the balance that is established between them has been demonstrated in experiments made on rats (Forbes, 1966).

Also in the case of humans, the experiments revealed an positive correlation between K and Mg concentrations while the K and Mg concentrations showed an negative correlation with Na and Ca (Tavichakorntrakool, 2007).

The trace elements investigated in this paper were: Fe, Zn, Cu and Mn. The homeostatic modifications produced by sodium nitrate solutions administrations in different concentrations (20xMCL and 40xMCL) are presented in table 3.

Table 3. The quantum of muscle trace elements in rabbits after administration of $NaNO_3$ solutions in different concentrations

Group	UM	n	Fe $\overline{X} \pm DS$	Zn $\overline{X} \pm DS$	Cu $\overline{X} \pm DS$	Mn $\overline{X} \pm DS$
C	μg/g	10	9,82 ±1,50	12,93 ±1,43	2,03 ±0,31	0,39 ±0,04
$E_{A(1)}$	μg/g	10	11,32 ±1,85	14,03 ±1,46	1,91 ±0,23	0,37 ±0,04
$\Delta X_{A(1)}$			+ 1,50	+ 1,1	- 0,12	- 0,02
$E_{A(2)}$	μg/g	10	12,51 ±1,01*	15,05 ±1,12*	1,84 ±0,21	0,34 ±0,05**
$\Delta X_{A(2)}$			+ 2,69	+ 2,12	- 0,19	- 0,05

* $p<0,01$; ** $p<0,05$

The obtained analytical results reveal an increase of Fe and Zn and a decrease of Cu and Mn. Significant variations according to Student test are found only in the case of sodium nitrate administration at 40xMCL in the case of Fe, Zn and Mn. The variations are direct proportional with the concentrations of the sodium nitrate solutions.

The increase of Fe and Zn in muscles may be a consequence of the observed behavior of rabbits. The instauration of hyperkinesia leads to a increase of the blood flow through the muscle tissue and therefore to the increase of iron concentration as iron is a constituent of blood heme.

Also Zn is a known component of many metalloenzymes such as lactate dehydrogenase which has an important role in the muscle activity generating lactic acid from piruvate (Maltin et al, 1983).

Piruvate conversion to lactic acid appears especially in hypoxia due to muscle intense exercise. Also muscle hypoxia may also be a result of methemoglobin formation.

CONCLUSIONS

Sodium nitrate administration reveals important dyshomeostatic effects on serum creatinine and muscle metallograms with implications in muscle physiology, effects that are manifested in the behavior of rabbits through a state of hyperkinesia.

After the administration of sodium nitrate serum creatinine levels increase direct proportional with the concentration of sodium nitrate solutions.

Sodium nitrate administration leads to dyshomeostatic modification of macro elements metallograms. A disequilibrium between extracellular electrolytes (Na, Ca) and intracellular electrolytes (K, Mg) with implications in the mechanism of muscle contraction and distension is revealed. The variations of macro elements concentration are directly dependent with the sodium nitrate concentration.

The analytical results shows an increase of Fe and Zn concentration consecutive to sodium nitrate administration, while Cu and Mn reveals a depression of their quantum in the muscle tissue. The dyshomeostatic effects of sodium nitrite are positive correlated with the nitrate solution concentration.

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NEW LIPOSOMES CONTAINING METAL OXIDES: ORIGINAL METHOD FOR EVALUATION OF COMPOSITION

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ABSTRACT

The paper presents an original method for evaluation of the composition of liposomes (natural or artificial lipidic mixtures) containing metal oxides. Two types of lipidic mixtures were used in order to obtain liposome nanocapsules: egg lecithin (phosphatidylcholine 50-60%, phosphatidylethanolamine 10-20%, phosphatidyl-inositol 4-6%, phosphatidic acid 3-5%, on the basis of HPLC analysis) and artificial lipidic mixture (L-α-phosphatidylcholine 86%, cholesterol 6%, stearylamine 8%). Liposomes containing metal oxides (undoped and Ag^+/Au^+ doped TiO_2) were obtained by using ultrasonication method and they were analyzed by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and energy-dispersive X-ray spectroscopy (EDS). TEM analysis indicate that the lecithin liposomes are multilamellar and relatively unstable, while lipid mixture liposomes are unilamellar and more stable, with diameters up to 500 nm and up to 300 nm, respectively. EDS-SEM analyses were used for evaluation of titanium dioxide and liposome mixture concentrations; titanium dioxide was more concentrated in unilamellar liposomes (up to 7%), comparatively with the case of multilamellar liposomes (1-2%).

Key words: liposomes, titanium dioxide, nanoparticles, nanocapsules, SEM, TEM, EDS

INTRODUCTION

The most used mixture compounds for protection and controlled releasing of various organic compounds (especially bioactive compounds) are liposomes (Kim and Baianu, 1991; Grabielle-Madelmont et al., 2003; Heurtault et al., 2003; de Leeuw et al., 2009; Malam et al., 2009). They are micro- or nanospheres which contain empty cavities, resulted by phospholipids assembling in water. Their membrane is

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formed by two or more double lipid layers, which contain the same water phase as in the exterior. These characteristics of liposomes reveal the possibility to encapsulate hydrophobic molecules in the double layer membrane or hydrophilic compounds in the interior cavity, as well as amphiphilic compounds (Nussinovitch, 1997).

Many applications result for these types of complex structures (de Leeuw et al., 2009; Malam et al., 2009): models for biological membranes, carriers for drugs or other bioactive compounds.

Historically, the first type of liposomes was multilamellars and was obtained by lipid-water interaction in different ratios. Modern liposomes are unilamellars, with well defined characteristics. Obtaining methods belong to the inverse phase evaporation (large unilamellars vesicles – LUV, with diameters of 100-1000 nm) and ultrasonication (small unilamellar vesicles – SUV, with diameters of 25-100 nm) (de Leeuw et al., 2009). Some liposomes are formed as polymer/liposome composites, in order to enhance the bioavailability and stability (Heurtault et al., 2003).

Liposomes have a great number of applications in the pharmaceutical (Grabielle-Madelmont et al., 2003; Derycke and de Witte, 2004; Bramwell et al., 2005; Ebrahim et al., 2005; Jaracz et al., 2005; Lee and Yuk, 2007) and food fields (Kim and Baianu, 1991). Stealth liposomes (Nussinovitch, 1997) have applications in the encapsulation of some anticancer, antifungal, antiviral, or antibiotic drugs, and in the food field liposomes are used for encapsulation of proteinases or other enzymes, antioxidants, food dyes, and vitamins (Heurtault et al., 2003).

Metal oxides (such as titanium dioxide or cobalt ferrite) have various biomedical applications. Thus titanium dioxide are used as UV absorbers in sunscreen products, cosmetic powders, creams, toothpaste, and in the cosmetics industry (Scott and Jones, 2002; Zou et al., 2003). Their most important properties are their lack of toxicity, compatibility with skin and mucous membranes, and good dispersibility in organic and inorganic solutions and binders. Cobalt ferrite nanoparticles can be used in the pharmaceutical field due to their hyperthermia effect (in anticancerigene formulations) (Jordan et al., 1999; Tedesco et al., 2004; Tanaka et al., 2005). Bioavailability of these water insoluble compounds can be enhanced by micro- or nanoencapsulation in matrices such as lipid mixtures for obtaining liposomes (Francescangeli et al., 2003; Hadaruga et al., 2009; Antimisiaris et al., 2009; Igarashi et al., 2009; Lazau et al., 2009; Caizer et al., 2010; Hadaruga et al., 2010; Popescu et al., 2010; Tardi et al., 2010).

In this study we investigated the possibility to encapsulate various metal oxides in natural or artificial lipid mixtures in order to obtain liposomes and to enhance the bioavailability of these inorganic compounds and try to elaborate a new technique for evaluation of the composition of liposomes containing these types of inorganic oxides with biomedical applications.

MATERIALS AND METHOD

Materials. Titanium dioxide doped with 1% Au^+/Ag^+ ions was obtained previously by sol-gel route according to (Hadaruga et al., 2009; Lazau et al., 2009; Hadaruga et al., 2010); the nanocrystals have dimensions between 10 and 40 nm. Cobalt ferrite ($CoFe_2O_4$, 10-20 nm) was obtained according to a previous study (Caizer et al., 2010). Natural lecithin (from egg, 35% phosphatidylcholine, PC, 25% phosphatidylethanolamine, PE, 15% phosphatidylinositol, PI, 7.5% phosphatidic acid, PA, and other phospholipids on the high performance liquid chromatography –

HPLC, analysis; Agilent 1100, Zorbax SB-C18, 250 x 4.6 mm x mm, 5 µm, 205 nm, Acetonitrile:Methanol 80:20, 0.8 mL/min; Figure 1) and artificial lipid mixtures (Sigma-Aldrich; each vial contains ~58 mg lyophilized powder, with a composition of 63 µmoles L-α-phosphatidylcholine, 9 µmoles cholesterol, and 18 µmoles stearylamine) were used for liposome synthesis.

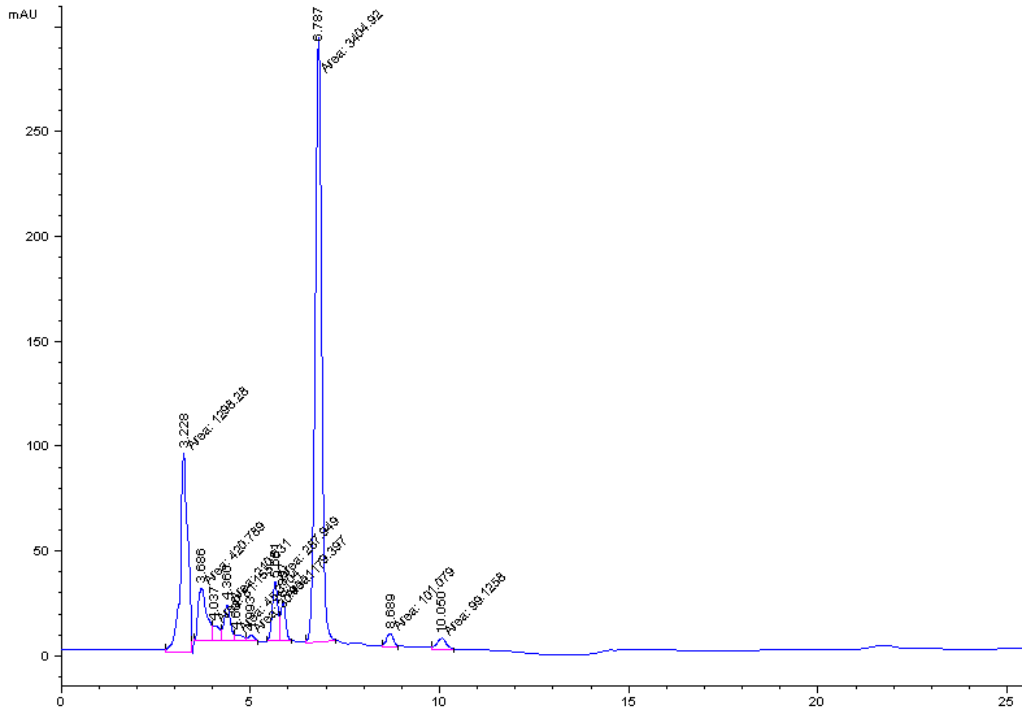


Fig. 1. HPLC analysis of natural lecithin used for obtaining liposomes

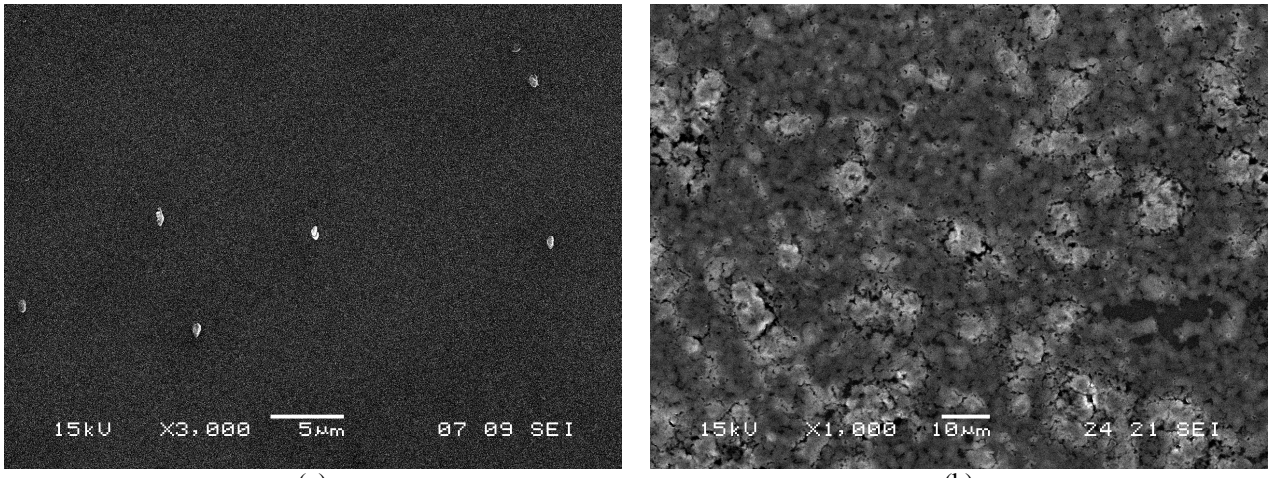
Obtaining and analysis of the liposomes containing metal oxides nanoparticles. Lecithin and lipid mixture liposomes containing titanium dioxide or cobalt ferrite were obtained by ultrasonication method. First, 0.025 g metal oxides (TiO_2 undoped or doped with 1% Au^+/Ag^+ or $CoFe_2O_4$ were suspended in 10 ml distilled water and the suspension was ultrasonicated in a flask (under cooling on ice) by using an Ultrasonic Liquid Processor Vibra Cell VC 505, 500 W, with the following conditions: amplitude 80%, ultrasonication time 15 minutes, pulse on 30 s, pulse off 15 s. Liposomes contain metal oxides were obtained from 0.0145 g lecithin/lipid mixture which are ultrasonicated in the same conditions with 4 ml distilled water and 1 ml metal oxides suspensions. After decantation, the liposome suspension was separed and analyzed by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and energy-dispersive X-ray spectroscopy (EDS) analyses. *SEM/EDS analysis*. Morphological and dimensional analysis of the liposomes containing metal oxides nanoparticles was performed by using a JEOL JSM 5510-LV apparatus coupled with EDS system, voltage of 15 kV, 300-150000× magnitude level. SEM analysis was performed on the non-covered and carbon-coated liposomes for EDS analysis. Carbon deposition was performed by using a JEOL JEE 4B vacuum evaporator, at a vacuum of 10^{-5} torr. *TEM analysis*. TEM was performed on a JEOL JEM 1010 apparatus, with a Mega View III CCD camera for acquisition of images and an acceleration voltage of 100 kV.

Evaluation of liposome composition. Original method for the evaluation of liposome composition (lipid mixture composition, metal oxide concentration, and

water) on the basis of SEM/EDS analysis was developed. Thus, the phospholipids (for natural lecithin) and L-α-phosphatidylcholine/cholesterol/stearylamine mixture contents (on the basis of compound concentrations from HPLC analysis or furnished by provider, respectively), the undoped or doped titanium dioxide nanoparticles or cobalt ferrite, as well as the water concentration in the final liposomes could be calculated by knowing the relative concentration of the main elements: C, Ti, Co, Fe, and O from EDS analysis. For example, Ti is present only in titanium dioxide nanoparticles, O is present in all components, but C is present in organic mixture used for obtaining these liposomes as well as on the surface of liposomes (EDS was performed on the carbon-coated samples; hydrogen is neglected), but the concentration of C from the surface of liposomes could not be established. Metallic ions used for doping the titanim dioxide nanoparticles are neglected. If C from the liposome surface is neglected, the lipid mixture content could be evaluated by using the percentage of C from EDS analysis. By knowing the Ti (or other metals) percentage the approximate metal oxide concentration of the resulted liposomes can be established. Finally, the concentration of water results from the percentage of O, after the excluding of the O percentage corresponding to the already known lipid mixture and metal oxide concentrations. The concentration of Au^+ and Ag^+ ions in the final liposomes cannot be calculated on the basis of EDS due to the very low concentration in the final liposomes, but they can be evaluated by knowing the initial composition of doped titanium dioxide nanoparticles (we presume that the metallic ions concentration are not modified by nanoencapsulation process).

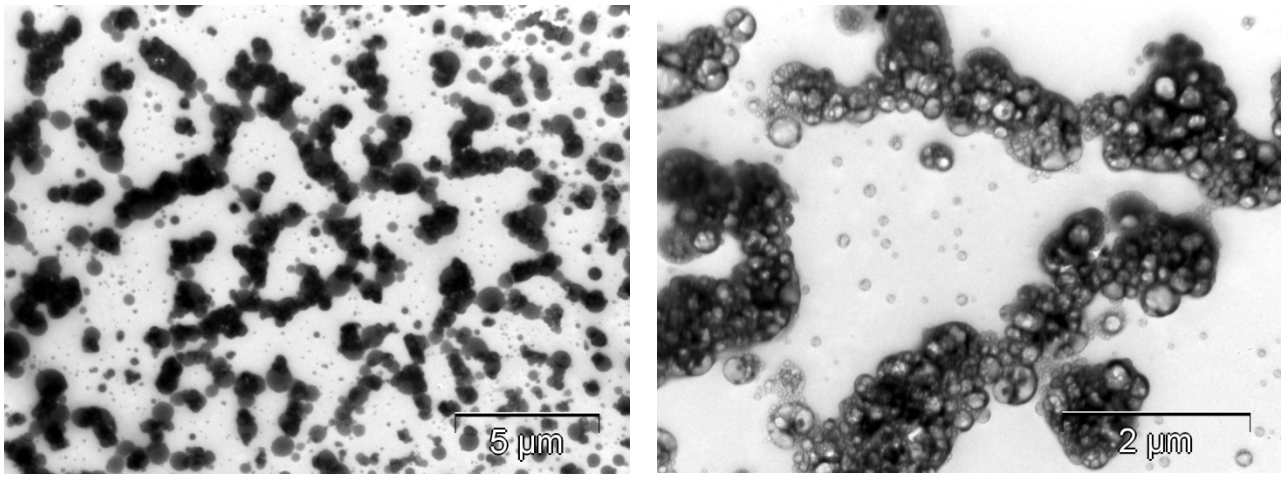
RESULTS AND DISCUSSION

Significant macroscopic differences were observed in the case of liposomes containing metal oxides: lecithin liposomes were obtained as cream-colored and opaque suspensions and lipid mixture liposomes containing even undoped titanium dioxide or Au^+/Ag^+ ions doped titanium dioxide were translucent (after the gravimetric decantation of the resulted suspension). SEM analysis of the uncoated and carbon-coated liposomes revealed that the lecithin liposomes are multilamellar and have non-spherical shapes with dimensions from 100 to 500 nm, while lipid mixture liposomes are unilamellar with spherical shapes and a relatively higher dimensional uniformity, 50-300 nm (Figures 2 and 3). More relevant images are obtained by TEM analysis of liposomes containing metal oxide nanoparticles. Thus, for the lecithin liposomes most of the nanocapsules appear as conglomerated structures in arcuated formations or in a chaotic disposition, which are formed by agglomerated liposomes with various diameters; some liposomes have attached electrondensely formations, probably metal oxides nanoparticles. Lipid mixture liposomes indicate that some of unilamellar particles contain metal oxide nanocrystals, but some of larger crystals only adhere to the liposomes (Figures 4 and 5).



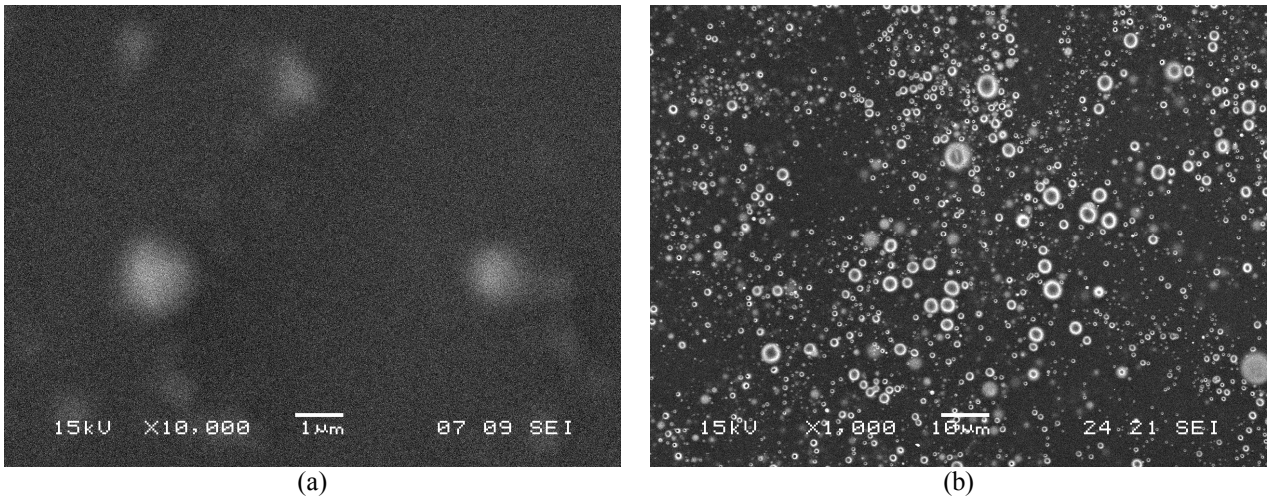
(a) (b)

Fig. 2. SEM (normal and carbon-coated) images (a and b) of lecithin liposomes containing titanium dioxide



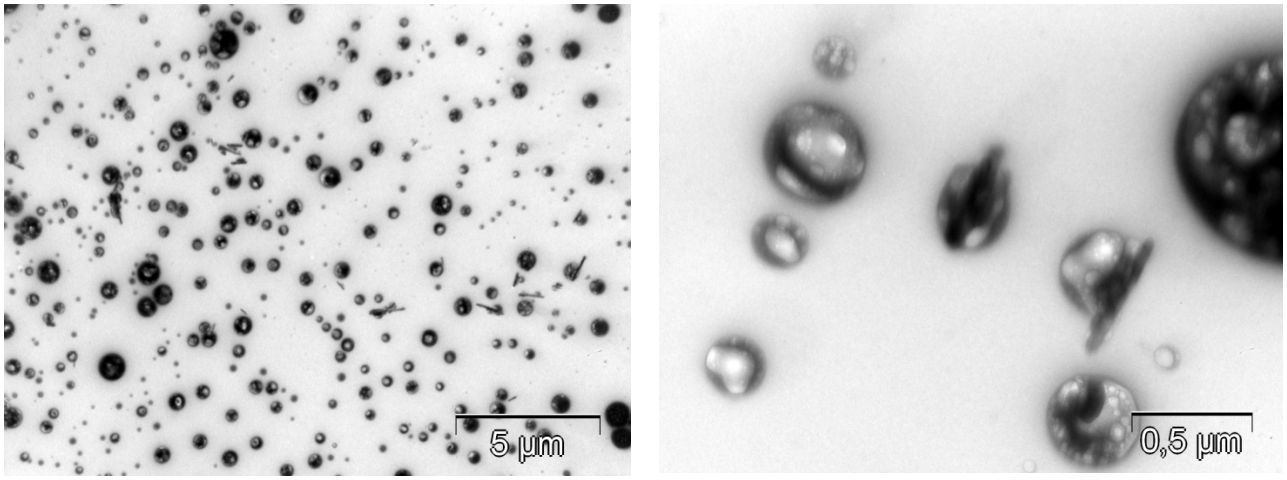
(a) (b)

Fig. 3. TEM images for lecithin liposomes containing titanium dioxide (a and b)



(a) (b)

Fig. 4. SEM (normal and carbon-coated) images (a and b) of lipid mixture liposomes containing titanium dioxide



(a) (b)

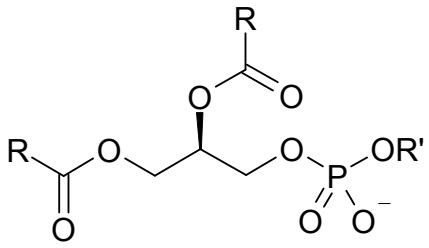
Fig. 5. TEM images for lipid mixture liposomes containing titanium dioxide (a and b)

Evaluation of the metal oxide concentration in liposomes, as well as lipid and water concentrations, a new calculation technique based on the EDS analysis was developed. Following conditions were considered in order to evaluate the metal oxide, lipid mixture, and water concentrations in liposomes:

- carbon used for coating of liposomes as well as hydrogen atoms from organic structures were neglected (carbon concentration from the liposome surface has insignificant concentration in comparison with the carbon from lipid mixture)
- concentration of metallic ions (such as Au^{+}/Ag^{+}) used for doping of metal oxides was neglected; it can be determined on the basis of initial concentration (1% for these case);
- liposomes are formed by the following components: lecithin (phospholipid mixture with known concentration) or artificial lipid mixture, metal oxide (titanium dioxide or cobalt ferrite), water.

The following steps for the evaluation of liposome compound concentrations were used:

1. Adjusted concentrations for phospholipid mixture of lecithin used were calculated on the basis of initial HPLC concentration (PE 25%, 7.5%, PI 15% şi PC 35%). The atomic percentage of lecithin were determined by knowing the molecular weight and molecular formulae of phospholipids; then, the adjusted atomic percentage (hydrogen was neglected), and the partial atomic percentages (for every component) were determined (Table 1). The same calculus were performed for the artificila lipid mixture (85.7% PC, 6.0% C, and 8.3% SA) (Table 2).



R - fatty acid moiety
OR' = choline moiety (phosphatidylcholine)
OR' = ethanolamine moiety (phosphatidylethanolamine)
OR' = inositol moiety (phosphatidylinositol)

2. The metal oxide concentration in liposomes can be evaluated by knowing the metal and oxygen concentrations and from EDS analysis. Thus, for titanium dioxide these percentages were 60% and 40% for Ti and O, respectively. C, Ti, and O concentrations in liposomes are indicated by EDS analysis (Figure 6), and after evaluating the lecithin concentration (on the C basis), the remaining O concentration is attributed to water.

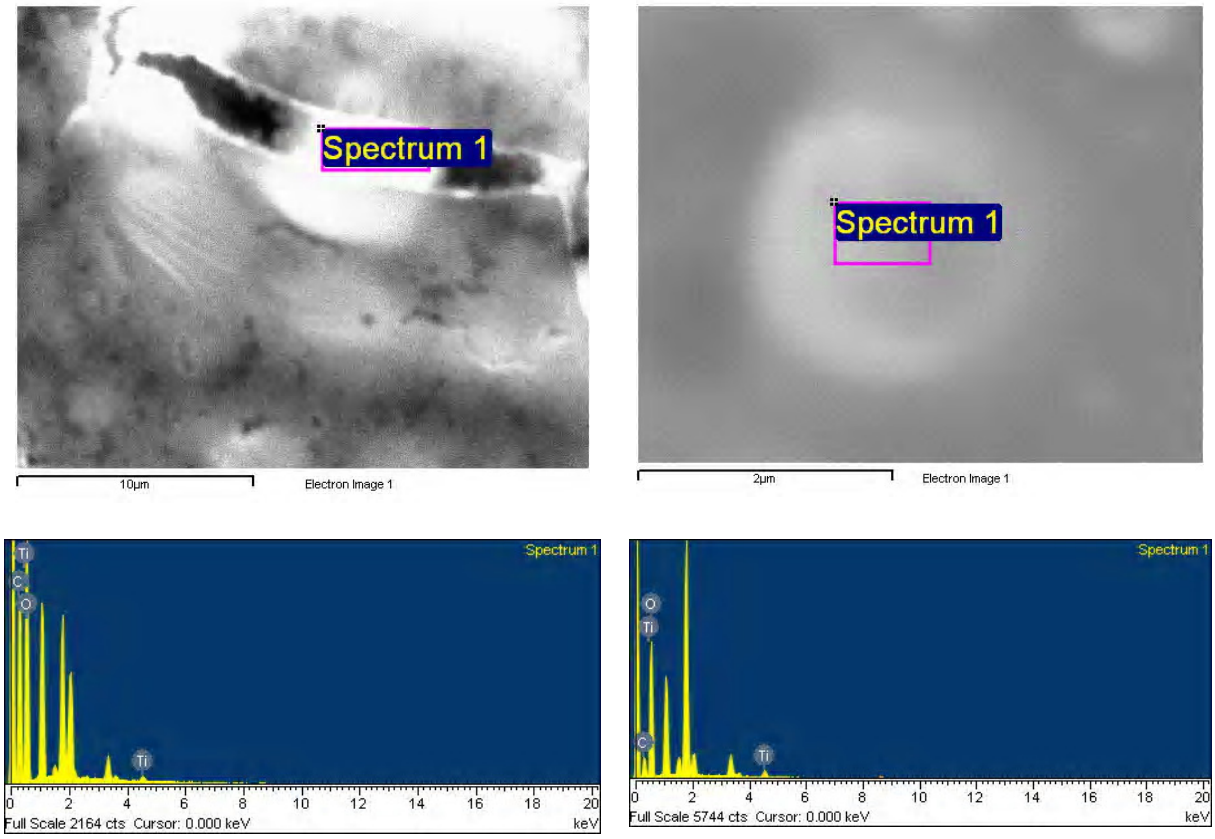


Fig. 6. EDS analyses of lecithin (left) and lipid mixture (right) liposomes containing metal oxides

Table 1. Atomic percentage evaluation for lecithin

Concentration and adjusted concentration for phospholipids

Compound	Conc (%)	Adj. conc (%)	MW (g/ mole)	n(C)	n(H)	n(N)	n(O)	n(P)
PE (Phosphatidylethanolamine)	25	30.3	749.1	41	83	1	8	1
PA (Phosphatidic acid)	7.5	9.1	591.8	31	60		8	1
PI (Phosphatidylinositol)	15	18.2	867.1	45	87		13	1
PC (Phosphatidylcholine)	35	42.4	760.1	42	82	1	8	1
Total:	*82.5*	*100*						

Atomic percentage (up) and adjusted atomic percentage (down)

Compound	%C	%H	%N	%O	%P
PE (Phosphatidylethanolamine)	65.68	11.08	1.87	17.09	4.14
PA (Phosphatidic acid)	62.86	10.14	0.00	21.63	5.24
PI (Phosphatidylinositol)	62.28	10.03	0.00	23.99	3.58
PC (Phosphatidylcholine)	66.31	10.79	1.84	16.84	4.08
	%C'	**%H'**	**%N'**	**%O'**	**%P'**
PE (Phosphatidylethanolamine)	73.98	2.11	19.25	4.66	73.98
PA (Phosphatidic acid)	70.06	0.00	24.11	5.84	70.06
PI (Phosphatidylinositol)	69.32	0.00	26.70	3.98	69.32
PC (Phosphatidylcholine)	74.45	2.07	18.91	4.58	74.45

Partial atomic percentage for lecithin phospholipids

Compound	%C"	%N"	%O"	%P"
PE (Phosphatidylethanolamine)	22.42	0.64	5.83	1.41
PA (Phosphatidic acid)	6.38	0.00	2.19	0.53
PI (Phosphatidylinositol)	12.62	0.00	4.86	0.72
PC (Phosphatidylcholine)	31.57	0.88	8.02	1.94
TOTAL:	*72.97*	*1.51*	*20.90*	*4.61*

Table 2. Atomic percentage evaluation for artificial lipid mixture

Concentration and adjusted concentration for lipid mixture

Compound	Conc (%)	Adj. conc (%)	MW (g/ mole)	n(C)	n(H)	n(N)	n(O)	n(P)
PC (L-α-Phosphatidylcholine)	49.8	85.7	791.2	44	89	1	8	1
C (Cholesterol)	3.5	6.0	386.7	27	46		1	
SA (Stearylamine)	4.9	8.3	269.5	18	39	1		
Total:	*58.2*	*100.0*						

Partial atomic percentage for lipid mixture

Compound	%C	%H	%N	%O	%P
PC (L-α-Phosphatidylcholine)	66.74	11.25	1.77	16.18	3.92
C (Cholesterol)	83.79	11.90	0.00	4.14	0.00
SA (Stearylamine)	80.14	14.47	5.19	0.00	0.00
	%C'	**%H'**	**%N'**	**%O'**	**%P'**
PC (L-α-Phosphatidylcholine)	75.32	0.00	2.00	18.26	4.42
C (Cholesterol)	95.29	0.00	0.00	4.71	0.00
SA (Stearylamine)	93.91	0.00	6.09	0.00	0.00

Partial atomic percentage for lipid mixture

Compound	%C"	%N"	%O"	%P"
PC (L-α-Phosphatidylcholine)	64.53	1.71	15.64	3.79
C (Cholesterol)	5.70	0.00	0.28	0.00
SA (Stearylamine)	7.83	0.51	0.00	0.00
TOTAL:	*78.07*	*2.22*	*15.93*	*3.79*

The concentration of the relevant elements for the case of lecithin liposomes containing metal oxides were C 19.8%, O 79.4%, and Ti 0.8% for the first duplicate and 38.2%, 60.5%, and 1.2%, for the second. According to the calculation technique presented above, the lecithin concentration is 25.5%, titanium dioxide 1.3%, and water 73.2%, for the first case and 49.1%, 2%, and 48.9%, for the second, respectively.

The concentration of metal oxides in the final lipid mixture liposomes was 7.4±2.00% (triplicates), for undoped nanocrystals, while the Au^{+} and Ag^{+} doped titanium dioxide containing liposomes have only 2.2% and 0.8% metal oxides.

CONCLUSION

The following conclusion among the evaluation of metal oxides concentration in liposomes can be drawn: (1) the lecithin liposomes containing metal oxides (such as titanium dioxide) have irregular shapes, are multilamellar, and the capsule diameters are in a wide range (100-500 nm), and the metal oxide/lecithin ratio in the obtained liposomes is approximately 0.05; (2) Unilamellar liposomes with higher uniformity, stability, and diameters up to 300 nm, having an approximate metal oxide concentration of 2-7% were obtained by using artificial lipid mixtures; (3) EDS analysis is a good tool for evaluation of metal oxides in complex products such as liposomes.

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STATISTICAL ANALYSIS OF WATER SAMPLES IN THE IMPACT AREAS OF DOMESTIC WASTE IN SUCEAVA COUNTY

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ABSTRACT

This paper presents briefly the results obtained after the implementation of a project on achieving a telematics system for the on line management and supervision of areas within city limits degraded following the uncontrolled waste storage. Our investigations had in view the determination of physico-chemical parameters (e.g. pH, conductibility), anionic compounds (nitrates, sulphates, chlorides etc.) and of some cationic compounds (Cr, Pb), all being important in ecobiology and xenobiochemistry. Achieving the ZoneMAP project was inscribed, given its main objective, in Directive 1999 / 31 / EC on storing waste and it referred to the implementation of measures, procedures and recommendations for preventing or diminishing the negative effects on the environment and health of population determined by the inadequate waste storage activities (EcoForum, 2008).

Key words: composition of water samples – statistical analysis

INTRODUCTION

Problems related to water and the compounds dissolved in it are of interest both for the terrestrial habitual external environment and for the environment of aquatic plants and animals (Adriano, 2001) by its involvments on biogeochemistry. In this framework the ecotoxicological problems are of main interest (Callow, 1993; Callow, 1994).

The pollution of ecosystems as an ecotoxicological problem (Moriartry, 1983) is more complex because water can carry both anionic mineral compounds (nitrates, chlorides, sulphates, phosphates etc.) and cationic compounds (heavy metals, some with toxicogen potential). By this way, through water, chemical compounds with toxicogen potential can penetrate in the tap water and in foods (Desphande, 2002; Omaye, 2002).

From ecotoxicological point of view the composition of water involves not only the soil where can appear pollutant substance but also the whole trophic chain plat-animal-human (Walter et al., 1996). Evident, the problem of water pollutanst is also the object of industrial toxicology (Lawerys, 1999).

In our investigations, presented in previous papers, we approached problems related to the harmful role of sulphur and heavy metals present in environment

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(Holban et al., 2002) and aspects related to the presence of transitional metals in food products (Holban, 2002). Existence of heavy metals in food products constitutes a real danger for health (Arowold, 2004).

Since past decades numerous scientific papers based on experimental works and biomedical observations evidenced the mutahgene and carcinogen effects of metals (Kazantzis and Lorna, 1979).

The object of this work is to present data concerning the distribution on anionic and cationic minerals (ions of heavy metals) in water.

MATERIALS AND METHODS

By the analyses achieved following the supervision, I wanted to assess the pollution level induced by the unarranged waste storage in the location area, at the limit of its perimeters (respectively the Radauti city waste storage)

None of the 7 city waste storages in Suceava County-Roumania, classified according to the waste types stored, as non-dangerous storages (class b) complies with the provisions in Directive no. 1999 / 31 / CE on storing waste (National legislation on environment protection).

According to the commitments assumed by Romania in the negotiation process of Chapter 22 – Environment, concerning the implementation of the Directive for storing waste, transposed in our legislation by the Government Decision No. 349 / 2005, transition periods were granted for the non-conform city storages as it follows (table 1). Data concerning the biochemical oxygen consumption at 5 days (BCO-5), the chemical oxygen consumption-tested with potassium dichromate method (COC-Cr) and the chemical oxygen consumption-tested with potassium permanganate method (COC-Mn) are presented in table 1 and will be discussed below.

Table 1. City waste storages in Suceava County that were granted a transition period

No.	County	Storage name	Area (ha)	Scheduled year for activity cessation
1	Suceava	Siret town storage	0.80	2008
2		Buliceni / Vatra Dornei town storage	1.70	2008
3		Suceava city storage	11.5	2008
4		Rădăuți city storage	4.43	2009
5		Antileşti / Fălticeni town storage	1.00	2010
6		Hurghiş / Câmpulung Moldovenesc town storage	1.62	2011
7		Gura Humorului town storage	2.12	2011

Closing the waste storages is achieved according to the provisions in the Government Decision no. 349 / 2005 and Order of the Ministry of Environment and

Waters Management no. 757 / 2004 for approving the Technical Normative on waste storage (Standards series “Environment management systems” - ISO 14.000)

The waste storage under survey is located in southeast part of Radauti city, approximately 5 km from the city, being achieved without foundation waterproofing and without wastewater draining, collection and cleaning systems. The platform’s neighbors to north, northeast and west is an agriculture land and to the south and southeast a cleaning station of the city having a perimeter of 900 m.

The waste storage operates since 1984. According to the commitments assumed by Romania, the storage was closed in 2009 following its reclamation procedure.

The storage exploitation was achieved by the surface deposit with work face advancement and compressing the waste with special plant that led, at the same time, to disseminating, leveling and pressing waste, thus diminishing the volume and increasing the density up to 0.8 - 1 t / cm.

Due to the fact there is not draining, collecting and cleaning system of the waters infiltrated in the waste mass, they are removed in the soil thus favoring the infiltration of miscellaneous pollutants (organic substances, pathogen germs, chemical substances resulting from different industrial waste, etc.) derived from garbage storage (Standards series “Environment management systems” - ISO 14.000).

1. STATISTICAL ANALYSIS OF WATER SAMPLES FROM POZEN CREEK, UPSTREAM AND DOWNSTREAM THE RĂDĂUTI CITY GARBAGE STORAGE

For emphasizing the quality of surface water and the effects created by the storage, the beneficiary took two water samples from Pozen creek (Table 2), one upstream and one downstream the location (Standards series “Environment management systems” - ISO 14.000). There are presented data regarding the total dissolved salts (TDS).

After comparing the results with the values imposed by the legislation in force, the following conclusions have been reached:

1. values of pH, upstream and downstream the storage are the allowed range 6.5- 8.5;

2. values of indicators referring to oxygen consumption : biochemical oxygen consumption BCO-5 ; chemical oxygen consumption-potassium dichromate method (COC-Cr); chemical oxygen consumption-potassium permanganate method COC-Mn.

3. values referring to anions : chlorides (Cl^-), total phosphates (PO_4^{3-}), sulphates (SO_4^{2-}) and values referring to cations (Pb^{2+}, Cr^{2+}) upstream and downstream the storage corresponding to the IInd quality class;

4. values of ammonium (NH_4^+) indicator, upstream and downstream the storage corresponding to the IIIrd quality class;

5. values of nitrates (NO_3^-) indicator, upstream and downstream the storage corresponding to the IVth quality class.

Table 2. Quality of water in Pozen Creek – samples taken upstream / downstream the landfill

No.	Indicator	Measurement unit	Indicator value	
			Pozen Creek - upstream	Pozen Creek - downstream
1.	pH	unit. pH	7.66	7.28
2.	COC-Cr	mg O_2/L	9.46	13.23
3.	COC-Mn	mg O_2/ L	5.39	7.21
4.	CBO-5	mg O_2/ L	2.62	3.44
5.	Nitrates	mg/ L	6.38	9.84
6.	Ammonium	mg/ L	0.53	0.50
7.	Chlorides	mg/ L	29.07	30.49
8.	Sulphates	mg/ L	64.48	71.89
9.	Phosphates	mg/ L	0.16	0.027
10.	Conductivity	µS/cm	621	632
11.	TDS	mg/ L	434	443
12.	Total Cr	µg/l	0.272	0.346
13.	Total Pb	µg/l	2.247	3.76

The analysis was made for the water samples taken from upstream and downstream the landfill located near Pozen Creek. The analysis was made for each component or components group if they concern the same chemical matter.

The data subject matter to statistical interpretation come from the parameter values sent by the system installed during the project implementation, from laboratory analyses of some samples taken during the project development period and from historical data made available by the authorities (Environment Protection Agency Suceava).

Histograms for the data collected (Figures 1, 2, 3, and 4) are presented below.

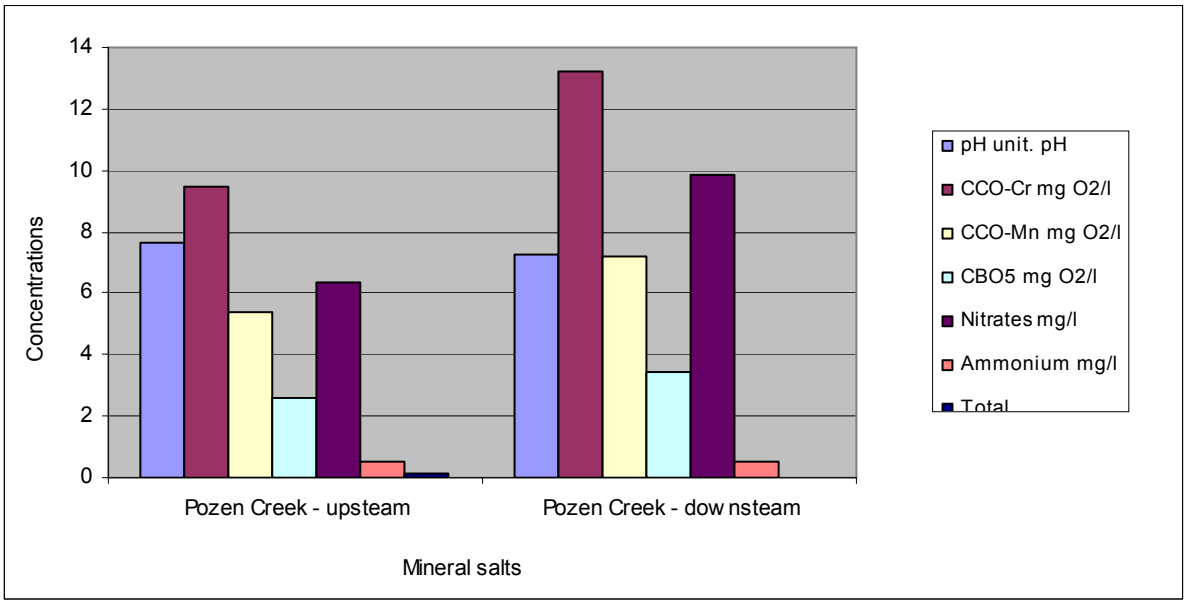

Fig. 1. Mineral salts / Concentrations Histogram.
Pozen Creek – upstream and downstream

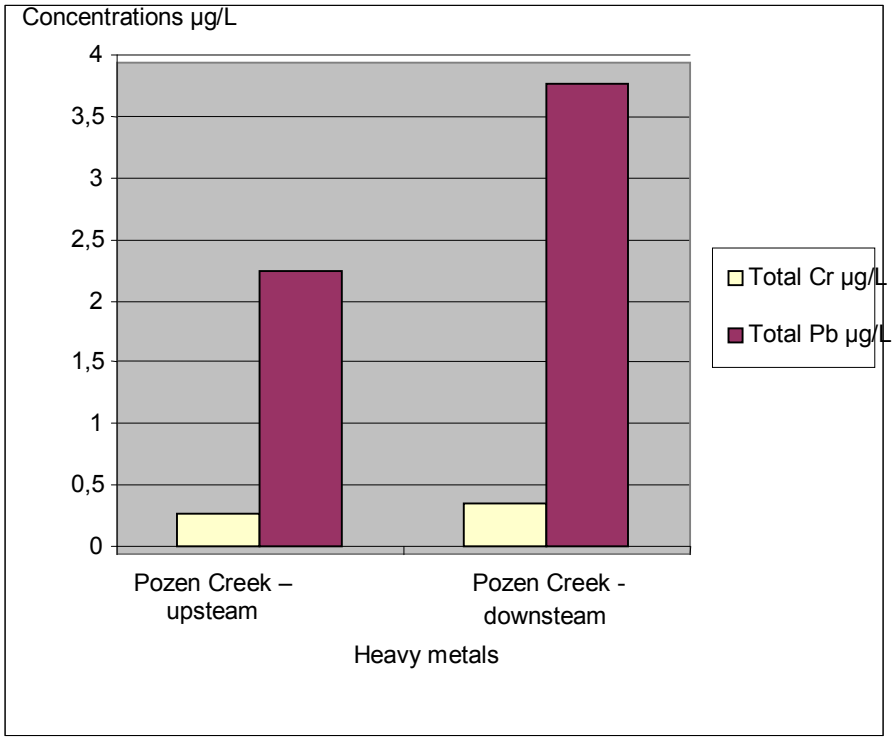

Fig. 2. Conductivity, TDS Histogram
Pozen Creek – upstream and downstream

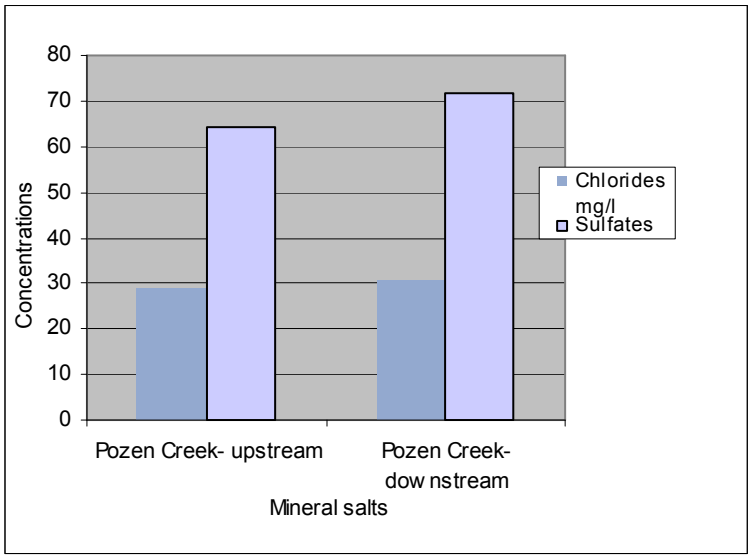

Fig. 3. Heavy metals / Concentrations Histogram.
Pozen Creek – upstream - downstream

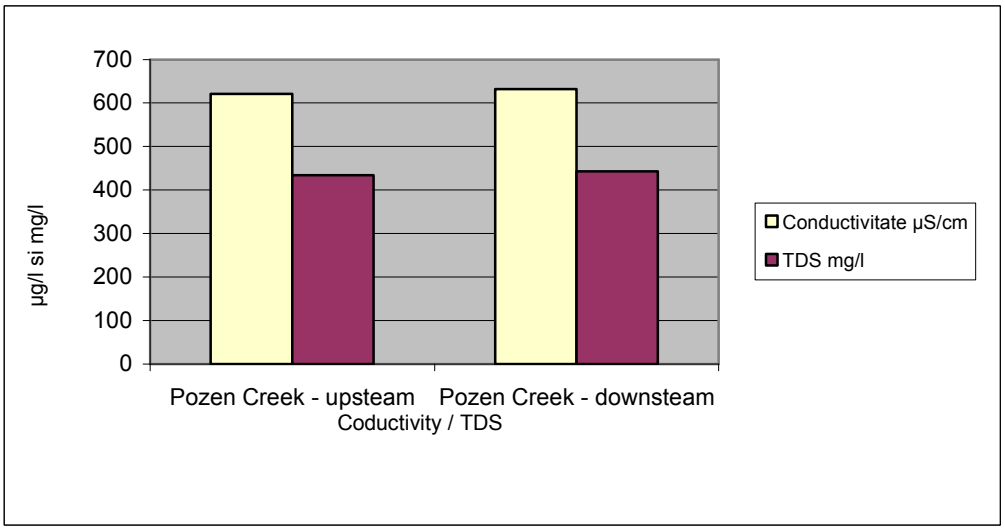

Fig. 4. Mineral salts (chlorides, sulphates) / Concentrations Histogram.
Pozen Creek – upstream - downstream

The conclusions presented from now on are obtained following a comparative quantitative analysis, without considering the qualitative aspects ands without reference to chemical type causes that led to those effects.

Downstream Pozen Creek was found an increase of concentration as against the upstream for the following mineral salts:

1. COC-Cr increased by approximately 20%
2. Nitrates increased by approximately 30%
3. Total Cr increased by approximately 60%
4. Chlorides increased by approximately 10%

The remaining mineral salts have insignificant concentration modifications in downstream Pozen Creek as against the samples taken upstream.

2. STATISTICAL ANALYSIS OF UNDERGROUND WATER SAMPLES FROM UPSTREAM AND DOWNSTREAM THE LOCATION OF THE RADAUTI CITY LANDFILL

For emphasizing the quality of underground water and the effects created by the landfill, two samples were taken from the control drills located upstream and downstream the location, on the flowing direction of underground water. The beneficiary took the samples.

The results of analytical trials (Report on the progresses registered at the time of preparing Romania for the accession to the European Union during September 2002 – June 2003, Government of Romania, June 2003) are presented in Table 3.

Table 3. Quality of water in Pozen Creek (analysis methods). Drill upstream / downstream the landfill

No.	Indicator	Measure-ment unit	Analysis method	Indicator value	
				Drill 1 - upstream	Drill 2 - downstream
1	pH	unit. pH	SR ISO 10523 / 97	7.15	7.09
2	COC-Cr	mg O_2/L	SR ISO 6060 / 96	57.02	19
3	COC-Mn	mg O_2/ L	STAS 9887 / 74	25.63	10.56
4	Sulphates	mg/L	STANDARD METHODS / 95	69.65	279.37
5	TDS	mg/L	SR EN 27888 / 97	803	1195
6	Nitrates	mg/L	SR ISO 7890-3 / 2000	0.248	0.414
7	Ammonium	mg/L	STAS 8683 / 70	6.76	6.35
8	Chlorides	mg/L	STAS 8663 / 70	56.72	181.52
9	Phosphates	mg/l	SR EN 1189 / 2000	0.242	0.07
10	Conductivity	µS/cm	SR EN 27888 / 97	1147	1707
11	Cr	µg/L	SR ISO 9174 / 1988	1.06	0.502
12	Pb	µg/L	SR ISO 8288 / 2001	2.95	1.371

After comparing the results with the values imposed by the legislation in force, the following conclusions were reached:

2.1. Drill F_1- upstream

Value of pH is inscribed in the approved values;

Value of conductivity is above the value allowed;

Values of the following indicators: ammonium, sulfates, COC-Cr, COC-Mn, indicate a significant pollution;

Values of the following indicators: nitrates, chlorides, Pb, Cr indicate an insignificant pollution.

2.2. Drill F_2- downstream

Value of pH is inscribed in the approved values;

Value of conductivity is above the value allowed;

Value of the following indicators: ammonium, sulfates, COC-Cr, COC-Mn, indicate a significant pollution;

Value of the following indicators: nitrates, chlorides, Pb, Cr indicate an insignificant pollution.

The significant pollution with ammonium, phosphates, and COC-Cr revealed from the drill located *upstream* the storage occurred given the influence of some cleaning station's installations and ducts located near the drill.

- The values of indicators ammonium, phosphates, COC-Cr diminish in the drill from *downstream* as against that in upstream.

These conclusions are obtained following a comparative quantitative analysis, without considering the qualitative aspects and without reference to the chemical type causes that caused those effects. Histograms for the data collected (Figures 5, 6, 7, 8) are presented below.

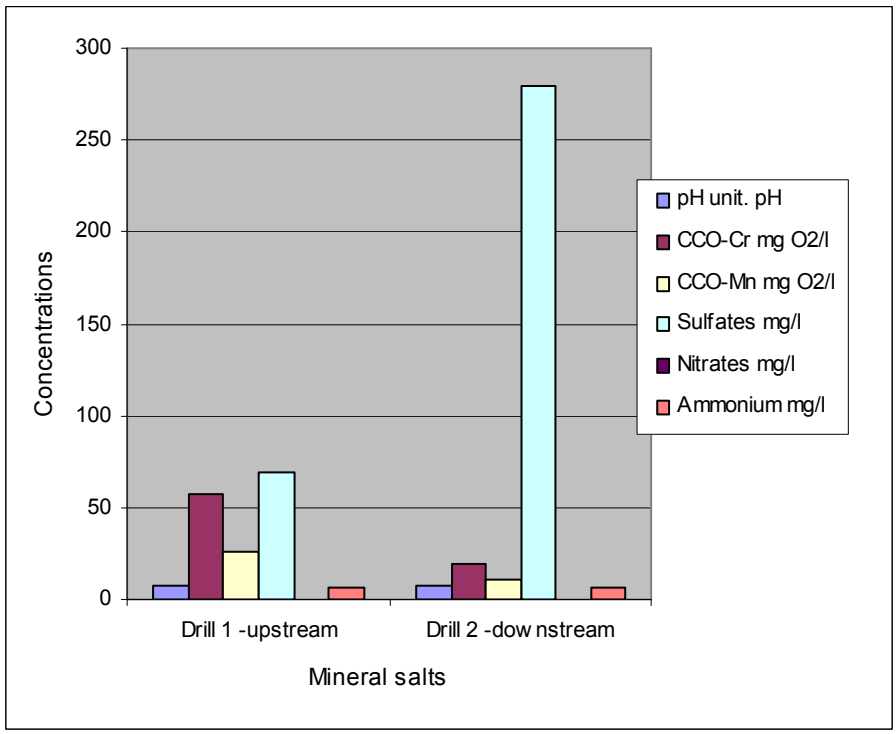

Fig. 5. Salts / Concentrations Histogram
Pozen Creek – upstream and downstream the landfill

After the interpretation of these ones, the conclusions reached are :

For the drill downstream the following were found:

- An increase by approximately 400% of the sulfates quantity,
- A diminishment by approximately 300% of the COC-Cr concentration,
- A diminishment by approximately 200% of the COC-Mn concentration

For the drill downstream, insignificant modifications were found for pH and ammonium.

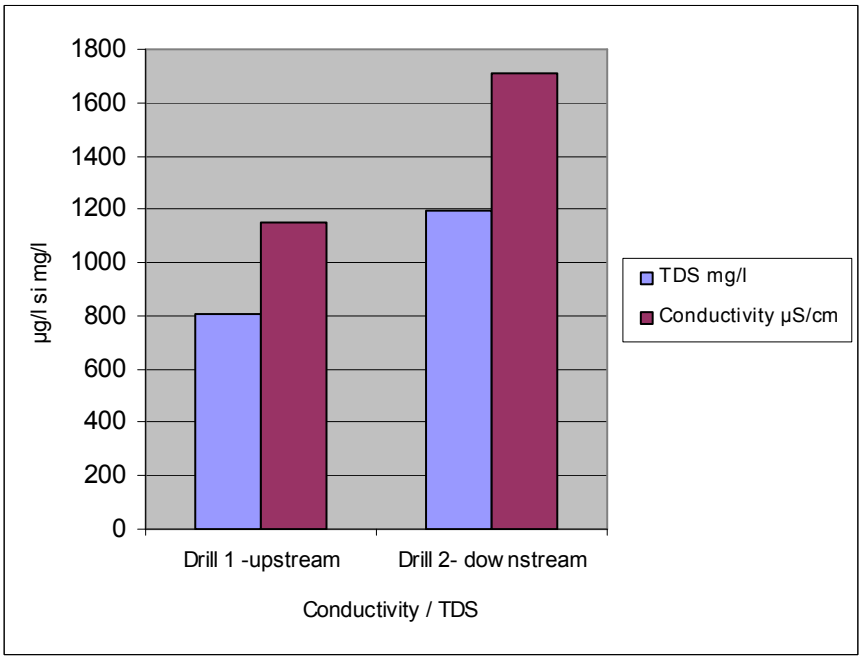

Fig. 6. Conductivity, TDS Histogram
Pozen Creek – upstream and downstream the landfill

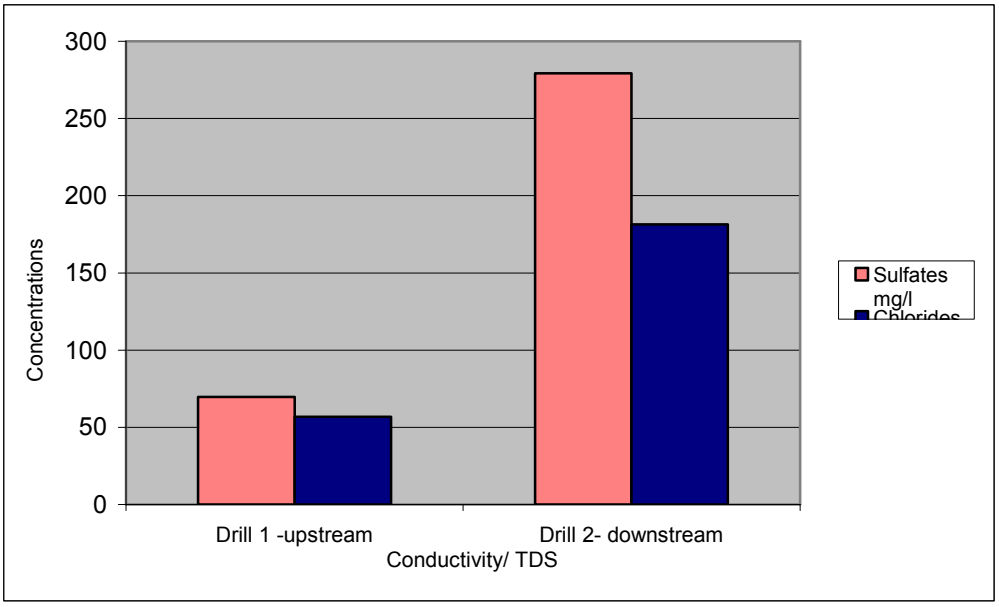

Fig.7. Sulphates and chlorides / Concentrations Histogram
Pozen Creek – upstream and downstream the landfill

The conclusions reached are the following:

For the drill downstream it was found
- An increase by approximately 50% of the TDS concentration;
- An increase by approximately 40% of conductivity,
- An increase by approximately 450% of sulfphates concentration,
- An increase by approximately 300% or chlorides concentration

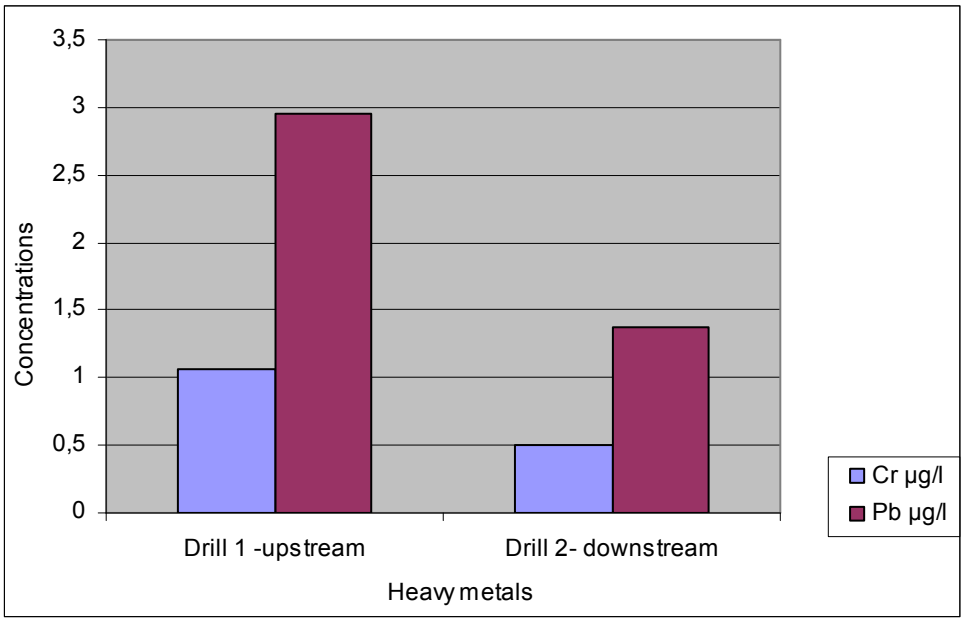

Fig. 8. Heavy metals / Concentrations Histogram
Pozen Creek – upstream and downstream the landfill

The conclusions that were reached are the following: for the drill downstream the following were found for the heavy metals analyzed:

- A diminishment by approximately 100% of the Cr concentration
- An increase by approximately 120% of the Pb concentration

CONCLUSIONS

The decomposition of solid waste in the domestic waste dumps occurs under the influence of some chemical, physical and biological processes. Following the decomposition processes, solid, liquid and gas derivate products occur that pose serious problems to the landfills management and environment factors protection. One of these problems is connected to generating leachate and its potential to pollute underground water (Report on the progresses registered at the time of preparing Romania for the accession to the European Union during September 2002 – June 2003, Government of Romania, June 2003).

At the time of designing an ecologic waste dump, it is necessary to know the leachate quality and the quantity.

The quantity of leachate generated depends on the moist content of the waste at the time of storage and the water quantity that may enter the waste dump after their storage. The quantity and quality of leachate generated in a landfill depends of the location, type of waste and manner for exploiting the waste dump. Consequently, the estimation of leachate quantity and quality is extremely difficult because it requires a high volume of data that must be acquired on field.

The leachate is the main contaminator of underground and surface waters' quality from the area of city waste storages.

♦ In modern storages, the products resulting from the stabilization process are retained inside the waste mass and their release in the environment is made in determined conditions after a certain treatment.

♦ In old-type landfills that have no systems for retaining the products resulting from waste decomposition, there are low control possibilities as regards the release of pollutants in the environment, this being by covering the waste mass, achieved thus that it diminishes the direct infiltration of water, air and gas emission.

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STACKING INTERACTIONS BETWEEN PHENANTHROLINE LIGANDS IN CRYSTAL STRUCTURES OF SQUARE - PLANAR METAL COMPLEXES

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ABSTRACT

Stacking interactions of phenanthroline square-planar complexes in crystal structures were studied analyzing data from the Cambridge Structural Database (CSD). In most of the crystal structures, two phenanthroline complexes were oriented "head-to-tail". Phenanthroline complexes show large range of diffrent overlap geometries in stacking interactions, however, the short metal-metal distances were not observed in stacking interactions of phen complexes. These results could help to understand interactions of phen complexes with DNA.

Key words: stacking interactions, phenantroline

INTRODUCTION

Noncovalent interactions of π-systems, including stacking interactions, have been extensively studied in recent years. Stacking interactions are generally studied between aromatic organic molecules or fragments. However, it was shown that other planar molecules and fragments can be also involved in stacking interactions.

Stacking interactions between chelate and C_6-aromatic rings were recognized by analyzing the data in crystal structures of square-planar transition metal complexes from the CSD. In the crystal structures there are mutual slipped-parallel orientation of these rings, similar to the orientation of two benzene rings. Recently we showed the existence of chelate-chelate stacking interactions in crystal structures from CSD.

Phenanthroline (1,10-phenanthroline-N,N') (phen) molecule coordinating to a metal ion forms large planar system of four rings: two pyridine fragments, one C_6-ring and one chelate rings. This planar system has propensity to form stacking interactions with the π -system of various aromatic groups. Tendency for stacking interactions is important for using these complexes in biochemistry, supramolecular and medicinal chemistry. Interactions between aromatic rings play key role in structure of biological systems such as DNA and proteins, and their interactions with small molecules. It is well known that these stacking interactions are occurring in the

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vertical interactions of nucleotid bases. Phenanthroline complexes interact with DNA by intercalating between base pairs of DNA.

To understand better stacking interactions of phen complexes, here we analyze the geometry of stacking interactions between phen square planar metal complexes in crystal structures from the CSD.

MATERIALS AND METHODS

The statistical study is based on the crystal structures archived in the CSD. The crystal structures involving phen complexes with coordination number 4 were screened for intermolecular contacts. In order to find intermolecular stacking interactions between phen complexes, we used the criterion where the distance between centroids of the rings was below 4.6 Å, 61 structures with 172 stacking interactions of phen square-planar complexes were found.

The geometric parameters used for analysis of the stacking interactions of phen ligands are presented in Fig. 1.

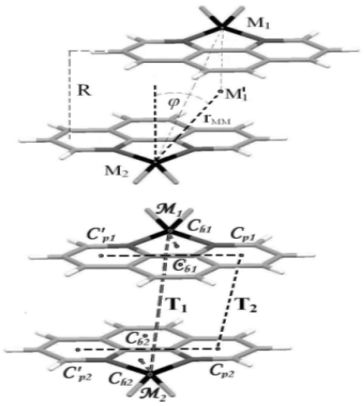

Fig. 1. Geometrical parameters describing interactions.

RESULTS AND DISCUSSIONS

The distribution of the normal distances of the interacting pyridine fragments shows pick at 3.3-3.5 Å, while in large number of interactions the normal distance is 3.2-3.7 Å (Fig. 2). These normal distances are typical for stacking interactions. Similar distribution of normal distances were observed for the stacking interactions on terpyridine complexes.

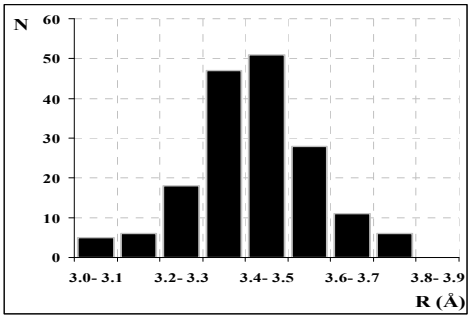

Fig. 2. Histogram of the distributions of the normal distance R for interactions of square-planar phen complexes

The distribution of T_1 torsion angle values shows preferred orientation with the angle from 170° to 180° (head-to-tail orientation) (Fig. 3). The number of the interactions with the angle from 0° to 10° (head-to-head orientation) is quite small, only 8 interactions. Also the number of the interactions with the T_1 between 10 and 170° is very small.

The distribution of T_2 torsion angle shows two preferred orientations; the first orientation with T_2 values of 0° to 10° and the second one with 170° to 180° (Fig. 3). There is a small number of the interactions with the T_2 between 10° and 170°. The values of T_2 torsion angle of 0° to 10° correspond to the interactions with overlap of large part of the phen ligand, while the values of 170° to 180° correspond to only partial overlap of phen ligands. The interactions with the values of T_2 in the range of 0° to 10° are encountered more often.

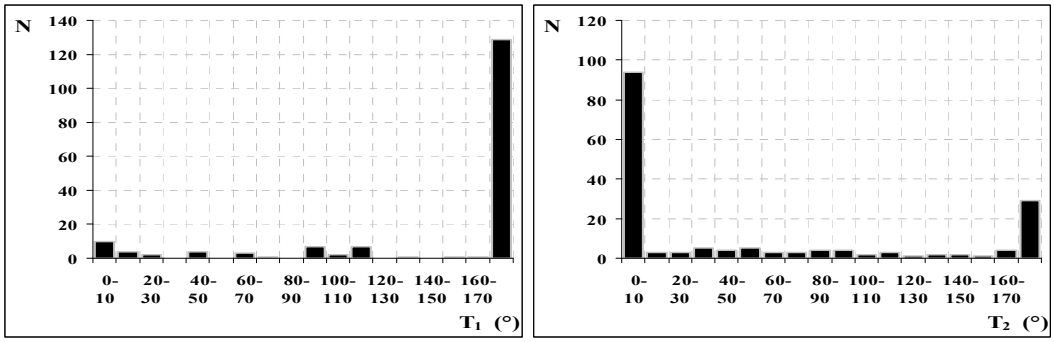

Fig. 3. Histograms showing the distribution of torsion angles T_1 and T_2, for interactions of square-planar phen complexes.

Because of the preference for the one value of the torsion angles T_1 and two values of T_2 two possible combinations of these torsion angles can describe most of the intermolecular stacking interactions of phen square-planar complexes. The values of T_1 angle are close to 180° and complexes are oriented head to tail, while T_2 values can be close to 0° (group I), or close to 180° (group II).

The mutual displacement of two interacting phen complexes was measured by two parameters: angle φ and offset r_{MM}. In our previous work we showed that these two parameters were important for the description of the mutual orientation of terpy complexes and we used it to analyze the orientations of the interacting phen complexes. The scattergram for a correlation between the angle φ and r_{MM} displacement are shown in Fig. 4 for both groups together. The interactions in two groups differ by angle φ; the interactions of group I have quite small values of angle φ, below 20º, while the interactions of group II have larger values of angle φ, mainly above 20º (with a several exceptions) and up to 70º. Both groups have large range of the r_{MM} displacement, however, the values are lower for group I. The values of the r_{MM} displacement for group I are from 3 to 10 Å, while for group II from 4 to 11 Å. The two groups overlap in the region of the angle φ about 20º.

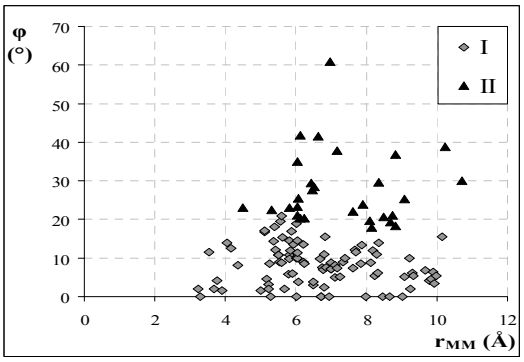

Fig. 4. The plot of the angle φ *vs*. the offset r_{MM} for interactions in group I and II.

The group I, with T_2 values close to 0°, is larger group; it includes 47 structures with 94 interactions. For this group angle φ values are less than 20° and values of r_{MM} displacement are in the range from 3.0 to 10.0 Å, and by visual inspection of the interactions we found out that in most of interactions, mutual overlaps of both pyridine rings exist. Example for this group is shown in Fig.5(left).

The group II of interactions, with torsion angle T_1 close to 0°, and T_2 close to 180° is smaller than group I, it includes 22 structures with 25 interactions. In this group phen ligands only partially overlap; these overlaps always include at least one pyridine ring. Example for this group is shown in Fig.5(right).

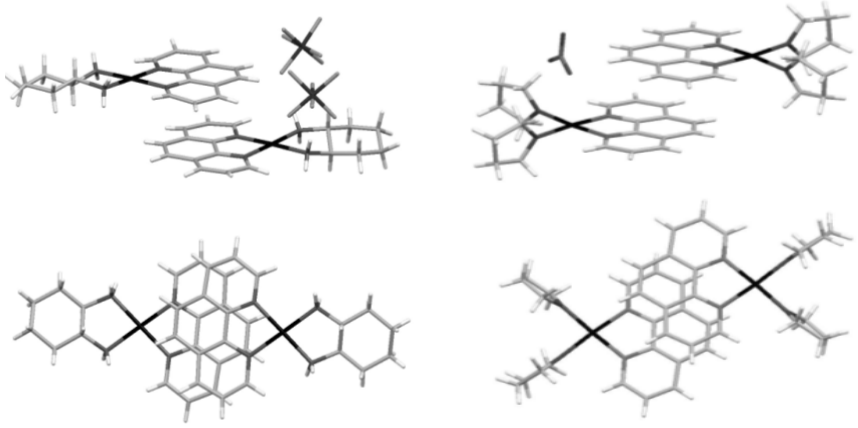

Fig. 5. Left:Two projections presenting stacking interaction in crystal structure BEBCAN
Right: Two projections presenting stacking interaction in crystal structure ILAHAE

CONCLUSIONS

In the Cambridge Structural Database (CSD) 61 structures with 172 stacking interactions of phen square-planar complexes were found. The distribution of the torsion angles T_1 and T_2 values show that in most of the interactions two interacting complexes are oriented “head-to-tail”, with the large area of phen ligand involved in the overlap. Phen complexes show large range of diffrent overlap geometries in stacking interactions, however, short metal-metal distances were not observed. These results could help to understand interactions of phen complexes with DNA.

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MONOFUNCTIONALISED CALIXPYRROLES. SYNTHETIC AND THEORETICAL APPROACH

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ABSTRACT

Novel substituted calix[4]pyrrole were designed and obtained, as precursors in the synthesis of thiol-substituted calyx[4]pyrrols. Their physical-chemical characterization is presented. Theoretical calculations were also carried out in order to explain the reactivity of meso-octamethylcalix[4]pyrrole towards electrophiles.

Key words: functionalized calix[4]pyrroles, electrophilic substitution,transition metal ligands, computation

INTRODUCTION

Calix[4]pyrroles are formed of four pyrrole rings bonded with four carbon atoms *meso*-disubstututed. Calix[4]pyrroles substituted in position *meso*- can not be oxidised to form a porphyrynic structures. This was explained with the fact that the pyrrole rings are neutral and in the structure of the calix[4]pyrroles there is no delocalisation of electrons like in the porphyrynic structures.

The lack of delocalisation in the calix[4]pyrrole structures causes an absence of absorption in visible area, or emission bands after exposing on fluorescence. These facts make studying calix[4]pyrrols with optical techniques impossible. The ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy is the most suitable method for characterising and studding this class of compounds (Bayer, 1886; Dennstedt and Zimmerman, 1887; Dionne et al., 1996; Cristea et al., 2002).

Calix[4]pyrroles offer a cup-shaped skeleton, analogous to that of calix[4]arene derivatives, in which pyrrole hydrogen bond donors are ideally preorganised for anion and ion pair binding which is readily adorned with redox/photo signal transducing functionality as required for advanced ion and small molecules applications. The metal-ion chemistry of calix[4]arenes with appended ligand donor groups is currently a subject for intensive investigations because of the new opportunities in diverse areas such as nano-scale coordination cavitand based chemistry, sensor design, biomimicry and entropic trapping of reagents for catalysis. Surprisingly and in a sharp contrast to the spectacular recent advances in the coordination chemistry of calixarenes functionalized with donor groups, no corresponding chemistry for calix[4]pyrroles substituted with simple donor groups has been reported (Schriver, 2002).

In this contribution, we report the study of new calix[4]pyrrole derivatives, designed with the purpose of tuning the anion binding affinity of these receptors by substitution at the *β*-position of the pyrrole rings, as well as preparation of *meso*-"expanded" calix[4]pyrroles. This was proposed in order to obtain an intermediate for the syntheses of thiol derivatives of the calix[4]pyrrole. Thiol derivatives can be used as ligands in organometallic chemistry.

MATERIALS AND METHODS

All manipulations were carried out using standard Schlenk and vacuum line techniques under an atmosphere of dry nitrogen. This required also the use of dry and oxygen-free solvents. THF was dried over sodium wire/benzophenone and distilled under an atmosphere of dry argon. Some deuterated solvents needed for NMR spectroscopy were used as purchased and kept under inert atmosphere over molecular sieves (DMSO). $CDCl_3$ was dried over $LiAlH_4$, distilled and kept over molecular sieves.

NMR spectroscopy: Proton 1H-NMR and 13C-NMR specrta used in the caracterisation of the products were recorded on BRUKER AVANCE, 300MHz spectrometar.

Theoretical calculations: The calculations were performed at the PM3 (Stewart, 1989) semiempirical level of the theory, using the Spartan package of programs [Wavefunction Inc.18401 Von Karman Avenue, Suite 370 Irvine, CA 92612]. A vibrational analysis was also carried out in order to ensure that all the geometries found correspond to minima.

Synthesis of *meso*-octamethylcalix[4]pyrrole: To a solution of pyrrole (4mL, 57 mmol) in acetone (40mL, 54 mmol), a methanesulfonic acid (2mL, 0.031mol) was added drop by drop. The reaction is very violend and exothermic. The reaction mixture was stirred at a room temperature over night. A white precipitate was formed which was isolated with filtratration under vacuum and purified with recrystalisation in ethanol. Yield: 5.11 g, 11.9 mmol, (~83%, based on the pyrrole); M.p.=296^{o}C; ^{1}H-NMR data (398K, δ=ppm): 7.01 (4 H, br s, NH), 5.89 (8 H, d, β-pyrrole), 1.50 (24 H, s, *meso*-CH_3).

Synthesis of lithium salt of *meso*-octamethylcalix[4]pyrrole, tetraanion: *meso*-Octamethylcalix[4]pyrrole (1g, 2,3mmol) was dissolved in 70 mL of dry THF in a 250 mL round-bottomed flask with stirring under inert atmosphere. The solution was cooled to -78^{o}C using a liquid N_2/ethanol bath. 15% n-Buthillithium (6 mL, 9.3mmol,) was added dropwise to the solution of *meso*-octamethylcalix[4]pyrrole, and the resulting mixture was stirred for an additional 1h. The lithium salt was not isolated from the mixture, and was used as such in the next reaction.

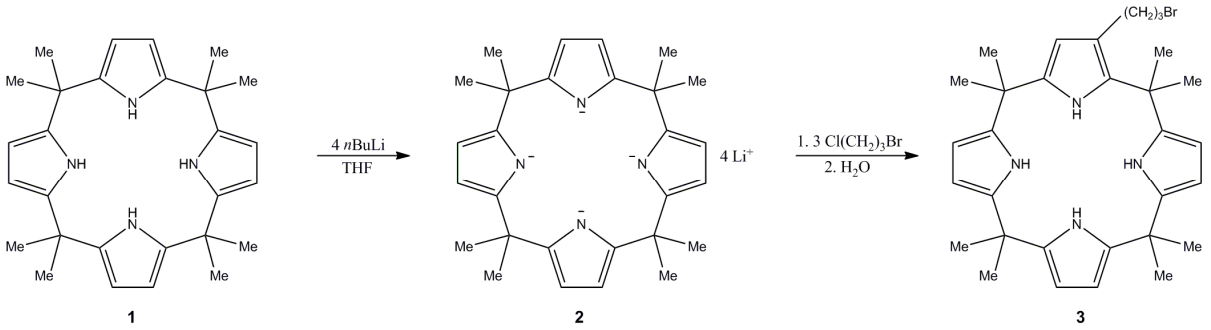
Synthesis of 1-bromopropyl- *meso*-octamethylcalix[4]pyrrole: 1-chloro-3-bromopropane (0.7 mL, 6.9 mmol) in THF (7 mL) was added dropwise to the reaction vessel containing lithiated calix[4]pyrrole. The solution was cooled to -78^{o}C using a liquid N_2/ethanol bath. Once the addition was over, the reaction mixture was stirred for additional 15 min, before being allowed to worm to room temperature. After 1h the reaction mixture was cooled down to 0°C and quenched by slow addition of water (30 mL) and saturated aqueous $Na_2S_2O_3$ (30 mL). The reaction mixture was treated with ethyl acetate (150 mL) and washed with birne. The organic layer was separated and dried over Na_2SO_4. The solvent was removed in vacuum and precipitate was characterised by NMR spectroscopy. ^{1}H NMR data (398K, δ=ppm): 6.90 (4H, br s,

NH), 5.82 (7H, m, CH pyrrole), 3.61 (2H, t, propyl CH_2), 3.49 (2H, t, CH_2Br), 2.21 (2H, q, propyl CH_2), 1.40-1.50 (24H, overlapping singlets, *meso*-CH_3).

RESULTS AND DISCUSSION

The starting material, *meso*-octamethylcalix[4]pyrrole **1** was obtained by the facile acid-catalyzed condensation of acetone and pyrrole according to previously reported procedures (Rothemund and Gage, 1955). This material was then lithiated with 4 equiv of n-butyllithium in hexane in THF at -78°C (Scheme 1) to give polyanion **2**, of undetermined structure (Pavel et al., 2000).

The calix[4]pyrrole anion obtained is susceptible to electrophilic attack. Thus, 1-chloro-3-bromopropane was successfully employed as an electrophile to give 1-bromopropyl-*meso*-octamethylcalix[4]pyrrole. Attempts were made with other 1-halo-propanes, but bromine or iodine-substituted derivatives were found to be ineffective in the synthesis of where the calix[4]pyrrole polyanion **2** was used as a nucleophile.



Scheme 1. Formation of a polyanion with undetermined structure by the lithiation of *meso*-octamethylcalix[4]pyrrole

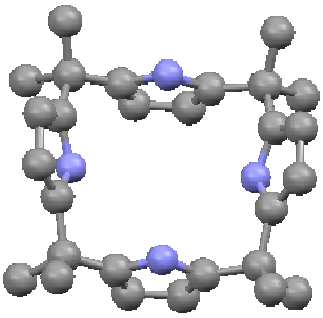
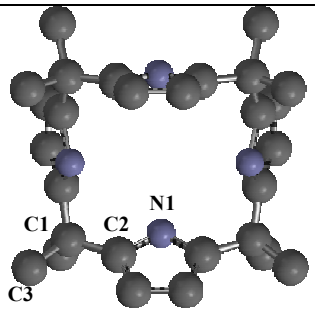
Using the method described in Scheme 1, the formation of mono-, di-, tri- and tetrasubstituted calix[4]pyrroles was often observed, due to the very strong electrophile. The reaction conditions were optimised in order to get the highest yield of monosubstituted derivatives with a very low contamination of disubstituted derivatives. That was achieved by using lower than stochiometric amount of electrophile.

The experimental results were supported with theoretical calculations performed at the PM3 semiempirical level of the theory, using the Spartan package of programs. The calculations showed that the experimentally proposed method satisfies all the predicted steps of the reaction and provides the syntheses of many calix[4]pyrroles that could prove useful in the construction of calix[4]pyrrole-based anion sensors which are effective and selective in their guest binding properties.

The calculations were performed at the PM3 (Stewart, 1989) semiempirical level of the theory. A vibrational analysis was also carried out in order to ensure that all the geometries found correspond to minima.

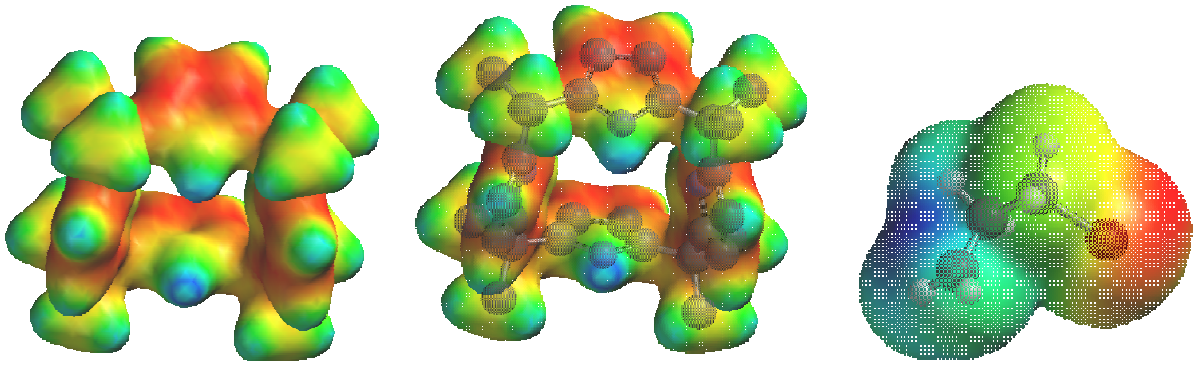
The optimized geometry of the *meso*-octamethylcalix[4]pyrrole is presented in Table 1, together with some selected geometrical parameters. The solid state structure of the *meso*-octamethylcalix[4]pyrrole dimethyl-sulfoxide solvate has been reported in the literature (Lynch et al., 2001) and it is also given for comparison.

Table 1. Selected geometrical parameters for the synthesised (left) and calculated structure (right) of *meso*-octamethylcalix[4]pyrrole

Molecule		
C1-C2 (Å)	1.504	1.503
C2-N1(Å)	1.373	1.399
C1-C3(Å)	1.519	1.532
C1-C2-N1 (°)	123.76	123.14

It can be seen that although the over-all geometry is similar for the two molecules, which means that the alternate orientation of the pyrrole rings is kept both in gas and in solid state. The bond angles calculated by the PM3 are in fair agreement with the experimental data. However, the bond lengths involving the nitrogen atoms in the solid state structure are larger than those calculated, due no doubt to the packing effects and N-H···O hydrogen bonds with the dimethylsulfoxide molecules.

The geometry of the model compound has been optimized at a semiempirical (PM3) level and the electrostatic potential density on the molecule is represented below (at an isovalue of 0.05), together with that of the cation $BrCH_2CH_2CH_2^+$.

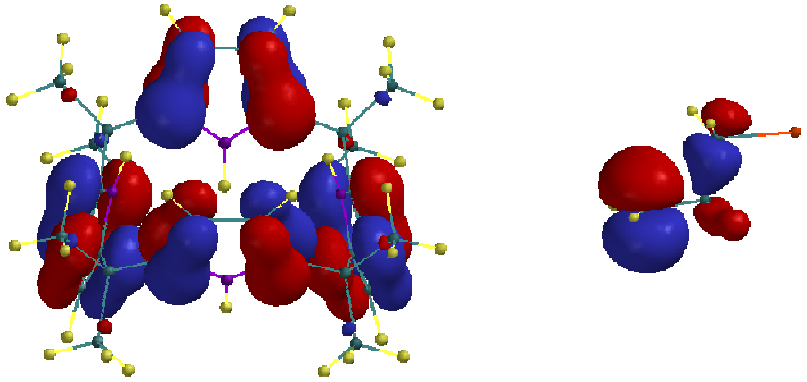


a **b** **c**

Fig. 1. Electrostatic potential densities for *meso*-octamethylcalix[4]pyrrole (a, b) and $BrCH_2CH_2CH_2^+$ (c)

Red areas represent a high value of electronic density, while blue areas are electron-poor zones. It can be seen from the charge distribution in the molecule that the carbon atoms in β to the nitrogen atoms are the ones that are more disposed to an electrophilic attack from the carbocation. As expected, the electrophilic centre of the carbocation is on the terminal carbon atom. The electrophilic substitution mechanism in β with respect to the nitrogen atom is also supported by the energies of the HOMO orbital on the calixpyrrol (-8. eV), close to that of the LUMO on the

carbocation (-8.81eV). The shape of the orbitals involved is shown below; it can be noticed that the HOMO of the calyxpyrrole is localized on the C=C bonds in the rings and it has an antibonding character.



a b

Fig. 2. HOMO orbital of the meso-octamethylcalix[4]pyrrole (a) and the LUMO orbital of the $BrCH_2CH_2CH_2^+$ cation (b)

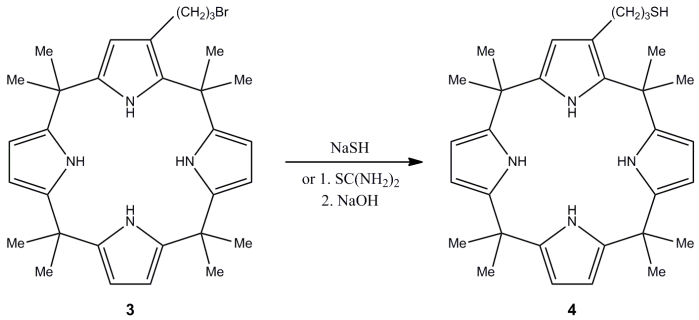
In addition, we have calculated the model compounds for mono-, di-, tri- and tetrasubstituted *meso*-octamethylcalix[4]pyrrole. Comparing the energies of the four possible products it was found that the tetrasubstituted derivative is the most preferable to be formed. The trend goes as expected: mono- > di- > tri- > tetra-1-bromopropyl-*meso*-octamethylcalix[4]pyrrole.

Table 2. Energies of formations calculated at the PM3 level

Derivative	monosubstituted	disubstituted	trisubstituted	tetrasubstituted
$\Delta_f H$ (kcal/mol)	37.211	25.682	13.557	2.540

CONCLUSIONS AND PERSPECTIVES

Compound **3** can be an useful intermediate in the synthesis of other novel functionalized calyx-pyrrols. For instance, the reaction with sodium hydrosulphide or thiourea to replace the lead to the formation of a thiol (**4**) (Scheme 2.).



3 4

Scheme 2. Convertion from an alkylhalide to thiol

After full characterisation of compounds **3** and **4**, their anion binding affinity will be investigated. Furthermore, compound **4** offers a well defined and readily accessible –SH group with a potential for synthesis of thiolates and complexation with a range of metal ions. The bromine derivative **3** can also be used as a ligand in organometallic chemistry.

Acknowledgements. The authors thank the SOE Programme for the financial support.

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COMPUTATIONAL MODELING METAL-PROTEIN INTERACTIONS: CISPLATIN

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ABSTRACT

The present work represents a computational study on the performance of molecular mechanics, semiempirical and density functional theory methods in accurately describing the interaction of platinum atom with a protein linked to copper homeostasis (Atox1). Although the UFF method is not among the best in describing model alpha-helical structures, it fares distinctly better than other methods in describing platinum-protein bonding within the test case model employed in this study. Generally speaking, however, the six-coordinated structure previously reported in the crystal structure of the Atox1-cisplatin adduct cannot be reproduced with methods as advanced as the M062x density functional with triple-zeta basis sets and relativistic corrections.

Key words: Cisplatin, metal-protein interaction, molecular modelling.

INTRODUCTION

The interaction of platinum complexes with proteins is of real interest when studying the properties and possible medical applications of such adducts. Cisplatin (cis-diamminedichloro platinum(II)) is a widely used anticancer drug. Clinical studies have demonstrated a link between cisplatin resistance and the human copper homeostatic protein Atox1. The structure of a dimeric cisplatin-(Atox1)2 adduct is known (pdb code 3lWL3)[1]. The Pt(II) ion is coordinated by the two Cys_{15} residues while the two Cys_{12} residues are at a greater distance. Two ammine ligands are also present, interacting with Thr_{11} and Cys_{12}. This leads to a 6-coordinated geometry at the Pt(II) ion, which is relatively unexpected. As part of our on-going experimental and computational efforts to investigate the effect of cisplatin on [protein structure and function, we report here the first computational study on the Atox1-cisplatin adduct, using the experimentally-determined crystal structure and focusing on the unusual distorted-octahedral coordination sphere at the platinum. The results will demonstrate that this unusual structure cannot be reproduced with any of the reliable methods currently available, and hence that the crystal structure may contain a superposition of two or more structures differing in ligation around the platinum, which may have led to the apparent crowding of ligands around the metal

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MATERIALS AND METHODS

Theoretical calculations (molecular mechanics, semiepirical[2] and density functional theory (M062x/lanl2dz)[3] with Gaussian[4] and Mopac[5]) of the Pt-Atox1 metal center are reported in order to study the precision/feasibility of the simulation. For higher-level computations an active site of about 10 Å around the metal center has been chosen, with the border atoms being frozen in fixed positions during geometry optimization.

RESULTS AND DISCUSSION

First a molecular mechanics optimization of Atox1 monomer using the Amber forcefield has been performed (Fig.1) and a RMSD value of 1.36 is reported as compared to the initial structure.

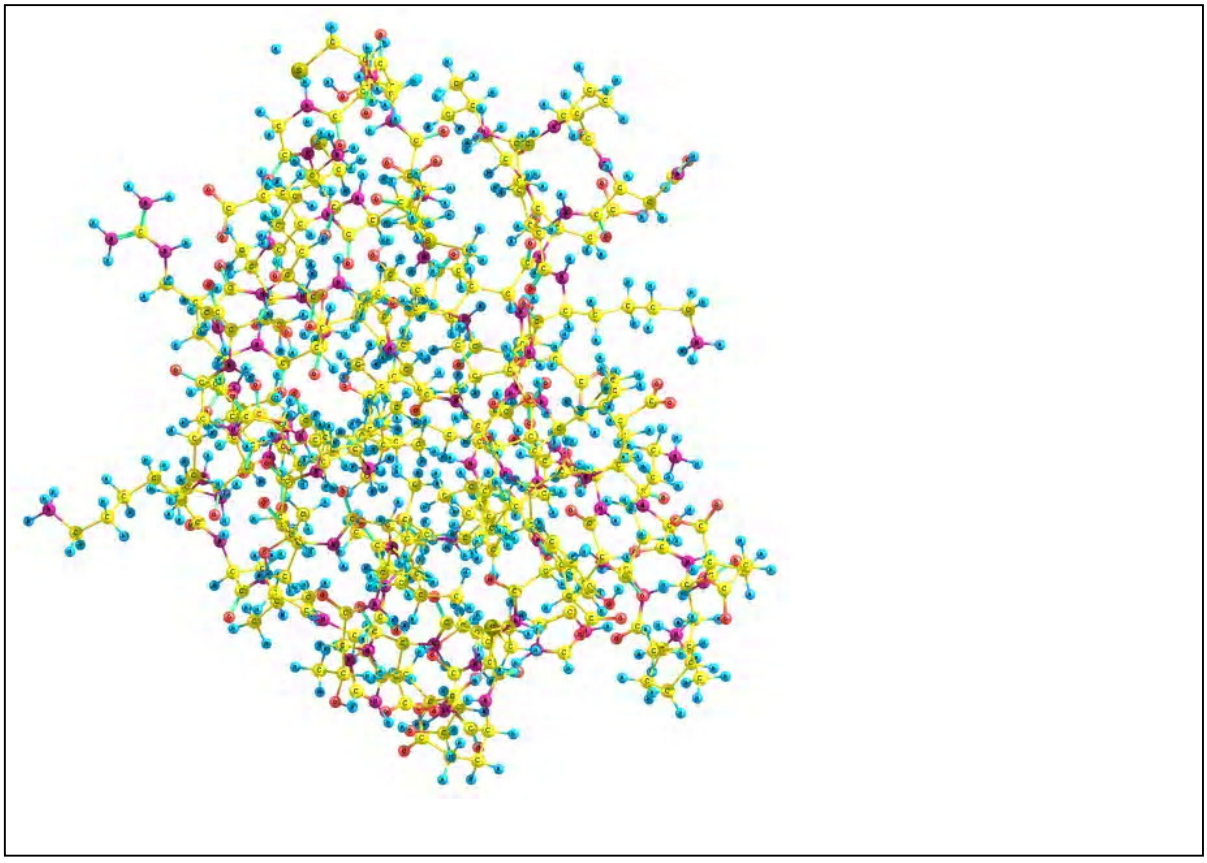

Fig. 1. Molecular mechanics (Amber force field) optimized geometry of the Atox1 protein.

As the Amber force field implemented in Gaussian lacks parameters for Platinum, UFF forcefield was further considered in an attempt to optimize the entire Pt-(Atox1)2 adduct (Fig.2). RMSD compared to the original values: 1.57. The relevant Pt-S bond distances are presented in Table 1.

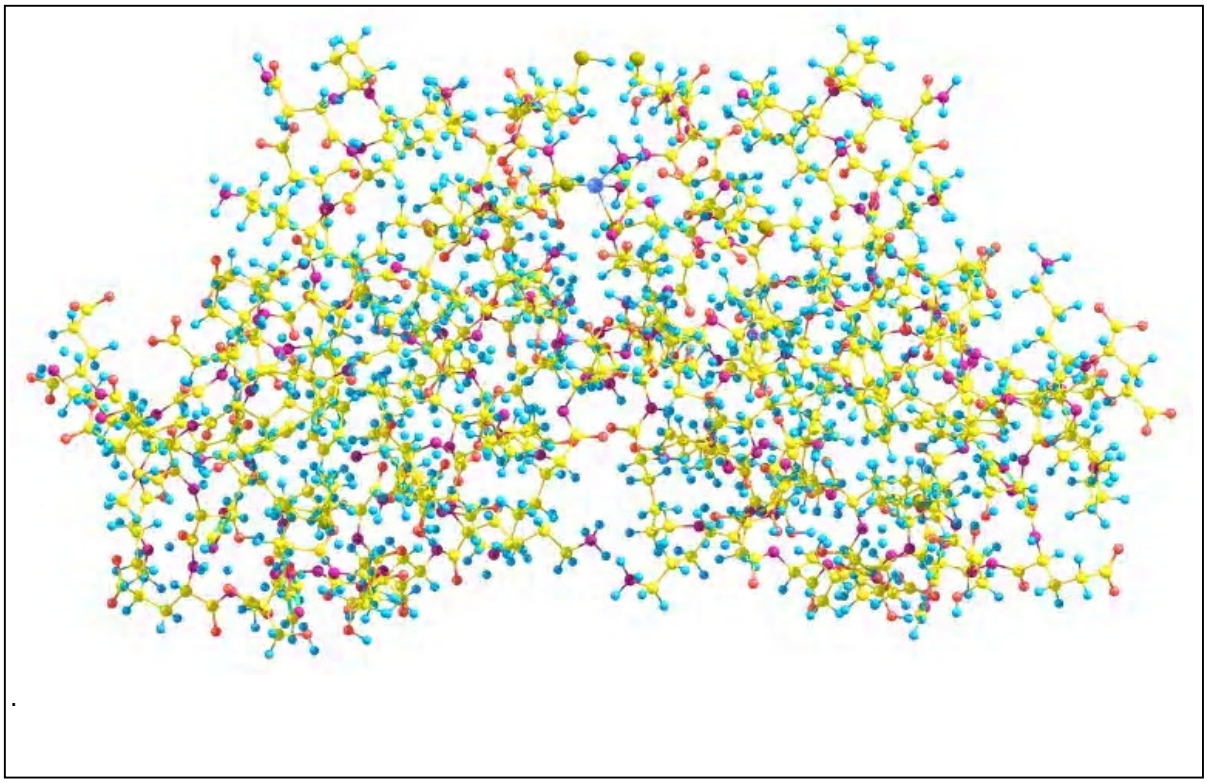

Fig. 2. Molecular mechanics (UFF force field) optimized geometry of the entire Pt-(Atox1)2 adduct. The Platinum atom is visible in the upper part, between the two Atox1 fragments

For testing higher level methods an approximately 10-Å fragment with the terminal atoms frozen has been selected. Three methods have been considered: the PM6 semiempirical implementation of Gaussian, the PM6 solvated model implemented in Mopac and a high density functional theory level, namely the M062X functional with the lanl2dz basis set implemented in Gaussian. All these optimized geometries are presented in Fig. 3. The relevant platinum-sulfur bond distances are depicted in Table 1

Table 1. Pt-protein bonding distances.

Bond values	Initial Pdb	Optimized UFF	10Å site UFF	10Å site PM6	10Å site PM6-water	10Å site M062X/lanl2dz
Pt-S(Cys15)1	2.10	2.41	2.23	2.32	2.33	2.40
Pt-S(Cys15)2	2.30	2.43	2.45	2.32	2.34	2.41
Pt-S(Cys12)1	2.46	6.19	2.48	4.92	5.11	4.32
Pt-S(Cys12)2	2.48	7.5	2.47	4.23	4.61	4.23

A strong distortion is noticed in all these cases and the intial Pt-S(Cys12) bonds are broken in almost all cases (i.e., elongated to 4-7 Å). Thus, there is a clear tendency of the platinum to return to its cisplatin-like square-planar geometry.

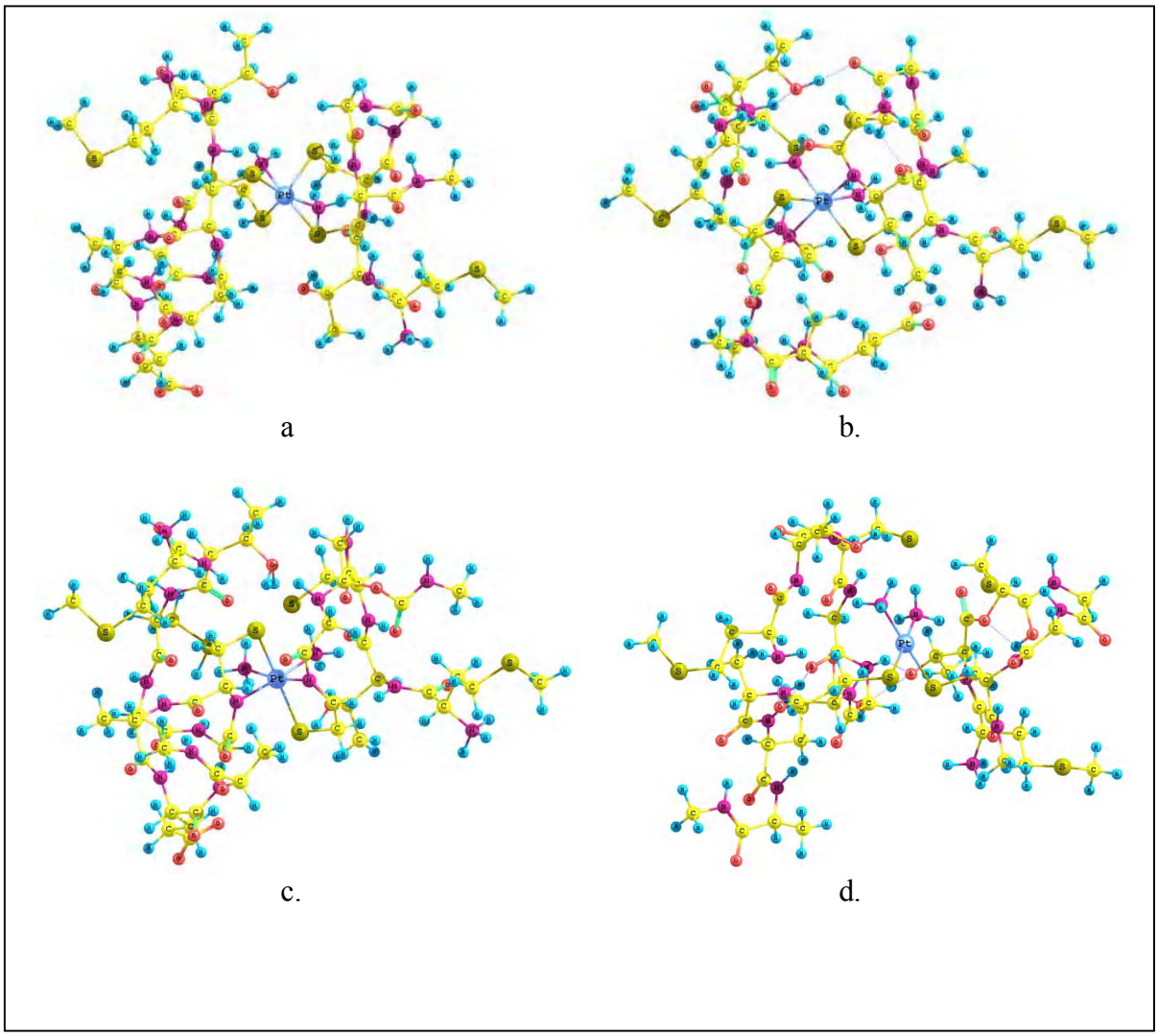

Fig. 3. a) Initial; b.) PM6 (in vacuum) semiempirical; c) PM6-solvated (water) semiempirical; d) Density functional theory (M062x/lanl2dz) optimization of the metallic region.

A model where the cysteinate ligands were reduced to methylthiolates has been optimized at a higher theory level and the results are presented in Figure 4.

This model retains all six ligands around the platinum, suggesting that the cleavage of platinum-sulfur bonds simply upon geometry optimization in the models summarized in Table 1 is not simply due to sterical or electronic problems in the first coordination sphere of the metal, but rather to constraints imposed by the second coordination sphere and the overall protein structure.

To conclude, the cisplatin adduct of a copper-trafficking protein (Atox1) has been examined with computational methods, using the experimentally-known crystal structure as starting point. The distorted-octahedral coordination environment around the platinum, seen in this crystal structure, cannot be reproduced by high- and

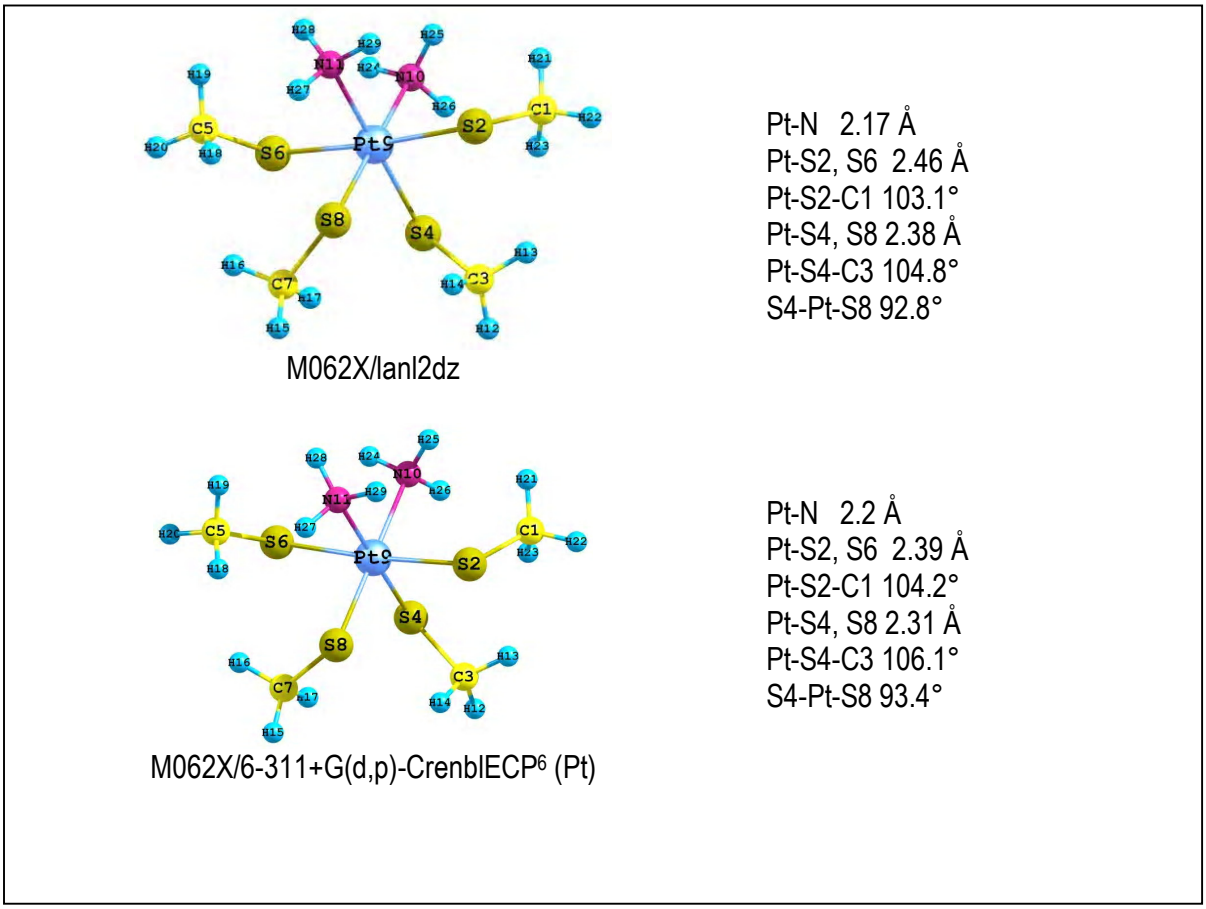

Fig. 4. Optimized geometries for a $Pt(NH_3)_2(SCH_3)_4$ model at different levels of theory.

medium-level computational techniques (semiempirical or density functional with latest-generation functional and basis sets as large as triple-zeta with relativistic corrections). One possible interpretation is that the crystal structure in fact represents a superposition of two or more different structures, differing between them in terms of platinum coordination sphere. While both structures would exhibit the expected square-planar geometry around the metal, a superposition of them would lead to the false impression of a sterically-crowded distorted-octahedral coordination sphere, as apparently seen in the Atox1-ciplatin crystal structure.

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STUDY OF OH…π INTERACTIONS BETWEEN COORDINATED WATER MOLECULE AND AROMATIC RING

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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 OH/π (MLOH/π) interactions, or C-H groups of aromatic fragment can interact with oxygen forming C-H···O interactions. Here we present the results of intermolecular OH/π interactions between coordinated water molecules and aromatic ring, and also compare it with interactions of non-coordinated water with the same π-system. Study of interactions was based on analysis of crystal structures. Crystal structures archived in the Cambridge Structural Database involving coordinated water molecules and aromatic ring were screened for intermolecular contacts. The analysis showed that coordinated water tends to establish bifurcated OH/π interactions with aromatic ring more frequently than non-coordinated. When the interaction is bifurcated, O-atom of the water molecule tends to be above the center of the aromatic ring. Coordinated water participates in bifurcated OH/π interactions with shorter contacts than non-coordinated. Also, non-coordinated water is able to establish the wider range of positions toward the aromatic ring.

Key words: OH…π interactions, coordinated water, bifurcated interaction.

INTRODUCTION

The conformations and functions of molecules depend on interactions with surrounding solvent, in particular with water molecules. The interactions of water molecule with aromatic groups are important as interactions of polar solvent with nonpolar molecules or fragments. The investigation of OH/π interactions between water molecules and the aromatic groups of amino acids in crystal structures of proteins confirmed relatively frequent occurence of aromatic interactions (Zarić et al., 2000).

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 (Milčić, Zarić, 2001), 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 (Milčić, Zarić,

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2001). Here we present results for intermolecular OH/π interactions between coordinated water molecules and aromatic ring and compare it with the results for interactions of non-coordinated water and aromatic ring. Study of the interactions was based on statistical analysis of the geometries in crystal structures from the Cambridge Structural Database (CSD).

MATERIALS AND METHODS

The statistical study is based on the crystal structures archived in the Cambridge Structural Database (CSD version 5.31, updates May 2010). The crystal structures involving coordinated water molecules and aromatic ring were screened for intermolecular contacts. We also derived structures with similar interactions involving non-coordinated water in order to compare the results with interactions of this type of water molecules.

We searched for structures in which the distance between one H-atom of the water molecule and the center of the aromatic ring Ω (d distance) is less than 3.5 Å, the angle α is larger than 110°, and the angle β is smaller than 30° (Figure 1). Among the CSD crystal structures we found 58 structures with 127 short intermolecular contacts between coordinated water and aromatic ring which satisfy these criteria. There were 315 structures with 644 contacts between non-coordinated water and aromatic ring within these criteria.

For the purpose of this analysis, we used parameters (Figure 1) which were directly retrieved from CSD (distance d, angles α and β).

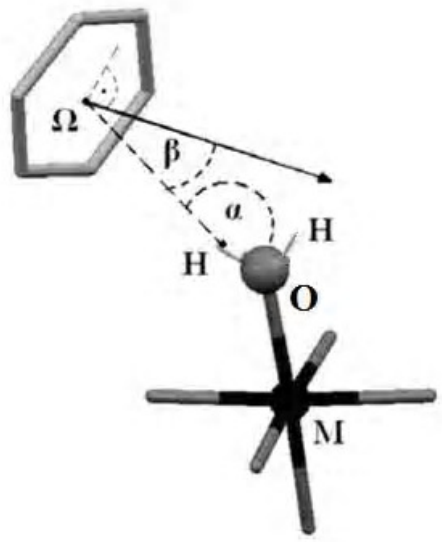

Fig. 1. Geometric parameters used for the description of OH/π interactions

RESULTS AND DISCUSSIONS

Among 127 interactions of coordinated water with aromatic ring, there were 46 bifurcated interactions (36.2%). For the purpose of detailed analysis we separated the results in two sets – bifurcated and non-bifurcated interactions.

The histogram for distribution of β angle (Figure 2) has maximum between 20° and 25° for both bifurcated and non-bifurcated interactions. Also, the histogram of α

angle values distribution (Figure 2) has maximum between 110° and 120° for both bifurcated and non-bifurcated interactions. These results indicate that in structures with bifurcated interactions the projection of O-atom is closer to the Ω point than the projections of H-atoms. This implies that O-atom prefers to be in the position above the center of the π-system.

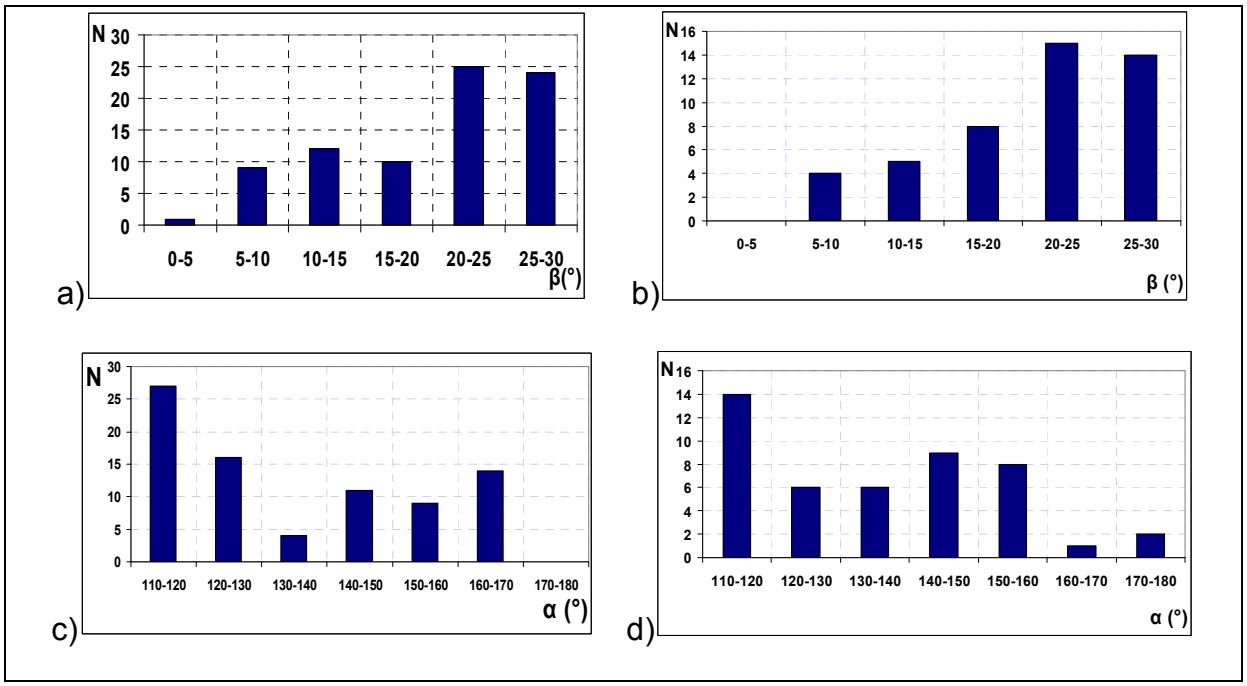

Fig. 2. Frequency distributions of β angle in *a)* non-bifurcated and *b)* bifurcated interactions and α angle in *c)* non-bifurcated and *d)* bifurcated interactions for coordinated water

The distribution of the d distances (Figure 3) among bifurcated OH/π interactions shows that H---Ω distances occur in interval from 2.2 Å and 3.5 Å overall, with the maximum between 2.3 and 2.4 Å, and also relatively large number of interactions is at the distance between 2.7 and 2.8 Å. Only a small number of non-bifurcated interactions is established at this distance, since the maximum is at 3.3-3.4 Å. This indicates that bifurcated interactions are more stable, because aromatic ring is interacting with two hydrogen atoms.

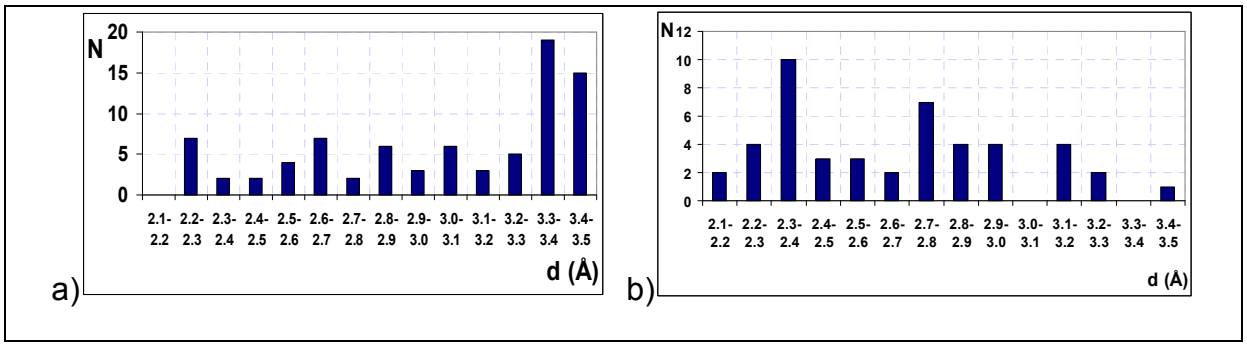

Fig. 3. Frequency distributions of d distances in *a)* non-bifurcated and *b)* bifurcated interactions for coordinated water

In Figure 4 and Figure 5 we present two structures that contain OH/π interactions between coodinated water molecule and aromatic ring.

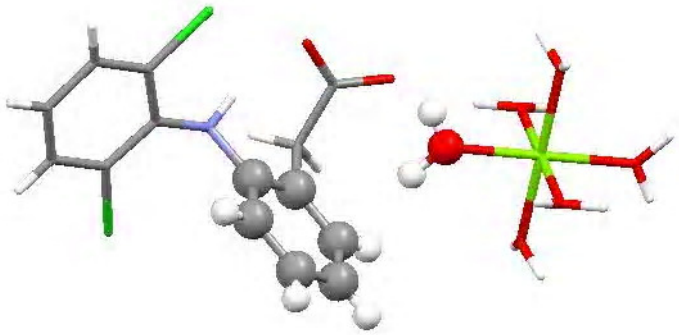

Fig. 4. Structure GOLPIG (non-bifurcated OH/π interaction of coordinated water molecule and aromatic ring)

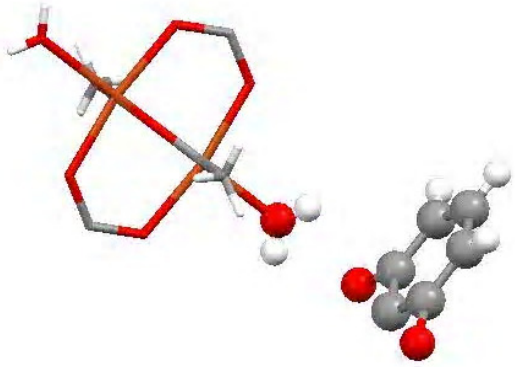

Fig. 5. Structure DEGYIX (bifurcated OH/π interaction of coordinated water molecule and aromatic ring)

Non-coordinated water molecules posses less tendency for establishing bifurcated interactions, since only 108 bifurcated interactions were found (16.8%).

Larger percent of interactions of non-coordinated water is established at d distances shorter than 2.8 Å in comparison to coordinated water (Figure 6). This implies that steric effect is very important, since coordinated water is the part of voluminous specie. This is especially favored among bifurcated interactions. However, bifurcated interactions of coordinated water show maximum at 2.3-2.4 Å, while non-coordinated water interacts at 2.4-2.5 Å, which means that there is slightly.

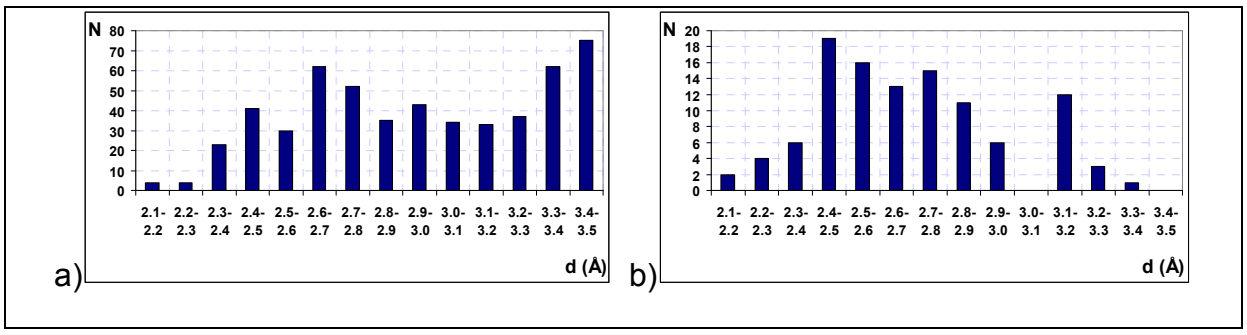

Fig. 6. Frequency distributions of d distances in a) non-bifurcated and b) bifurcated interactions for non-coordinated water

stronger preference for interacting with aromatic ring when metal ion is included, probably because H-atoms are more available to π-system when metal ion is withdrawing negative charge.

Among the interactions involving non-coordinated water molecules, values of α angle are in the wider range (110° to 140°) and also there is a higher percentage of structures with β angles smaller than 20° (Figure 7). This is the consequence of the fact that non-coordinated water molecules are more flexible in their positioning than voluminous complex species that contain coordinated water.

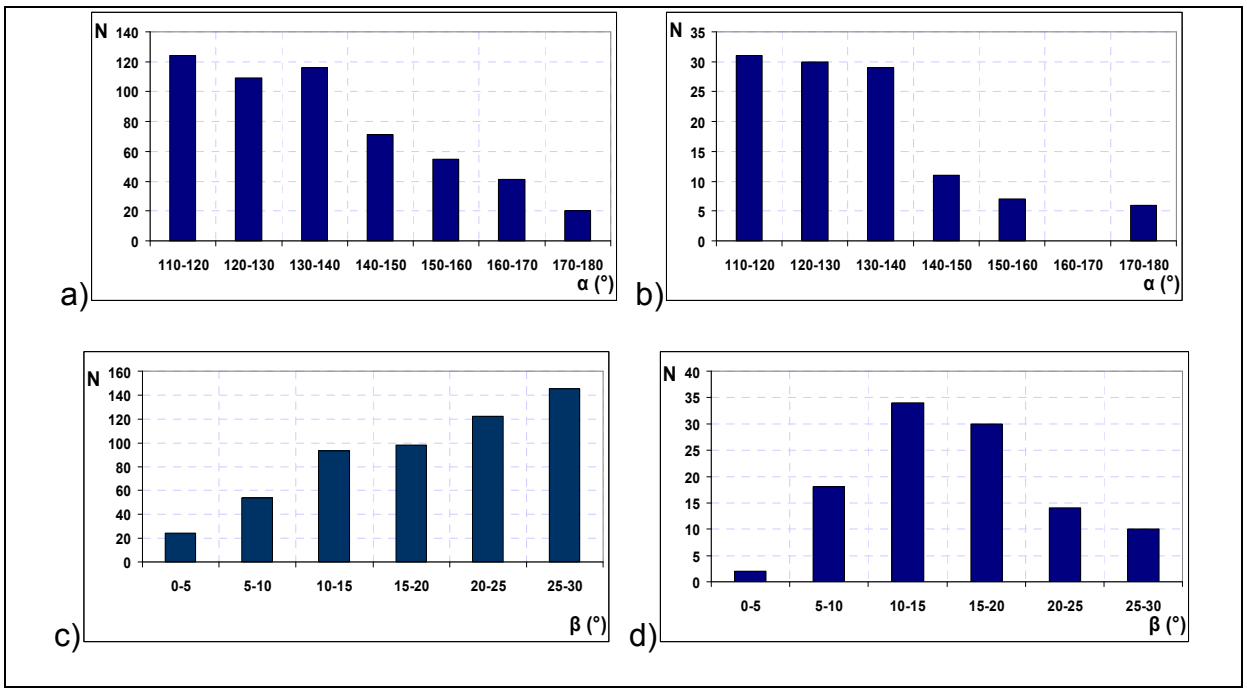

Fig. 7. Frequency distributions of α angle in a) non-bifurcated and b) bifurcated interactions and β angle in c) non-bifurcated and *d)* bifurcated interactions for non-coordinated water

In Figure 8 and Figure 9 we present two structures that contain OH/π interactions between non-coodinated water molecule and aromatic ring.

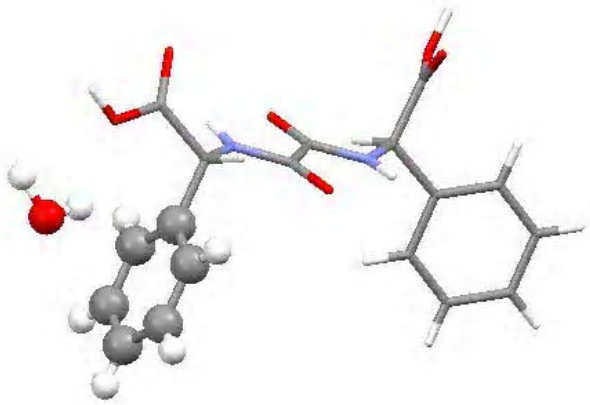

Fig. 8. Structure ACETEH (non-bifurcated OH/π interaction of non-coordinated water molecule and aromatic ring)

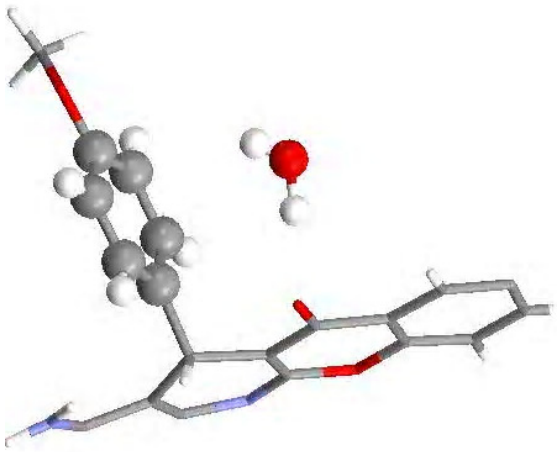

Fig. 9. Structure DOBJAG (bifurcated OH/π interaction of non-coordinated water molecule and aromatic ring)

CONCLUSIONS

According to the percentage of established bifurcated interactions, coordinated water molecules prefer to build this type of OH/π interaction with aromatic ring to non-coordinated. Since H---Ω distances in bifurcated interactions are shorter than in non-bifurcated (2.3-2.4 Å, in comparison to 3.3-3.4 Å), it is obvious that bifurcated interactions are more stable, because aromatic ring is interacting with two hydrogen atoms. Derived results for α and β angles show that O-atom of the water molecule tends to be above the center of the π-system.

The higher percentage of the d distances shorter than 2.8 Å implies that non-coordinated water interacts with aromatic ring at shorter distances than coordinated. This leads to the conclusion that steric effect is very important. The distribution of values of α and β angles confirms the importance of steric effect by showing the wider range of the angles. These data are the consequence of the fact that non-coordinated water molecules are more flexible in their positioning than voluminous complex species that contain coordinated water. The preference for stronger interaction of coordinated water is the consequence of larger partial positive charge of H-atoms.

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SEPARATION, IDENTIFICATION AND DETERMINATION OF SOME HARD METALS FROM *ACHILLEA MILLEFOLIUM L.*

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ABSTRACT

The Achillea millefolium samples from Copsa Mica, Romania, were subjected to thin layer chromatography and atomic absorption spectrometry analysis.The following metal ions were separated and quantitatively determined by these methods: Cu (II), Pb(II), Zn(II), Cd(II), Ni(II), Cr (III) and Co(II).

Key words: *Achillea millefolium*, extract, metal ions

INTRODUCTION

Yarrow (*Achillea millefolium L.*) is an herbaceous plant with pinnate, hairy leaves and white or rosy flowers, original from Europe and western Asia. It grows from the plains to the sub-Alpine area on haymaking fields, pastures, forest borders, railways and road sides, on relatively moist sandy soils (Munteanu 1996, Wood 1997, Tămaş 1999). It has been a medicinal plants ever since Ancient times, drawing its name from the Trojan legendary hero Achilles who used it to treat the wounds of his soldiers. It is also known as common yarrow, devil's nettle, gordaldo, milfoil, nosebleed plant, old man's pepper, sanguinary, soldier's woundwort, thousand-leaf, thousand-seal, etc. (Pârvu et al. 1998).

Its inflorescences (Millefolii flos) its aerial parts with inflorescences (Millefolii herba) contain volatile oil (0.1-0.4%) with chamazulene (about 20% of the oil), and a bitter substance, achileine. Besides this, it also contains organic matter such as formic acid, ascorbic acid, auteic acid, aconitic acid, folic acid, probionic acid, salicylic acid, valerianic acid, linoleic acid, myristic acid, oleic acid, palmitic acid, stearic acid, succinic acid, traces of caffeic acid, flavones, sugars, amino acids, proteins, tannins, inorganic substances such as iron, manganese, zinc, copper, cadmium, nickel, cobalt, silica, calcium, zirconium, etc. (Pârvu et al. 1998, Rohloff et al. 2000)

The plant also contains anti-inflammatory, antirheumatic, antiseptic, antispasmoic, aromatic, astringent, carminative, colagenic, diaphoretic, digestive, expectorant, stimulant, tonic, vasodilatating, etc. principles.

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A large number of scientific works show that medicinal plants cultivated in areas polluted with heavy metals accumulate these elements above admitted levels to a point that makes them medicinally useless (Pais and Jones 1997, Măruțoiu et al. 2006).

The goal of this study was to analyse heavy metal content in the medicinal plant Achillea millefolium.

MATERIALS AND METHODS

Yarrow (*Achillea millefolium* L.) – see fig. 1 - was harvested from the railway road side near Copsa Mica, a heavy-metal heavily polluted area. A sample (1 g) of flowers was mixed with 6 ml of HNO_3 (65%), 2ml of H_2O_2 (30%), and then diluted with 10 ml pf deionised water. The sample digestion lasted 6 hours. Micro elements from the *Achillea millefolium* flowers harvested from both the polluted and the non-polluted areas were determined through atomic absorption spectrometry (AAS) using a Perkin-Elmer atomic absorption spectrometer.

We mineralised 3 g of flower sample to extract microelements in order to separate them through thin layer chromatography, since we need a certain ion concentration in visualisation reactions.

Separating metal ions from the extract was done on DEAE-cellulose R - silica gel R plates (1:1 w/w), using as a mobile phase the mixture isopropanol – methanol – chlorhydric acid 5N (5:5.1, v/v). Spot visualisation was done through the spraying of plaques developed with 0.1% pyridine-2-aldehyde-2-furoilhydrazone and through examination in UV at 366 nm.

Fig.1. Yarrow (Achillea millefolium L.)

RESULTS AND DISCUSSIONS

A number of 7 heavy metal ions (Pb(II), Cu(II), Ni(II), Co(II), Cd(II), Zn(II) and Cr(III)) were identified in *Achillea millefolium* harvested by the railway road outside the town of Copsa Mica. Retention values are shown in table 1.

Table 1. R_Fx100 values of metal ions presented in Achillea millefolium from Copşa Mică

No.	Metal ion	R_F x 100	
		Standard	Sample
1	Pb(II)	5	5.5
2	Cu(II)	45	45
3	Ni(II)	25	25.5
4	Co(II)	35	35
5	Cd(II)	21	21
6	Zn(II)	40	41
7	Cr(III)	0	0

Results obtained through atomic absorption spectrometry are shown in table 2.

Table 2. The quantities of metal ions detected in Achillea millefolium from Copşa Mică

No	Metal ion	Concentration (mg/Kg) Samples from npolluted area	Concentration (mg/Kg) Samples from polluted area
1	Pb(II)	0.5	10.5
2	Cu(II)	4.7	26.12
3	Ni(II)	1.85	21.85
4	Co(II)	0.57	18.57
5	Cd(II)	0.25	12.25
6	Zn(II)	30.9	130.9
7	Cr(III)	0.2	12

The concentration values for the sample harvested from non-polluted areas are within normal limits, but for Copsa Mica, where there used to be a lot of factories producing non-ferrous metals that polluted the area, concentration is above admitted limits. This shows that medicinal plants in the Copsa Mica area cannot be used to produce medicine or to be used by the population.

CONCLUSIONS

Medicinal plants in polluted areas accumulate heavy metals from the soil, water, and air: therefore, we need to analyse them before using them in the manufacture of

pharmaceuticals (herbal teas, medicines, tinctures, cosmetics, etc.) to identify heavy metals, pesticides, and toxic products.

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SPECTRAL STUDIES OF COPPER(II) COMPLEXES CONTAINING ANTIPYRINE DERIVATIVES AS LIGANDS

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ABSTRACT

Comparative analysis of the infrared and electronic spectra of a series of complexes of copper(II) containing antipyrine derivatives as ligands were performed. The compared copper(II) complexes were $[Cu(BAMP)(H_2O)](ClO_4)_2$, $[Cu(BAMP)](ClO_4)_2$, $Cu^{I}Cu^{II}(BAMP)I_3$, $[Cu(TAMEN)](ClO_4)_2H_2ODMF$, $[Cu(TAMEN)][Cu(NCS)_2Cl](DMF)_2$ *were BAMP=N,N-bis(antipyrilmethyl)piperazine and TAMEN=N,N'-tetra-(4-antipyrylmethyl)-1,2-diaminoethane. The geometry of the copper(II) is different in the analyzed complexes depending on the ligand and anion. In* $[Cu(BAMP)(H_2O)](ClO_4)_2$ *and* $[Cu(BAMP)](ClO_4)_2$ *the metalic ion(II) geometry can be described as a square-based pyramid with the* N_2O_2 *donor atoms of BAMP forming the basal plane whereas in the* $[Cu(TAMEN)](ClO_4)_2H_2ODMF$, *and* $[Cu(TAMEN)][Cu(NCS)_2Cl](DMF)_2$ *the geometry of copper(II) is distorted octahedral due to an Jahn Teller effect. For* $Cu^{I}Cu^{II}(BAMP)I_3$ *the geometry is pyramidal five-coordination for copper(II) and planar for copper(I). The IR spectra has revealed that BAMP acts as a tetradentate ligand, through nitrogen piperazine atoms and antipyrine oxygen atoms. TAMEN act as hexadentate ligand through the nitrogen atoms of ethylenediamine bridge and the oxygen atoms of antipyrine moieties. It can be observed that both ligands, BAMP and TAMEN, participate in coordination with all the potential donor atoms.*

Keywords: copper(II), antipyrine, infrared spectra.

INTRODUCTION

Antipyrine, 2,3-dimethyl-1-phenyl-3-pyrazolin-5-one, the first synthesized drug with fever and pain release effect, and its derivatives possess a large variety of clinical, biological and pharmacological effects. They have analgesic, antipyretic, anti-inflammatory, antibacterial, antitumor activity (Burdulene et al., 1999; Bondock et al., 2008; Sondhi et al., 2000; Sayed et al., 2003; Rosu et al., 2010; Turan-Zitouni et al., 2001; Sayed et al., 1992; Daoudi et al., 2003). Mannich bases obtained from antipyrine and its derivatives have been prepared with the aim to obtain biological active compounds (C. Mannich, B. Kather, 1919). In this respect, complexes of some first row metal ions with ligands containing the antipyrine moiety N,N-bis(4-antipyrylmethyl)-piperazine (BAMP)(figure 1) and N,N'-tetra-(4-antipyrylmethyl)-1,2-diaminoethane (TAMEN)(figure 2) have been studied in our group (Costisor et al. 1994, Costisor et al. 2002, Tudose et al. 2005, Tudose et al. 2006). The obtained

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complexes have been biological investigated (Alexandrova et al. 2004, Alexandrova et al. 2005, Alexandrova et al. 2006, Alexandrova et al. 2008, Popova et al. 2006) and some of them have been proved to have antimicrobial and antitumoral activity higher than the ligands. It was established that the biological activity depends on the nature of the obtained complexes (mononuclear or polinuclear), the nature of the ligand, the nature of the anion.

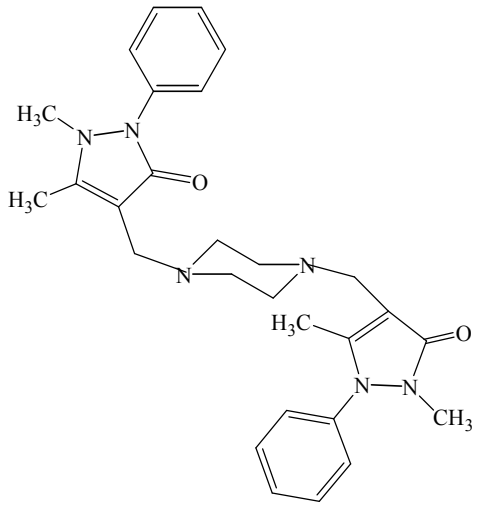


Fig. 1. N,N-bis(antipyrilmethyl)piperazine (BAMP)

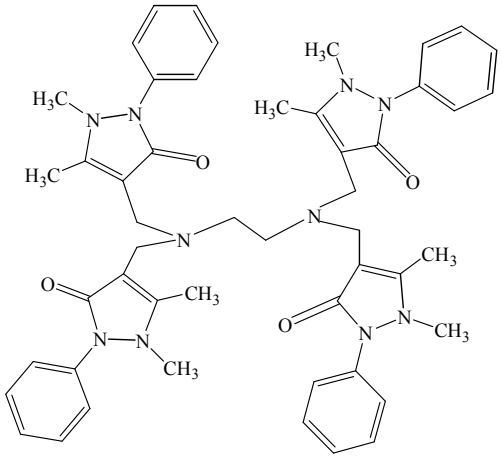


Fig. 2. N,N'-tetra-(4-antipyrylmethyl)-1,2-diaminoethane (TAMEN)

In this paper comparative analysis of the infrared and electronic spectra of a series of complexes of copper(II) containing antipyrine derivatives BAMP and TAMEN as ligands were performed. The compared copper(II) complexes were $[Cu(BAMP)(H_2O)](ClO_4)_2$, $[Cu(BAMP)](ClO_4)_2$, $Cu^{I}Cu^{II}(BAMP)I_3$, $[Cu(TAMEN)](ClO_4)_2H_2ODMF$, $[Cu(TAMEN)][Cu(NCS)_2Cl](DMF)_2$ were BAMP=N,N-bis(antipyrilmethyl)piperazine and TAMEN=N,N'-tetra-(4-antipyrylmethyl)-1,2-diaminoethane.

MATERIAL AND METHODS

All the needed chemicals have been purchased from commercial sources and were used without further purification.

All the copper(II) complexes were synthesized as published previously (Costisor et al. 2002, Tudose et al. 2003, Tudose et al. 2005, P. Weinberger et al. 2000).

IR spectra were performed with a Jasco FT/IR-430 spectrometer in the range 4000-400 cm^{-1} on KBr pellets. Electronic absorption spectra of the complexes were measured on a 10^{-3} M solution in DMF with a Lambda 12 Perkin Elmer spectrophotometer.

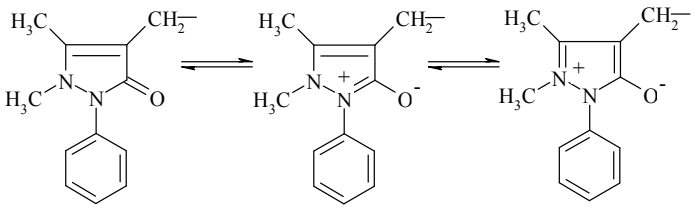
RESULTS AND DISCUSSIONS

As the figure 1 shows the ligand BAMP has four potential donor atoms, the two oxygen atoms of antipyrine fragments and two nitrogen atoms of piperazine bridge. The Mannich base TAMEN has six potential donor atoms, the four oxygen atoms of antipyrine fragments and two nitrogen atoms of ethylenediamine bridge. TAMEN can act as monodentate or tetradentate ligand, the obtained complexes are mononuclear or as bis-bidentate ligand, the obtained complexes are polinuclear. The electronic spectra of all the investigated complexes, recorded in 10^{-3} DMF solution have revealed that the metallic ion geometry is different as the table 1 shows. It was observed that in TAMEN investigated complexes the copper(II) ion is has an distorted octahedral geometry due to Jahn Teller effect, characteristic to an d^9 configuration (Lever et al. 1968). The copper(I) ion has an planar geometry in complexes $Cu^{I}Cu^{II}(BAMP)I_3$ and $[Cu(TAMEN)][Cu(NCS)_2Cl](DMF)_2$.

Table 1. The copper(II) ion geometry in complexes

Compound	λ_{max}(nm)	Geometry
$[Cu(BAMP)(H_2O)](ClO_4)_2$	709	square-planar
$[Cu(BAMP)](ClO_4)_2$	670	square-planar
$Cu^{I}Cu^{II}(BAMP)I_3$	703	pyramidal five-coordination
$[Cu(TAMEN)](ClO_4)_2H_2ODMF$	713	Pseudo-octahedral
$[Cu(TAMEN)][Cu(NCS)_2Cl](DMF)_2$	704	Pseudo-octahedral

IR spectra of the ligands and their copper(II) complexes were recorded and compaired. In the IR spectra of the Mannich bases, an intense band appear at 1662 cm^{-1} for BAMP and at 1658 cm^{-1} for TAMEN. These bands are assigned to the v(C=O) mode of antipyrine fragments. In the IR spectra of the complexes this bands are shifted as result of the involvement of the carbonilic oxygen atom of the antipyrine in coordination (Ferraro 1971; Nakamoto 1986). For all the investigated complexes the IR data suport the involvement of the antipyrine oxygen atom in the coordination, which suggests mesomeric structures II and III of antipyrine fragments.



I II III

Fig. 3. Mesomeric resonance structure of the antipyrine fragment of the ligand

Table 2. IR bands of the ligands and their complexes

Compound	v(cm^{-1})	Assignments
BAMP	1662	v(C=O)
	1429	Antipyrine –C=C– group
TAMEN	1658	v(C=O)
$[Cu(BAMP)(H_2O)](ClO_4)_2$	1556	v(C=O)+v(C=N)
	1108	Uncoordinated perchlorate ion (v_3)
	847	piperazine skeleton
	624	Uncoordinated perchlorate ion (v_4)
	512	v(Cu-O)
	454	v(Cu-N)
$[Cu(BAMP)](ClO_4)_2$	1561	v(C=O)+v(C=N)
	1174	v(C-O)
	550	v(Cu-O)
	419	v(Cu-N)
	1088	Uncoordinated perchlorate ion (v_3)
	624	Uncoordinated perchlorate ion (v_4)
$Cu^{I}Cu^{II}(BAMP)I_3$	1555	v(C=O)+v(C=N)
	600-615	v(Cu-O)
	495-507	v(Cu-N)
$[Cu(TAMEN)](ClO_4)_2H_2ODMF$	1587	v(C=O)+v(C=N)
	1438	antipyrine -C=C- group
	1145	v(C-O)
	1089	Uncoordinated perchlorate ion (v_3)
	624	Uncoordinated perchlorate ion (v_4)
	505	v(Cu-O)
	417	v(Cu-N)
$[Cu(TAMEN)][Cu(NCS)_2Cl](DMF)_2$	1599	v(C=O)+v(C=N)
	1434	v(C=C)
	812	v(C–S)
	505	v(Cu-O)
	495	δ(NCS)
	462	v(Cu–N)

CONCLUSIONS

It was observed that the geometry of the copper(II) is different in the analyzed complexes depending on the ligand and anion nature. In $[Cu(BAMP)(H_2O)](ClO_4)_2$ and $[Cu(BAMP)](ClO_4)_2$ the metalic ion(II) geometry can be described as a square-based pyramid with the N_2O_2 donor atoms of BAMP forming the basal plane whereas in the $[Cu(TAMEN)](ClO_4)_2H_2ODMF$, and $[Cu(TAMEN)][Cu(NCS)_2Cl](DMF)_2$ the geometry is distorted octahedral due to an Jahn Teller effect. For $Cu^{I}Cu^{II}(BAMP)I_3$ the

geometry is pyramidal five-coordination for copper(II) and planar for copper(I). The IR spectra has revealed that BAMP acts as a tetradentate ligand, through nitrogen piperazine atoms and antipyrine oxygen atoms. TAMEN act as hexadentate ligand through the nitrogen atoms of ethylenediamine bridge and the oxygen atoms of antipyrine moieties.

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THE EFFECTS OF MYCORRHIZAL FUNGI, STREPTOMYCETES AND PLANTS ON HEAVY METAL MOBILTY AND BIOACCUMULATION IN AN INDUSTRIALY ENRICHED SOIL: PRELIMINARY RESULTS OF A LYSIMETER EXPERIMENT

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ABSTRACT

We performed a lysimeter experiment to observe the influence of myccorhizal fungi and streptomycetes amendments on heavy metal mobility and plant uptake in a soil enriched with Pb (around 2000 mg/kg), Cu (around 250 mg/kg) and Zn (around 500 mg/kg) by atmospheric deposition from a battery factory near Bucharest, Romania. This study shows the results of the first stage in a series of four alternative Helianthus anuus and Secale cereale cultures on contaminated soil amended with Glomus intraradices fungi and Streptomyces acidiscabies and S. tendae bacterial inocula. Soil metal concentrations, Eh, moisture, temperature, pH and nutrient variation have been correlated with plant oxidative stress and microbial activity in an attempt to explain the influence of bacteria and fungi inocula on soil conditions and plant bioaccumulation.

Key words: lysimeter, heavy metals, Helianthus annuus L., bioremediation

INTRODUCTION

Heavy industrialization in the communist period has lead to the significant enrichment in heavy metals of soils in and near urban areas in Romania, with the highest concentrations near metal extraction and processing centers (Lucaciu et. al. 2004, Pope et. al. 2005, Damian et. al. 2008). Although most of the industrial processing installations generating atmospheric plumes or leachate have been closed or moved in recent times their close proximity to living quarters, highly frequented institutions and water sources, and the persistent nature of heavy metal pollutants in the soil mean that they still pose a threat to the environment and human health (Pope et. al. 2005, Lăcătuşu 2010). The former industrial areas east of Bucharest, the capital and largest city in Romania are hot spots of heavy metal enrichment of soils through atmospheric deposition and material/waste storage. Their close proximity to settlements causes heavy metals to enter local water systems through leaching into the water table and rivers, leading to noticeable health hazards, such as elevated lead concentrations in the blood of children in the area (Velea 2009).

In this context of persistent enrichment of certain areas with heavy metals, phytoremediation methods, that use the potential of plants and microorganisms to reduce the negative influence of these areas on humans and other organisms gain

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more an more interest (Kabata-Pendias 2001, Pilon-Smiths 2005, 2006, Vangronsveld 2009) as they are less disruptive and sometimes cheaper due to less extensive logistics (Neagoe et al. 2006). Heavy metals may exit from an enriched soil via either leaching or bioaccumulation, paths determined by solubility and bioavailability, which are in turn controlled by plant cover, microbial activity, organic mater and clay content, Eh, pH, drainage and mineral composition of the soil (Kabata-Pendias 2001, Iordache et.al. 2006). Rhyzosphere microorganisms influence metal mobility in the soil by changing soil conditions, like pH, Eh and organic matter content, secretion of chelating agents, accumulation and adsorption or by specific or non-specific biotransformation (Tabak 2005, Wenzel 2008). Moreover the rhyzosphere is host to complex relationships between plants, their associated mycorrhiza and bacteria, forming a network of interactions and substance exchanges known as the wood-wide web, linking many different individuals and insuring fast nutrient transfer and protection from toxic elements (Giovanetti 2006, Bonfante 2009). To know and stimulate these interactions, where they have been disrupted by man's influence, gives the possibility to direct remediation techniques for optimal results in regard to available financial resources and decisional factors' interest in area reclamation. Also, knowledge gathered from mezoscale experiments is useful in perfecting models of metal mobility at full scale in software applications (Iordache et.al. 2009), giving a powerful prediction tool to aid in the full scale directing of phytoremediation towards extraction or stabilisation of metals.

MATERIALS AND METHODS

Soil. Soil for the lysimeter installation was sampled from about 4km east of Bucharest, in the Pantelimon area that has been an industrial platform for decades. The main source of heavy metals enrichment was atmospheric deposition from the “Neferal” factory's chimney plume. The factory specialized in battery production and non-ferrous metals, since 1932. After 1995 the factory's activities were limited torecycling used batteries into lead, aluminum, and other non-ferrous material products. The soil near the chimney of the factory is richest in heavy metals, in concentrations close to 2000 mg/Kg for lead, 250 mg/Kg for copper and 500 mg/Kg zinc, with the highest concentrations at a depth of 0-15 cm. Soils predominating the area are reddish-brown preluvosoils, characterized as moderately acid, with a clayey texture, having small to average humus contents (1.8 – 3.7%) and total nitrogen (0,130 – 0,163%) and small amounts of soluble phosphorus (11 – 15 mg/kg) (Lăcătuşu 2008).

Experimental setting. The lysimeter installation (Figure 1.) consisted of 10 undisturbed soil monoliths, 30 cm wide by 60 cm tall, sampled from the same area to reduce heterogeneity.

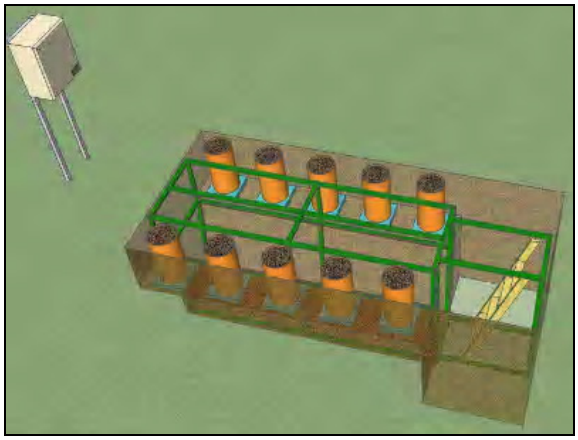

Fig. 1 Lysimeter instalation.

The top layer oh herbaceous plants and dense roots (ca. 5 cm of soil) was removed prior to sampling. The lysimeters were equipped with temperature, redox and humidity senzors and data was constantly monitored with a datalogger. Field tension was simulated at the bottom of the monoliths with a vacuum pump, also used to sample leachate. The installation was housed in a below-ground chamber for thermal insulation purposes. Plant cultures consist of 4 alternate successions of rye (planted in autumn, harvested in summer) and sunflower (planted in mid-summer and harvested in autumn).

Results will only be shown for the first culture of sunflower. Experimental variants consisted of 2 unamended, negative control replicates, 4 replicates amended with *Glomus intraradices* mycorrhizal inoculum in expanded clay (10%, mixed in the first 20 cm of soil), and 4 replicates amended both with *G. intraradices* and the streptomycetes: *Streptomyces acidiscabies* and *Streptomyces tendae* in liquid CSA growth medium, 10 ml per lysimeter.

Soil and water analyses.We analyzed key parameters for soil, water, plants and microorganisms. Soil analysis was performed at sampling in the immediate vicinity of each of the lysimeters from 0 to 60 cm depth in 10 cm increments, and after each plant harvest at 0 – 15 and 15 – 30 cm depths. Soil moisture was calculated after drying soil samples at 105ºC until constant weight. pH was measured in a soil water mixture (1:2.5). Soil samples were kept at 4ºC and processed within 24 hours after sampling. For nitrogen compounds, 20 g of soil were extracted with 100ml 0.2M KCl solution and for phosphate 5 g with 100ml 0.5 M $NaHCO_3$. Samples were analyzed through colorimetric methods: ammonium by Na nitroprussid, nitrate by sulphosalycilic method, nitrite N1 naphtyletylendiamine and sulphanilimide and phosphate with molid-ammonium and malachite green (Neagoe et al. 2005). Elements were analyzed on an Elan DRC-e ICP-MS from Perkin Elmer after digestion with aqua regia using an Anton Paar Multiwave 3000 digestion oven.

Leaching water was sampled after major rain events, leachate volumes and metal concentrations in leaching water were recorded.

Plant analyses. After harvesting, plants were measured weighed and separated into roots, shoots leafs and flowers. Roots were washed in tap water, distilled water and deionized water. Plant material was freeze dried, ground and stored at -45ºC. Fresh and freeze-dried biomass and individual heights were recorded.

For protein and enzyme assays, dry plant material (50 or 100mg) was homogenized in 4ml cold 100mM potassium phosphate buffer containing 2% polyvinylpyrolidone, 2mM EDTA and 2mM dithioerithrol and cetrifuged at 6000 rpm for 20 minutes at 4ºC. The supernatant was dialyzed overnight at 4ºC in 5mM K phosphate buffer. Protein concentrations were determined spectrophotometrically with alkaline copper reagent and Folin-Ciocaltau reagent against a BSA standard curve (Lowry 1951, Iordachescu 1980). Superoxide dismutase was measured through the inhibition of the rate of reduction of Cytochrome c by the superoxide radical, observed at 550 nm according to McCord and Fridovich (1969). Peroxidase activity was determined by spectrophotometrically measuring the transformation of guajacol to tetraguajacol in the presence of H_2O_2 according to Mascher et al (2002). the reaction mixture contained 33mM guajacol and 0,3mM H_2O_2 in 50 mM citrate/phosphate buffer.

For the chlorophyll and carotenoid assay, 50 mg of dry plant matter was homogenized in a buffer containing 80% acetone, 15% water and 5% NH_3 25% solution. Samples were then centrifuged to remove solids and spectrophotometrically measured at 480, 638, 645, 647, 663 and 664 nm and chlorophyll a, chlorophyll b and carotenoids were measured as described by Schöpfer (1989).

Microorganism analysis. Root fragments were cleared with KOH and colored with lactophenol blue for mycorrhiza differentiation. Roots were divided into approximately 1cm long fragments and around 20 fragments from each experimental variant were observed under a Nikon microscope.

Statistic analyses Statistic test were performed with the software “Statistica”.

RESULTS AND DISCUSSIONS

Plant biomass.

Biomass differed consistently between replicates (Figure 2). One of the unamended negative reference lysimeters (R1) and two of the replicates amended only with fungi (F2 and F4) showed very poor growth compared to other replicates of the same experimental variants. Differences in biomass between the negative reference variant and the one amended with *G. intraradices* mycorrhizal fungi were not statistically significant because of this high variation. Biomass differences between negative reference and fungi plus *S. acidiscabies* and *S. tendae* variant were close to statistic significance (p = 0.064), but still non-significant due to the usage of only 2 replicates for negative reference (due to financial constraints).

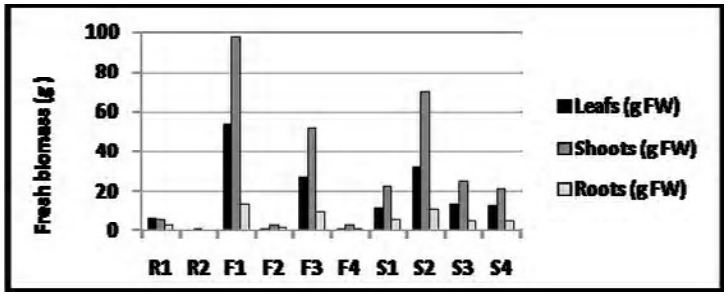

Fig. 2 Plant biomass.
R = unamended reference
F = amended with mycorrhizal fungi
S = amended with fungi and streptomycetes

Next, we shall focus on finding an explanation for the poor performance of the three replicates from the data provided by the studied parameters.

Plant health correlated with metal concentrations in plant tissue.

We focused mainly on Pb, Zn and Cu, as they were the major contaminants in the studied area. We were unable to corelate differences between mean plant tissue metal concentrations and stress for unamended reference against fungi amended and fungi and streptomycetes amended variants due to insuficient biomass for one of the R variants, however comparing the latter two yielded significant results. Most important, there were lower metal concentrations in the roots of fungi and streptomycetes amended replicates, but other parameters also varied in a significant manner (Figure 3).

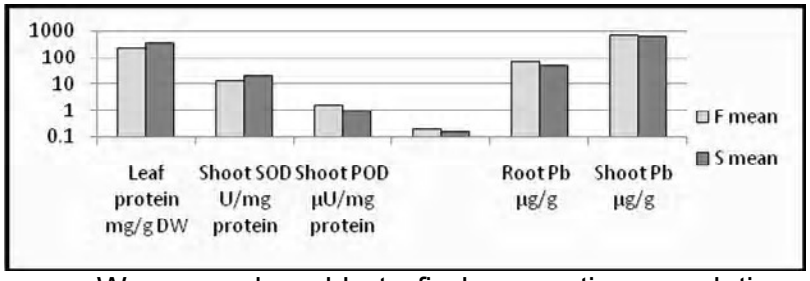

Fig. 3 The variation of metal concentrations and oxidative stress enzymes between F and S variants.
F = amended with mycorrhizal fungi
S = amended with fungi and streptomycetes

We were also able to find a negative correlation between metal and assimilating pigment concentrations when comparing fungi amended and fungi plus streptomycetes amended variants (Figure 4).

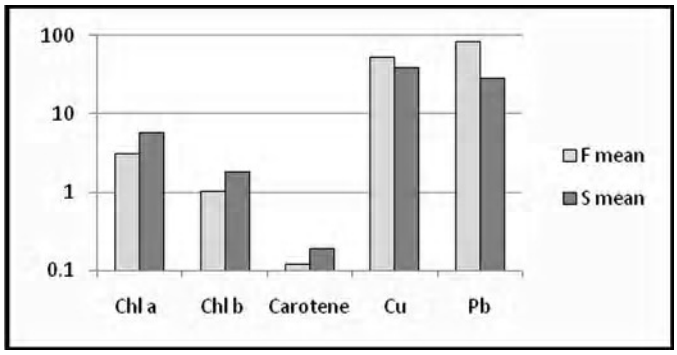

Fig. 4 Correlation between metal concentrations and assimilating pigments between F and S variants.
F = amended with mycorrhizal fungi
S = amended with fungi and streptomycetes

When investigating metal accumulation for each of the replicates we observed a linear relationship between logarithmic values of biomass and metal concentrations, suggesting metal accumulation did not increase with biomass, rather a dilution of metals in plant tissue occurring for plants with more biomass (Figure 5). The distribution of accumulated heavy metal concentrations closely mirrored that of biomass, again underlining the bad performance of the same three lysimeters.

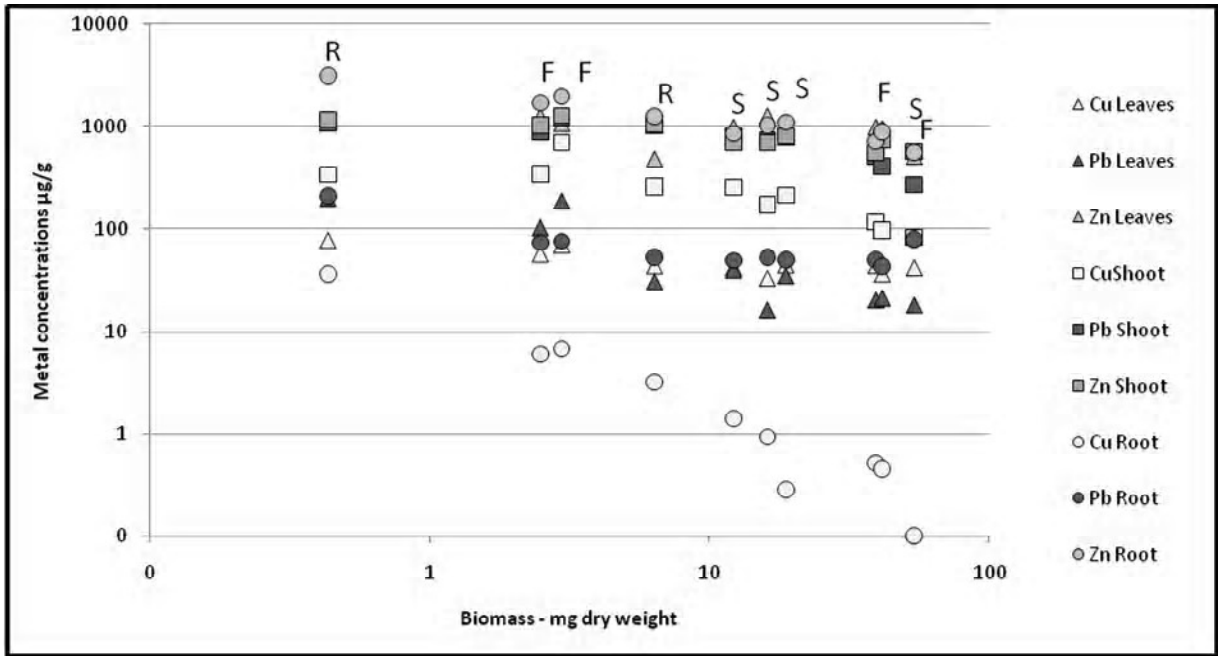

Fig. 5 Linear logarithmic relationship between total biomass of each of the lysimeters and their plant metal concentrations. R = unamended reference, F = amended with mycorrhizal fungi, S = amended with fungi and streptomycetes.

Factors influencing metal mobility and plant uptake.

Next, we looked at other factors influencing heavy metal bioaccumululation, such as total soil concentrations, microbial activity, soil pH, metal solubility and leaching and redox potential of the soil.

There were insignificant differences in soil pH between replicates after harvesting. Microbial activity was slightly higher in replicates amended with streptomycetes, as expected (Figure 6).

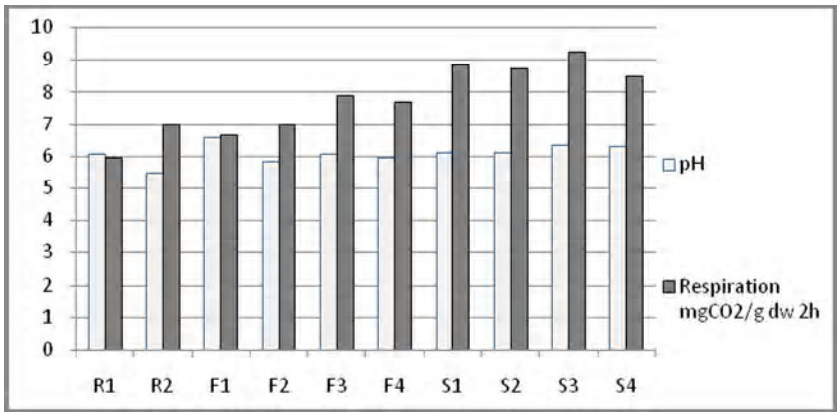

Fig.6. pH and microbial activity variation between experimental replicates after harvesting. R = unamended reference, F = amended with mycorrhizal fungi, S = amended with fungi and streptomycetes.

Pb, Cu and Zn soil concentrations in the soil at monolith sampling were decreasing with depth, but their variation pattern was not consistent with the three lysimeters with poor plant growth (Figure 7). After harvesting, concentrations of the same metals in the lysimeter soil also did not reveal a pattern explaining the dilemma (Figure 8). As total soil metal concentration were not the explanation, we turned to metal bioavailability. Parameters in the first 5 cm of soil prior to monolith sampling showed a high degree of heterogeneity, varying in all lysimeters and one parameter alone did not explain the poor growth of lysimeters R2, F2 and F4, but there might be complex conditions for plant inhibition due to different metal concentrations and small-scale microbial communities variation as suggested by initial nutrient data.

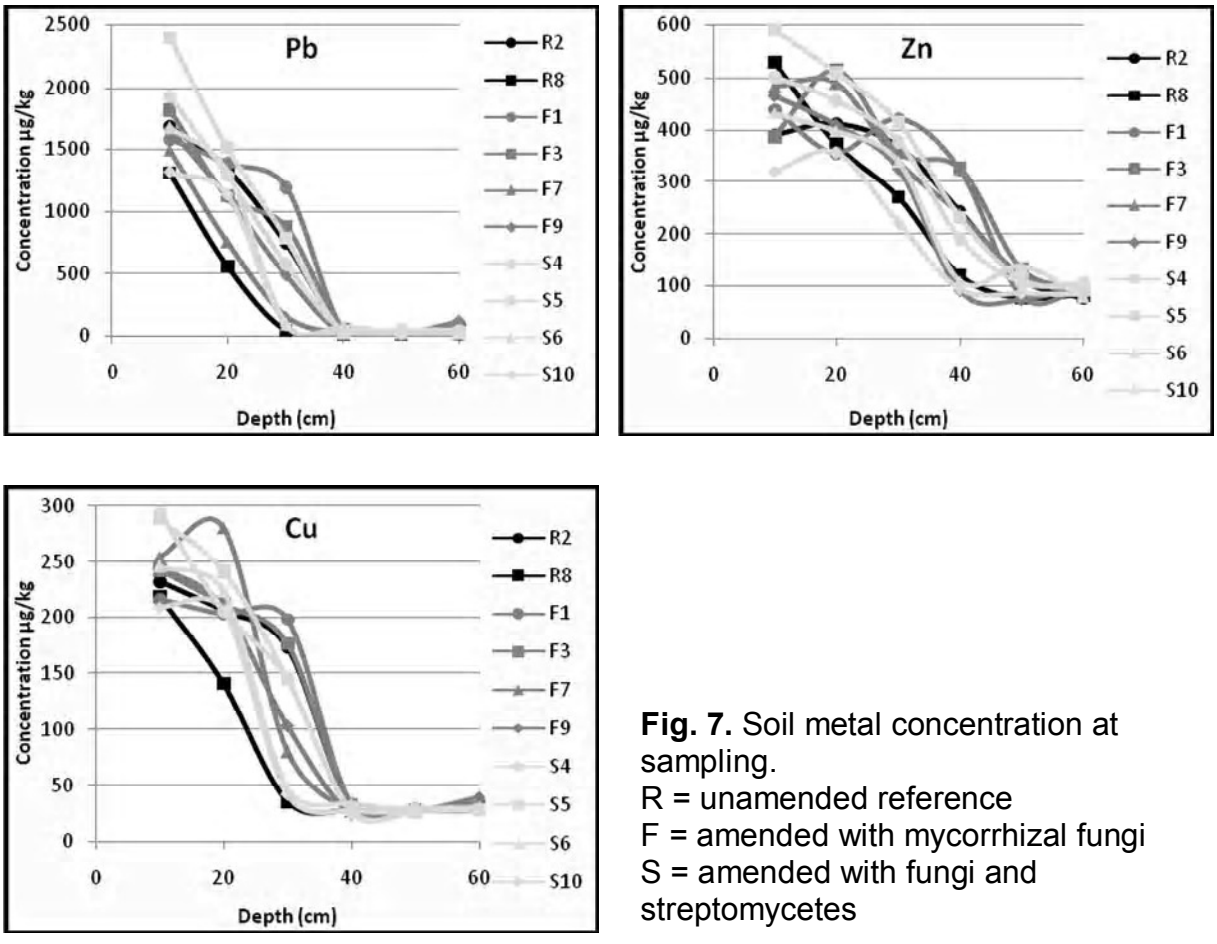

Fig. 7. Soil metal concentration at sampling.
R = unamended reference
F = amended with mycorrhizal fungi
S = amended with fungi and streptomycetes

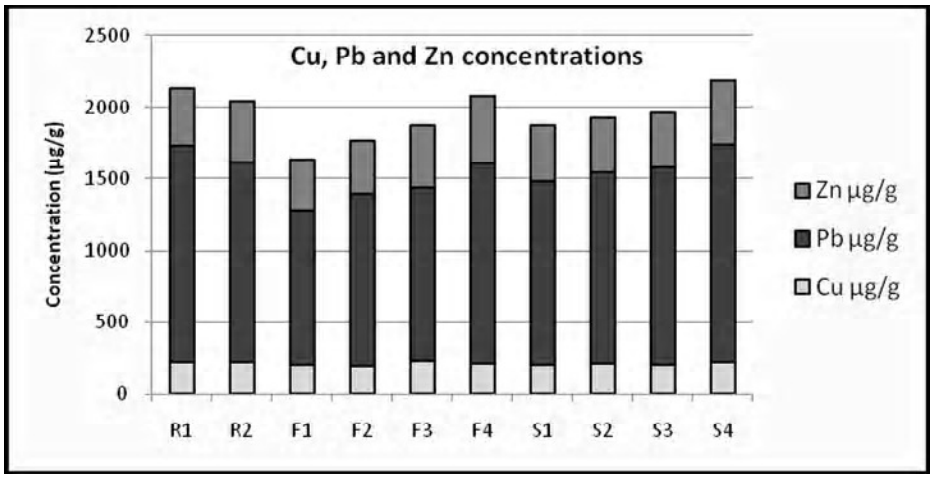

Fig. 8. Soil metal concentrations at plant harvesting. R = unamended reference, F = amended with mycorrhizal fungi, S= amended with fungi & streptomycetes

Metal solubility: metal exports through leaching

Lysimeter F2 had highest metal exports due to metal solubility and not higher permeability of substrate, showing highest metal mobility. R2 and F4, also poor growers, showed more metal solubility than F1 and F3, which had good plant growth (Table 1).

Table 1 Metal exports through leaching. R = unamended reference, F = amended with mycorrhizal fungi, S = amended with fungi and streptomycetes

	Cu (µg)	Pb (µg)	Zn (µg)	(ml)		Cu (µg/l)	Pb (µg/l)	Zn (µg/l)
R1	25.35	44.90	5469	11550	R1	2.19	3.89	473.5
R2	27.60	66.38	5141	10650	R2	2.59	6.23	482.7
F1	21.79	34.36	3335	6715	F1	3.24	5.12	496.7
F2	32.00	75.66	5032	8630	F2	3.71	8.77	583.1
F3	16.33	22.41	2043	11800	F3	1.38	1.90	173.1
F4	26.17	31.51	4179	12300	F4	2.13	2.56	339.8
S1	22.04	35.16	4518	13150	S1	1.68	2.67	343.6
S2	15.97	25.43	4186	11250	S2	1.42	2.26	372.1
S3	26.79	52.34	4264	10700	S3	2.50	4.89	398.5
S4	19.27	23.20	4885	10825	S4	1.78	2.14	451.3

Monitoring data: humidity and redox.

A two month monitoring of humidity variation showed drainage differences between some of the lysimeters. Drainage patterns were homogenous amongst replicates, with a decrease of moisture dynamics with depth, explained by the slowing down of the water flow as it infiltrated deeper into the monoliths after wetting or a meteorological event. Some differences existed: lysimeter F2, one of those with plant growth problems showed higher humidity dynamic at 30 cm than at 10 cm, showing a possible preferential flow in that area. Also, lysimeter S2 showed

constantly higher humidity at 50 cm, an indication of possible drainage problems (Figure 9).

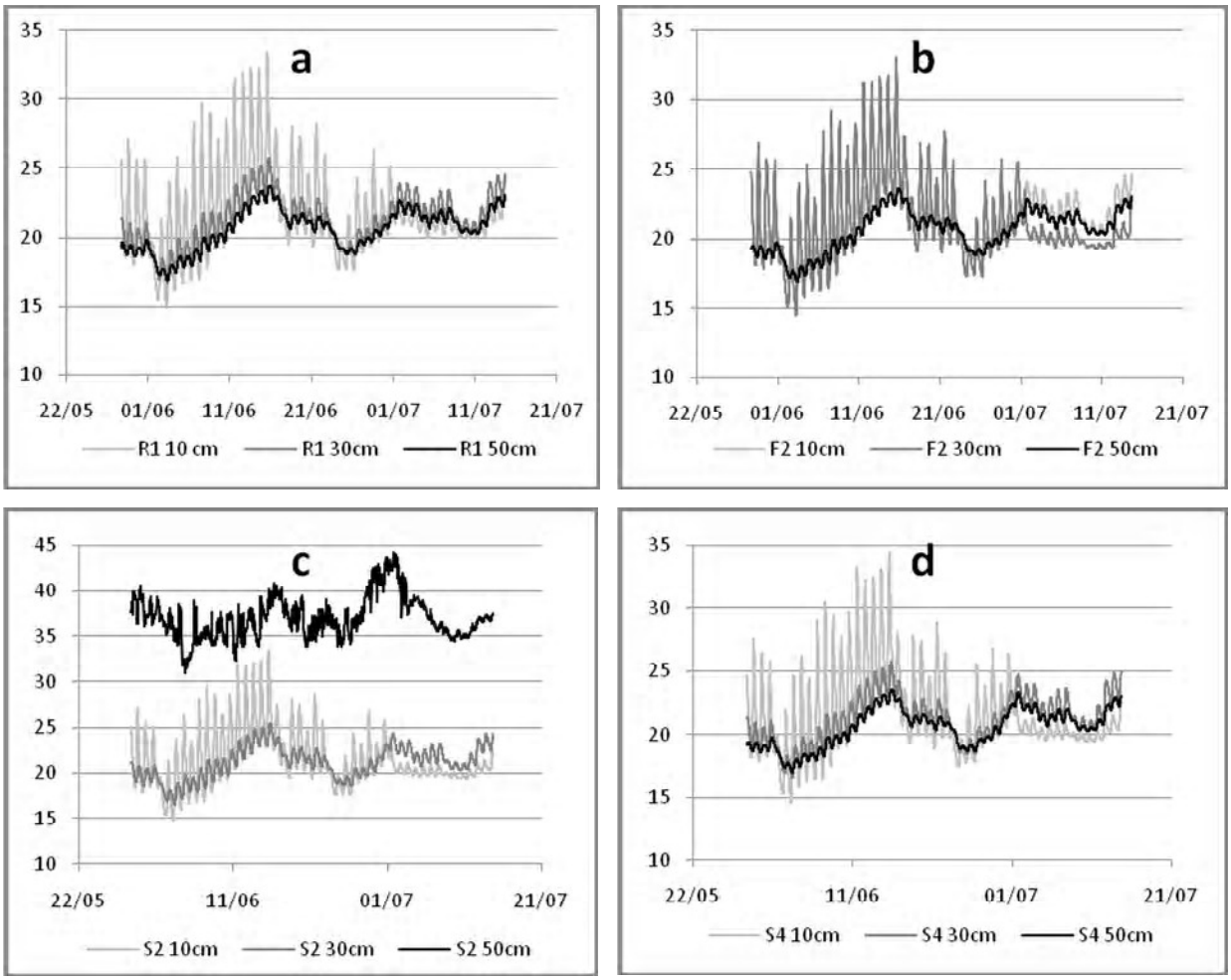

Fig. 9. Humidity monitoring data. a. normal dynamic in negative reference lysimeter R1. b. possible preferential flow in fungi amended lysimeter F2. c. drainage problems in fungi and streptomycetes amended lysimeter S2. d. normal dynamic in lysimeter S4.

The monitoring of redox evolution yielded the most interesting results concerning the low biomass production of replicates R2, F2 and F4. These lysimeters manifested a far stronger drop in redox potential in the first 10 cm of soil at rain events than the other lysimeters (Figure 10).

Mycorrhization.

We were unable to calculate statistically significant myccorhization degrees of plant roots due to the small biomass divided between the many lab tests. On the other hand, microscopic observations of colored root fragments revealed vesicles and arbuscules only in variants amended with mycorrhizal fungi inoculums, respectively F and S variants (Figure 11).

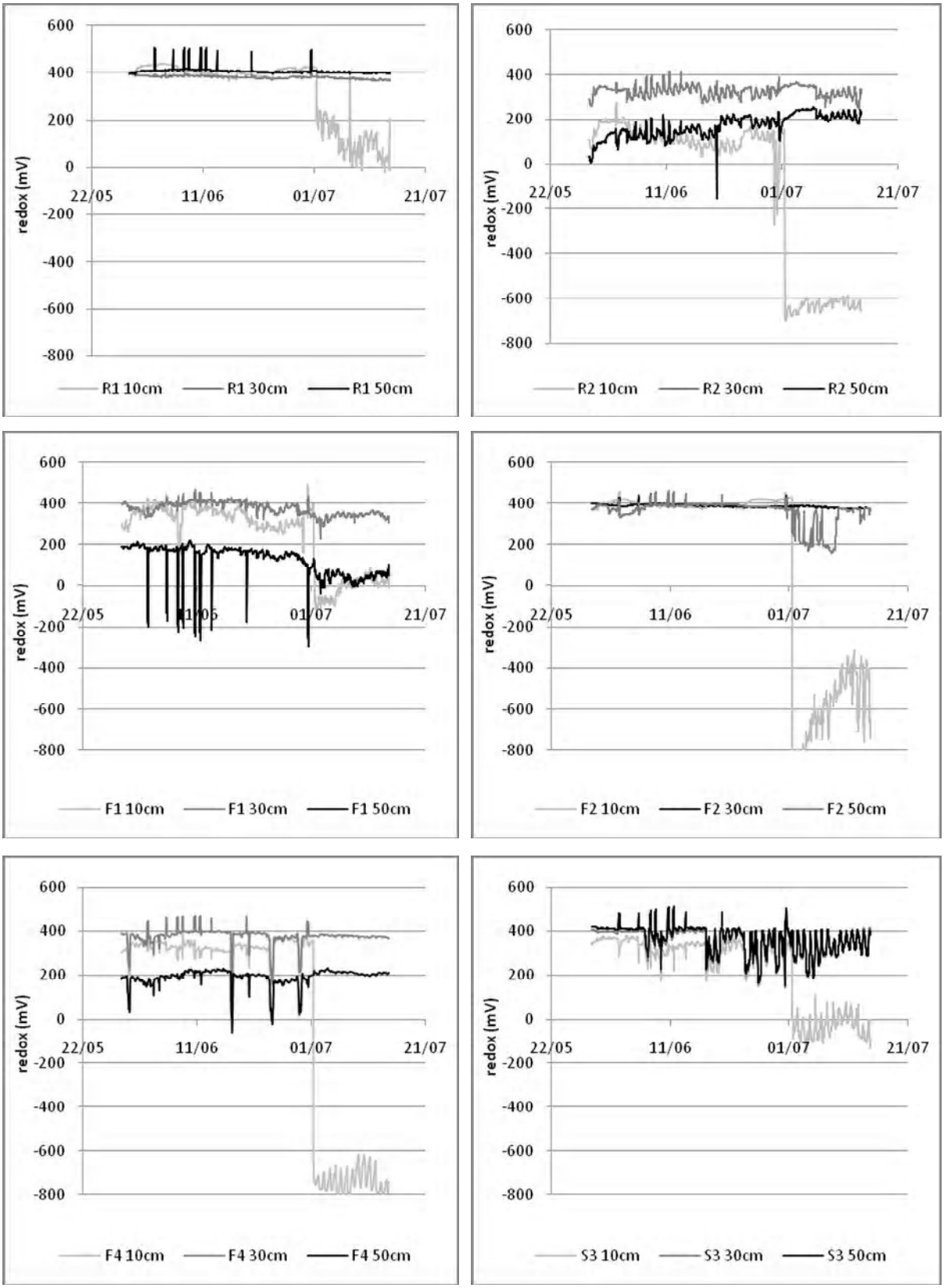

Fig. 10. Redox monitoring data. Lisimeters R1, F1 and S3 showing a normal redox evolution. Lisimeters R2, F2 and F4 showing stronger drop in redox at rain events.

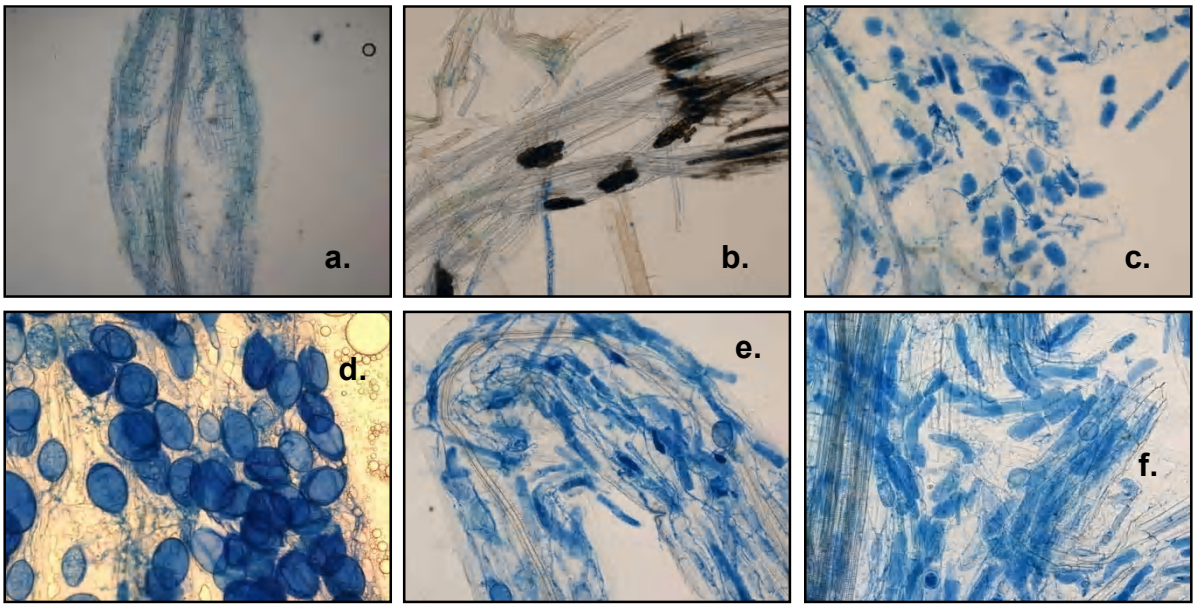

Fig. 11. Microscopic images of colored root fragments. a. and b. fragments from R plants, showing no myccorhiza, c. and d., fragments from F plants showing arbuscules and vesicles, e. and f., fragments from S plants showing arbuscules.

CONCLUSIONS

Using streptomycetes as an additional inoculum seems to significantly influence soil conditions, plant health and metal uptake.

As this is a multi-stage experiment, the data interpretation is limited to showcasing phenomena patterns within the different lysimeters, complete data interpretation being possible only after the cultures will be finished and all the data will have been gathered.

ACKNOWLEDGEMENTS

Funding was provided by the PNII CNCSIS Romanian Ideas grant 291/2007, the PNII CNMP Romanian grant 31012 / 2007 and the EU FP7 Project UMBRELLA. We would like to thank Dr. Frank Schindler for the streptomycetes strains, Prof. Dr. Katarzyna Turnau for the crash course in arbuscular mycorrhizal fungi and Dr. Florian Bodescu for the help in installing the experiment.

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STUDY OF STACKING INTERACTIONS BETWEEN COORDINATED PYRIDINES IN SQUARE-PLANAR METAL COMPLEXES

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ABSTRACT

Stacking interactions between coordinated pyridine molecules in square-planar metal complexes were studied in the crystal structures. Crystal structures archived in the Cambridge Structural Database involving coordinated pyridine molecules were screened for intermolecular contacts. The largest number of stacking interactions between pyridine ligands have head-to-tail orientation.

Key words: stacking interactions, pyridine, metal complexes

INTRODUCTION

Stacking interactions refers to parallel alignment of aromatic molecules, where interplanar distance of molecular planes is in interval from 3.3 Å to 3.5 Å (Janiak, 2000). These interactions are very similar in essence to interactions between nucleotide pairs in DNA (Wakelin, 1986) and to those that are responsible for stability of tertiary protein structure (Burley, Petsko, 1988). Coordination of the nitrogen of aromatic systems to a metal would increase property of electron withdrawal trough positive charge of the metal. In that way nitrogen aromatic systems should be very suitable for π-π interactions because of low of π-electron density. Furthermore, nitrogen as heteroatom increases affinity for stacking interactions.

Coordinated pyridine molecules, in terms of stacking interactions, can be oriented “head-to-head” or “head-to-tail”. It is also notable that rings are in most cases in parallel displaced orientation. Study of stacking interactions between coordinated pyridine molecules was based on statistical analysis of crystal structures archived in the Cambridge Structural Database.

MATERIALS AND METHODS

Study of interactions was based on analysis of crystal structures. Crystal structures archived in the Cambridge Structural Database (Allen, 2002) involving coordinated pyridine molecules were screened for intermolecular contacts. We searched for structures in which the distance between centroids of pyridine rings is

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less than 4.6 Å. Among the CSD crystal structures we found 86 structures and 102 short intermolecular contacts.

The geometric parameters used for analysis of the stacking interactions of coordinated pyridine are presented in Figure 1. The distance between centres of the pyridine rings is d. The distance from the other ring center projection to the average plane of the first one is referred to as r. The normal distance between the planes of the interacting rings is R. Torsion angle T is the Ω_1-N_1-N_2-Ω_2 angle. The distance between the center of one ring of the first pyridine, and the projection of the nitrogen atom of the second pyridine to the average plane of the first one, represents the horizontal displacement r_n (Figure 1). In addition to these parameters, it was also used the between the plane of the pyridine rings (which are hereinafter referred to as P_1/P_2 angle).

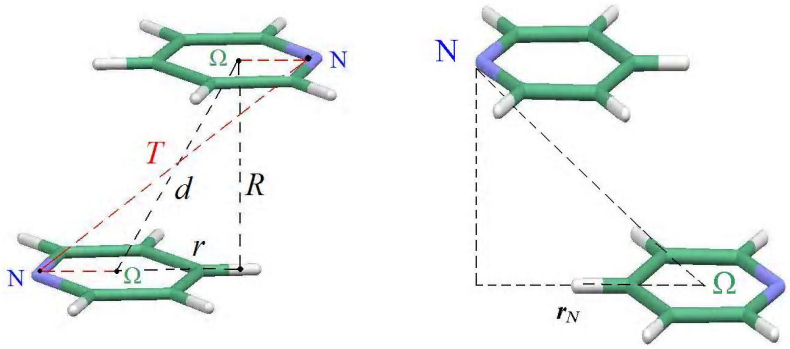

Fig. 1. The geometric parameters and atom labeling used for the description of stacking interaction.

RESULTS AND DISCUSSIONS

In the Cambridge Structural Database (CSD), crystal structures of 86 pyridine square-planar complexes with the distances between the two pyridine centroids (Figure 1) below 4.6 Å were found. In these structures 102 interactions between coordinated pyridines were identified.

The interactions were investigated analyzing geometrical parameters. The distribution of the P_1/P_2 angle between the planes of pyridine rings shows that in the majority of the interactions pyridine rings have parallel alignment (P_1/P_2 angle ranged from 0 to 10^0). Parallel alignment occurs in 102 contacts between coordinated pyridine molecules. In further analyze of stacking interactions only those contacts were used.

Analysis of geometric parameter R showed that distribution of normal distances are in interval from 3.2 Å and 4.0 Å (Figure 2) with maximum in interval from 3.3 to 3.5 Å, which is typical distance for stacking interactions (Janiak, 2000).

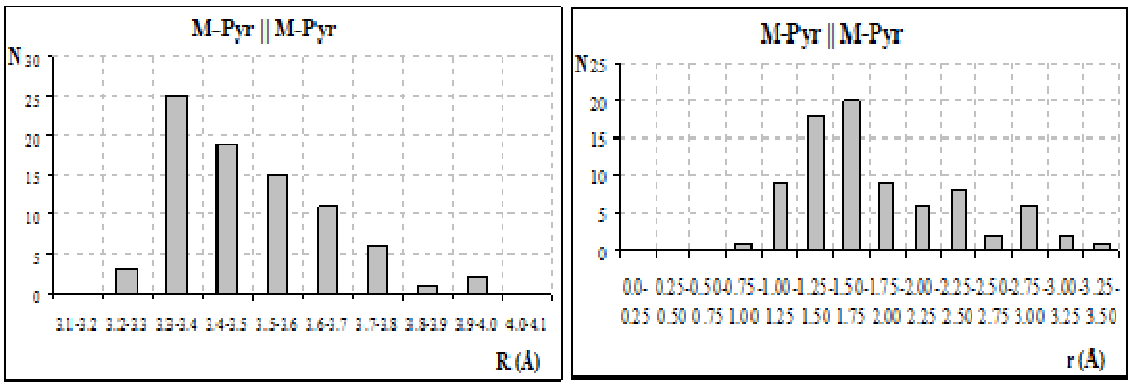

Fig. 2. Histogram of the distributions of the normal distance R and offset values r for the stacking interactions of pyridine molecules in square-planar complexes.

Histogram of the distribution for the offset values shows that rings are parallel displaced. Displacements, described with parameter r, are between 0.75 and 3.50 Å with a peak at 1.25 to 1.75 Å (Figure 2). Maximum distribution of r values for coordinated pyridine corresponds to the position when the projection of one pyridine ring center is located near the edge of the second ring.

The projections of nitrogen atoms on the second ring plane, in all contacts, are not located above the pyridine ring. The reasons for such distribution of r_N values (Figure 3) are interactions of pyridine ligands with the other ligands, from second interacted complex, or interactions with groups or molecules from the external sphere of the complex, these are located above other ligands or pseudo-coordinated to metal ion.

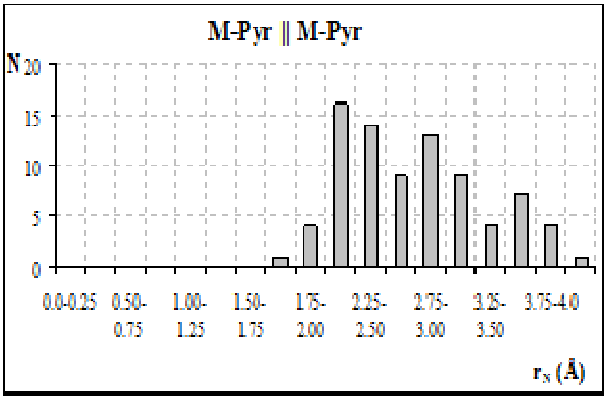

Fig. 3. The histogram of the distribution for r_N value

The largest number of stacking interactions, between pyridine ligands (71 contacts of 82), have head-to-tail orientation. There is only one interaction with the T value between 10° and 170°. The interactions with the head-to-head orientation are encountered in 10 contacts) as shown in Figure 4.

By visual inspection of the interactions we found out that head-to-head orientation occurs only in the complexes without voluminous ligands and if there are interactions between metal ions and other ligands or between the other ligands themselves.

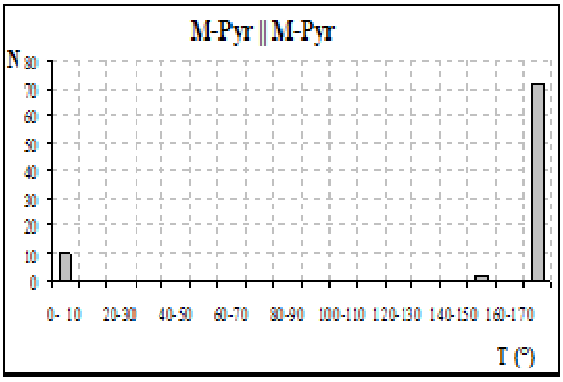

Fig. 4. Histogram showing the distribution of T torsion angle for stacking interactions between pyridine ligands in square-planar metal complexes.

CONCLUSIONS

In the Cambridge Structural Database (CSD), crystal structures of 86 pyridine square-planar complexes with the distances between the two pyridine centroids (*d*, Figure 1) below 4.6 Å were found. In these structures 102 interactions between coordinated pyridines were identified. Favored geometry for stacking interactions, between coordinated pyridines, is parallel displaced geometry. In the largest number of stacking interactions two pyridine ligands have head-to-tail orientation. The projections of nitrogen atoms on the second ring plane, in all contacts, are not located above the pyridine ring.

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DEUTERIUM DEPLETED WATER - CADMIUM SCAVENGER IN INTOXICATED MALE RATS

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ABSTRACT

The present work deals with the study of the deuterium depleted water (DDW) effect on male rats` antioxidant system, in cadmium (20 ppm Cd/ b.w. in single dose administration) induced oxidative stress. After 61 days of DDW treatment the malondialdehyde (MDA), registered slightly higher values as controls. Malondialdehyde, protein, alaninamino transferase (ALT) and aspartatamino transferase (AST) were determined by spectrometric methods. Cadmium was determined in male rats` liver and kidney by the graphite furnace technique with absorption spectrometry. In short time treatment (30 days), DDW had a prooxidant effect (MDA values are increasing) but after a longer time treatment (61 days), DDW could partially counteract the damages due to Cd intoxication by stimulating the cell antioxidant defense system. An important Cd scavenger role was observed at DDW treated groups. DDW had an important liver protective role.

Key words: cadmium, deuterium depleted water scavenger, rats

INTRODUCTION

It is well known that cadmium represents a dangerous environmental pollutant which is present in soil, water and air. A lot of scientist have brought in evidence that oxidative stress is installed in cadmium toxicity (Eybl et al, 2004, Eybl et al, 2006). As cadmium absorption is realized very quickly, its metabolism is very slow, which causes a tissue increasing in time. Cadmium realizes a protein binding to form a Cd-metallothioneine (MT-Cd). MTs have the capacity to bind both physiological (such as zinc, cooper, selenium) and xenobiotic heavy metals (cadmium, mercury, etc) through the –SH group of its cysteine residues (Sigel et al., 2009).

A central role in the uptake and disposition of many trace elements, especially the heavy metals has the liver (Ballatori, 1991).

The cadmium toxicity is due to the cell membrane lipids peroxidation, which causes the free radicals formation, the SH dependent enzymes and zinc enzymes inactivation. It was been also suggested that cadmium poisoning is caused by the disturbance of mithocondrial respiration, and probably the deficienty of zinc. This is an evidence of cadmium/zinc antagonism (Bogden, 2004).

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Free radicals, respectively the reactive oxigen species (ROS) in physiological concentration are stimulating the development and the cell division, but in very large doses, ROS lead to apoptosis (Dejica, 2001).

The last decade researches proved that the deuterium depleted water (DDW) have special influence on the whole animal organism respectively on cells and tissues development (Somlyai et al, 1998; Somlyai, 2001); a decreasing of the deuterium concentration in tissues or bodies, slow down the proliferation of a lot of types of cancer (Berdea P. et al, 1999, Somlyai, 2001, Manolescu et al, 2006,).

Some of the deuterium depleted water properties are: has a great influence in the animal and plant cells development and multiplication; in the cell transport phenomenon, in the DNA synthesis; has antioxidant properties. The cell water has less deuterium quantities (90 ppm) as the tap water (150ppm), so it is considered as structural water. (Bild et al, 2004, Eremia et al, 1997)

As there are quite few studies in this field, the objectives of the present work were: to study the effect of DDW administration in cadmium intoxication on liver function; to determin the cadmium level in male rats` liver and kidney and to determin the level of oxidative stress of rats` organism concerning the cells lipid peroxidation level (expressed as malondialdehyde production at 24 h after cadmium administration),

MATERIALS AND METHODS

The experiment was carried out on 60 adult Wistar male rats, with a body weight of 220-240 g, maintained in good physiological conditions. The male rats were divided in five groups. Each group included 12 rats.

L1- control, received tap water ad libitum during 61 days; L2 – received DDW (with a deuterium content of 30 ppm/l) ad libitum during 61 days; L3- received tap water during 30 days, in the 31 day, 20ppm Cd /kg b.w (as $CdCl_2$) single dose were administrated by gastric tubing and after 24 hours, L3 rats were sacrificed; L 4- pretreated with DDW ad libitum during 30 days, in the 31 day 20 ppm Cd/kg b.w as $CdCl_2$ single dose were administrated by gastric tubing and after 24 hours L4 rats were sacrificed and L5 - pretreated with DDW ad libitum during 30 days, in the 31 day 20 ppm Cd/kg b.w as $CdCl_2$ single dose were administrated and 30 days more they were treated with DDW ad libitum.

After 31 days from the beginning of the experiment (respectively 24 hours after Cd intoxication) blood was collected (on heparine), by cardiac punction and than sacrificed (liver and kidney were collected from L3 and L4); a second sampling took place at the end of the experiment (after 61 days), when blood and tissues samples were collected under general narcosis from L1, L2 and L5.

Malondialdehyde (MDA) was determined by the thiobarbituric acid reaction in plasma (Carbonneau et al, 1991). Protein ranges, alanin aminotransferase (ALT) and aspartat aminotransferase (AST) activities were determined by colorimetric methods in plasma (Ghergariu et al, 2000).

Cadmium content in liver and kidney was determined by atomic absorbtion spectrometry (AAS-Shimadzu 6200). Liver and kidney were digested in teflon containers in a microwave oven closed system (MARS X CEM).

All chemicals were supplied by Merck, Germany and Sigma- Aldrich, USA and were of analytical purity.

The investigations were carried out with the approval of the Local Ethics Committee according to the Romanian law 205 /2004, art.7, 18, 22 and the

regulations no. 143/400/2002 and 37/2002, concerning with the protection of vertebrate animals used for experimental and other scientific purposes.

The data are presented as means ± S.D. values. ANOVA and TTest were used to analyze mean differences between experimental groups for each parameter separately and between groups

RESULTS AND DISCUSSIONS

The results are presented in table 1 and figures 1-2.

Cadmium concentration. After 24 hours of a single Cd administration as cadmium chloride, the highest value was registered at L3 (Cd intoxicated) in liver and kidney. The 30 days DDW pre- treatment (L4), could maintain Cd at lower doses. There was observed a decreasing with 61.8% ($p< 0.001$) in the liver and a slightly increasing of Cd content in the kidney which means that DDW was capable to mobilize intracellular bound cadmium, DDW was acting as an effective Cd scavenger, as at L5 (DDW+Cd+DDW) there were registered 21.84 times lower values in liver as at L3 (Cd intoxicated) group ($p< 0.001$) and 5.75 times lower in kidney as in L3. In control and DDW treated rats, the concentration of cadmium was situated at similar values.

MDA concentration. The cadmium chloride administration caused a significant lipid peroxidation. Plasma MDA registered a significant increasing (188.9 %, $p < 0.001$) compared to control (L1) and higher as the DDW pre-treated group (L4). In the DDW pretreated and treated (61 days) and Cd administration group (L5) was registered a decreasing of MDA concentration (31.6%, $p<0.001$) in male rats` plasma, as in the Cd intoxicated groups (L3). The pre-treatment and the treatment with DDW after single dose Cd administration decrease significantly the lipid peroxidation. The results are presented in Table 1, figure 1.

Table 1. Liver and kidney Cd average values, serum protein and MDA average values and the activities of ALT and AST, in DDW treated male rats

Parameter	Cd µg/g organ		Protein (g%)	MDA (ηmol/mg)	ALT (UI)	AST (UI)
	liver	kidney				
L1- H_2O	0.02± 0.006	0.01± 0.005	5.33± 0.84	19.07± 1.80	23.88± 0.77	16.59± 1.31
L2 –DDW(61days)	0.045± 0.015***	0.029± 0.008***	7.56± 0.34*	27.61± 3.71***	19.47± 1.60***	18.76± 2.99**
L3 – H_2O (30 days) +Cd	45.65± 5.34***	7.88± 1.91 ***	5.05± 0.92	35.9± 1.99***	42.6± 0.91***	15.53± 1.18**
L4- DDW(30 days) +Cd	17.46± 3.48***	9.55± 1.22***	5.16± 0.63*	29.7± 2.14***	21.13± 1.47***	16.01± 1.16*
L5- DDW(30 days) +Cd+ DDW(30 days)	2.09± 0.59***	2.11± 0.31***	5.60± 0.12*	24.98± 2.14***	17.81± 1.39***	11.44± 0.83***

Note: Mean ± S.D.; n= 12 animals per group, * $p> 0.05$, ** $p< 0.05$, *** $p< 0.001$

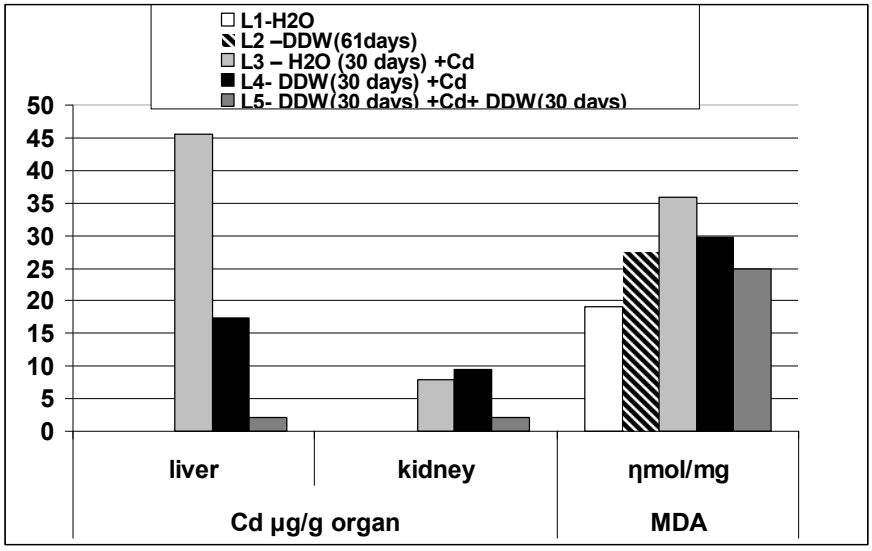

Fig. 1. MDA average values and liver and kidney Cd average values, in DDW treated and Cd intoxicated male rats

The lipid peroxidation was installed after Cd intoxication and there was observed that a preventive treatment with DDW reduced the MDA concentration at Cd intoxicated groups (Olariu et al. 2008).

The protein amount values were situated at similar ranges with a slightly increasing in the DDW treated groups ($p > 0.05$).

At the DDW (61 days) treated group (L2) without intoxication, a stimulation in the protein synthesis, was observed. This observation could be made even at L5 group (DDW pretreated and treated rats and Cd intoxicated group); slightly increased protein values as the control, were registered.

Alanin aminotransferase activity has an immediately response at the toxic. ALT registered the highest average value (42.6± 0.91 UI, $p < 0.001$) at the Cd intoxicated group (L3). At the other experimental groups, ALT activities registered similar values.

Aspartat aminotransferase activity was not so affected as ALT. AST activities registered similar ranges in both DDW treated and untreated groups. Significantly AST lower activities ($p < 0.001$) were registered at L5. (Table 1, figure 2)

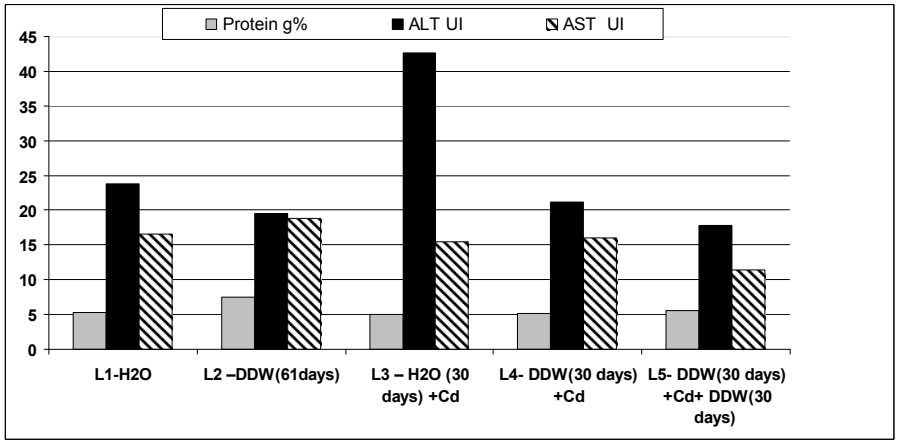

Fig. 2. Protein, ALT and AST average values in DDW treated and Cd intoxicated male rats

Both enzymes activities were situated in the range of the literature data (Meingassner et al.1992)

CONCLUSIONS

1. DDW (61 days) stimulated the protein synthesis
2. Liver function was not significantly affected; A significant decrease ($p< 0.001$) was registered in DDW 61 days administration group, but transaminases activities (ALT and AST) were maintained in normal ranges.
3. The DDW pre-treatment and the treatment after single dose Cd administration decreased significantly the lipid peroxidation; a MDA decreasing concentration (31.6%, $p<0.001$) in male rats` plasma, was registered
4. The 61 days DDW treatment (L5), could maintain Cd at lower doses in liver and kidney.
5. In liver, 21.84 times lower values at L5 as at L3 (Cd intoxicated) group ($p< 0.001$) and 5.75 times lower in kidney were determined
6. The results indicated that DDW was capable to mobilize intracellular bound cadmium
7. DDW was acting as an effective Cd scavenger
8. DDW had a liver protective role

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THE ANALYSIS OF CONCENTRATION OF HEAVY METALS AND MICROELEMENTS OF PLANT VINCA MINOR

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ABSTRACT

In the case of the vegetable product Herba from Vinca minor, the concentration of micro- elements can influence the concentration of alkaloids present, active ingredients directly responsible for the therapeutic action.We have studied two different geographical areas with similar altitude, one in Romania and one in Serbia. The minerals concentration was determined by atomic absorption spectrometry. The two lots of herbal products have similar concentrations of heavy metals and micronutrients. It is noted the concentrations close from the samples of the stem and leaves. In periwinkle leaves was determined a higher concentration of molybdenum (0.21 mg / kg) than in the stem (0.17 mg / kg) and lower of nickel (1.09 mg / kg) and chromium (1,139 mg / kg). Saschiu vegetable samples taken in the study not present heavy metal pollution

Key words: *Vinca minor,* minerals, spectrometry.

INTRODUCTION

The content of macro-and micronutrients of plants, is dependent on several factors: the specific structure of the soil and its geological origin, the lighting, the temperature system, the area of plant growth, the phylogenetics characteristic, and physiological factors: age and speed of development [Andersen 1990]. The many factors involved motivate the difficult realization of standard image concerning the composition of macro-and microelements in plants products (AOAC 1990, Butnariu 2006).

The accumulation of heavy metals in plants is different depending on plant species and its growth area. In terms of heavy metal accumulation, the plants are grouped into three categories: *Plants batteries* (accumulated in aerial parts of the plant) *Plants indicators* (a similar concentration in the aerial and underground parts) and *Plants with low content of heavy metals* (the concentration is not influenced by the amount presented in the soil) (Alexa 2003, Butnariu 2007).

The vegetables bioindicators are the plant species which have the advantage that they can provide a response to the combined effect of certain heavy metals. The *Sentinel*

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species are rapidly growing and react quickly to increase the heavy metal concentration; it is used to signal the early presence of this metal (Wolfe 2005, Ward 1995).

Vinca minor periwinkle, is a plant native to Europe. The leaves are evergreen and the flowers white or pale purple are solitary in the leaf axils. Ethnomedically are used to enhance blood circulation, including of the brain and to treat cardiovascular disorders (Stanescu 2004). We chose to study the heavy metal concentration of plant *Vinca minor*, because in the specialized literature the content of microelements is less present. In the case of Herba plant product from *Vinca minor*, the concentration of microelements can influence the concentration of presents alkaloids, active ingredients directly responsibles for the therapeutic action (Shallari 1998). We have studied two different geographical areas with similar altitude, one in Romania and one in Serbia. In order to achieve a comparative image regarding the concentration of microelements with other data and information from the literature, we chose a plant botanically related and studied *Catharanthus roseus* (L.) G.Don (*Apocynaceae).* Data on two different lots, Lot 1 and Lot 2 (a comparative study on the leaves, stem and flower) are shown in Table 1. (Lokeshwari 2006).

Table 1 The concentration of microelements expressed as (mg / kg, ppm), to vegetable products of *Catharanthus roseus*

Element	**Lot 1**	**Lot 2 Leaves**	**Lot 2 Stem**	**Lot 2 Flower**
K	6.504	3.791	2.925	5.425
Ca	29.098	6.373	5.653	2.775
Cr	24.6	1.43	2.64	1.83
Mn	183.7	63.72	93.42	44.02
Fe	424.9	79.51	37.52	24.91
Ni	6.8	3.27	4.02	4.02
Cu	3.7	4.35	8.51	3.77
Zn	54.6	50.12	49.51	31.02
Cd	-	2.61	1.33	1.58
Pb	-	4.44	1.43	2.93

European Pharmacopoeia VI ed. requires maximum concentration limits, in plant products for therapeutic use, for Pb (5 mg / kg), Cd (0.2 mg / kg) and Hg (0.1 mg / kg) (Ph.Eur.2008).

MATERIALS AND METHODS

The plant material used was the aerial part of *Vinca minor* plant harvested from two different geographical areas, in April 2010: Botanical Garden of Faculty of Agriculture Cluj Napoca (Ro), hills of the Western Serbia (SRB). Drying was done at room temperature, were kept in glass pharmaceutical containers with dark color. The Voucher samples were deposited in the Herbarium collection of the Faculty of Pharmacy. There were separate the stems of leaves, and were analyzed separately.

The ash and the water content were determined calcinations and hot drying procedure (Butnariu 2006, Peev 2006).

For the determination of mineral content of the two analyzed vegetal products were processed following the wet disintegration. For each sample set was achieved a control sample. Minerals concentration was determinate by atomic absorption spectrometry. Spectrophotocolorimetric determination is a physical – chemical method whose principle is based on comparing the intensity of staining intensity color sample for analysis of known concentrations and different solutions. The heavy metals were measured from the obtained hydrochloric solution by pulverization in the air-acetylene flame and measurement of the absorbance, respectively emission at the characteristic wavelength for each analyzed element. It was used an atomic absorption spectrophotometer, controlled by PC (AAS, Analytik Jena AG). For the spectrophotometer calibration were prepared sets of etalons of different concentrations in HCl 0,5N solution for each analyzed element, starting to the concentrated standard solutions. The concentration (C) for each determined element was calculated with the following formula:

$$C\ (mg/kg\ or\ ppm) = a \bullet f / m,$$

where: f = dilution factor; a = element content indicated by apparatus (mg/l);
m = sample initial weight. (Butnariu M. et al., 2010).
For each category of plant product have been working on five separate samples, calculating the average content of mineral elements and standard deviation.

RESULTS AND DISCUSSIONS

Containing metal concentration values studied in the leaves and stems of periwinkle of the two groups analyzed are shown in Table 2.

Table 2: Concentration of microelements in the leaves and stems of *Vinca minor* (mg / kg, ppm)-Values are means of triplicate samples (± SD)

Element	***Vinca minor* Srb**		***Vinca minor* Ro**	
	Content in leaves (mg/kg)	**Content in stem (mg/kg**	**Content in leaves (mg/kg)**	**Content in stem (mg/kg)**
Hg	0.063± 0.005	0.072± 0.007	0.070± 0.006	0.070± 0.007
Mo	0.29± 0.05	0.19± 0.02	0.21± 0.06	0.17± 0.03
Fe	0.971 ± 0.04	1.054 ± 0.05	0.965 ± 0.04	1.049 ± 0.05
Mn	12.01 ± 0.04	11.2 ± 1.5	10.71 ± 0.05	10.7 ± 1.5
Cu	2.118 ± 0.006	2.321 ± 0.006	2.105 ± 0.006	2.110 ± 0.005
Zn	3.7± 0.3	3.09 ± 0.24	3.5 ± 0.3	3.52 ± 0.21
Ni	1.062 ± 0.04	1.138 ± 0.04	1.090 ± 0.04	1.150 ± 0.05
Cr	1.206 ± 0.03	1.96 ± 0.08	1.139 ± 0.03	1.79 ± 0.07

Cd	1.09 ± 0.07	1.11 ± 0.08	1.02 ± 0.07	1.03 ± 0.09
Pb	0.06 ± 0.02	0.07 ± 0.04	0.04 ± 0.02	0.04 ± 0.03

It has achieved a screening of the heavy metal concentration in leaves and stems of sachiu harvested from two different geographical areas. The two lots of herbal products present the similar concentrations of heavy metals and microelements. It is noted the near concentrations in the case of samples from the stem and leaves. In periwinkle leaves was determined a higher concentration of molybdenum (0.21 mg / kg) than in the stem (0.17 mg / kg) and lower nickel (1.09 mg / kg) and chromium (1,139 mg /kg).Periwinkle leaves have a significantly lower content of Fe, Mn, Zn by comparison from leaves of *Catharanthus roseus*. This difference can be explained as due to different pedoclimatic conditions and species differences. The metals such as Cr, Ni, Cu and Cd concentrations have values close to vegetables products of the two species compared. Similar to *Catharanthus roseus* in the case of periwinkle was determined a lower concentration of nickel and chromium in the leaves comparatively to the stem.

CONCLUSIONS

The periwinkle vegetable samples taken in this study, not present heavy metal pollution. The vegetable products can be used as raw material for pharmaceutical industry.

Periwinkle leaves have a significantly lower content of Fe, Mn, Zn by comparison from leaves of *Catharanthus roseus*. It is noted the near concentrations in the case of samples from the stem and leaves.

Monitoring the quality of plants, soil and water represents an important direction in the maintenance and development of sanogenesis.

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ANALYSIS OF MINERAL CONCENTRATION OF PINE FOLIAR BUDS AND OF GEMMOTHERAPEUTIC EXTRACT

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ABSTRACT

Foliar buds are a category of plant products used in phyto-therapy and, more recently, in gemmotherapy. Plant products for pharmaceutical use should meet quality control standards concerning admitted concentration of heavy metals. This study aimed at monitoring mineral content analysis of dried pine foliar buds in *Pinus silvestris* L. and *Pinus montana* Mill. (*Pinaceae*) as well as that of the hydro-glycero-alcoholic extract.

Foliar bud samples under study show no heavy metal traces. Pine foliar buds, because of their tendency to concentrate and accumulate some heavy metals, could be a detector-type bio-indicator.

Key words: buds, trace elements, gemmotherapy

INTRODUCTION

Foliar buds are a category of plant products used in phyto-therapy and, more recently, in gemmotherapy (Pitera 2000, Stanescu 2004). Plant products for pharmaceutical use should meet quality control standards concerning admitted concentration of heavy metals (Lokeshwari 2006, Ph.Eur.2008).

The present study aimed at analysing dried pine foliar buds and hydro-glycero-alcoholic extract from the point of view of their mineral content. In phyto-therapy, they use foliar buds from *Pinus silvestris* L. (*Pinaceae*), and in gemmotherapy they use foliar buds from *Pinus montana* Mill. (*Pinaceae*) (Pitera 2000).

Pine leaves are known in literature: they are studied for their heavy metal content and are considered pollution indicators. Recent research have proven the fact that tree leaves are a more proper parameter unit for pollution bio-monitoring compared to tree bark and tree bark lichens (AOAC 1990, Stech 2004, Wolfe 2005).

Heavy metal accumulation in plants differs depending on plant species and on plant growth area. From this point of view, plants can be grouped into three

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categories: *accumulating plants* (plants accumulating heavy metals in their aerial parts), *indicator plants* (plants with equal heavy metal contents in their aerial and underground parts), and *low heavy metal content* (heavy metal content is not influenced by the amount of heavy metals in the soil) (Butnariu 2007, FDA 1993, Ward 1995).

Plant bio-indicators are plant species that can answer the combined effect of some heavy metals. *Sentinel species* are plant species that grow rapidly and that react quickly to heavy metal content increase; they are used to signal early heavy metal presence (Butnariu 2006, WHO 1998).

The values of heavy metal concentration in pine leaves from three areas differing in their pollution rate such as presented in literature are shown in Table 1(Wolfe 2005).

Table 1 . Pine leaves heavy metal content values (mg/kg, ppm)

Metal	Urban area pine leaves	High-way pine leaves	Industrial area pine leaves
Pb	39.8	62.3	42.6
Zn	14.46	18.49	24.16
Cu	7.93	15.43	10.6
Ni	10.16	16.7	13.2
Cr	2.04	3.15	3.97

The 6th edition of the European Pharmacopoeia asks for maximum admitted limits in Pb (5.0 mg/kg), Cd (0.2 mg/kg), and Hg (0.1 mg/kg)(Ph.Eur.2008)

MATERIALS AND METHODS

The plant material used was represented by pine foliar buds harvested late winter and early spring of 2008 (before full opening) in the Bihor Apuseni Mountains area. They were dried in environmental temperature and kept in dark coloured pharmaceutical recipients. We deposited voucher samples in the herbarium collection of the College of Pharmacy in Timişoara (Romania). We prepared hydro-glycero-alcoholic extracts (5% of dried plant products) from fresh foliar bugs of *Pinus montana* according to the 10th edition of the French Pharmacopoeia.

The ash and the water content were determined calcinations and hot drying procedure (Butnariu 2010, Peev 2006).

For the determination of mineral content of the two analyzed vegetal products were processed following the wet disintegration. For each sample set was achieved a control sample. Minerals concentration was determinate by atomic absorption spectrometry. Spectrophotocolorimetric determination is a physical-chemical method whose principle is based on comparing the intensity of staining intensity colour sample for analysis of known concentrations and different solutions. The heavy metals were measured from the obtained hydrochloric solution by pulverization in the air-acetylene flame and measurement of the absorbance, respectively emission at the characteristic wavelength for each analyzed element. It was used an atomic absorption spectrophotometer, controlled by PC (AAS, Analytik Jena AG). For the spectrophotometer calibration were prepared sets of etalons of different concentrations in HCl 0.5N solution for each analyzed element, starting to the

concentrated standard solutions. The concentration (C) for each determined element was calculated with the following formula:

$$C\ (mg/kg\ or\ ppm) = a \bullet f / m,$$

where: f = dilution factor; a = element content indicated by apparatus (mg/l);
m = sample initial weight. (Butnariu *et al*. 2010).
Apparatus. AA Spectrometer 4100, Perkin Elmer AS-70, HGA 700.

RESULTS AND DISCUSSIONS

The values of mineral concentration in pine foliar buds from the two species and the pine hydro-glycero-alcoholic extract, as well as the values of the micro-element extraction yield from the plant product into the pharmaceutical preparation are shown in Table 2.

Table 2 Values of Mg, micro-elements, and heavy metals content (mg/kg, ppm)

Metal	*P. silvestris* dried buds	*P. montana* dried buds	Hydro-glycero-alcoholic extract	Extraction yield
Mg	1723	1406	38.9	55.33
Mn	342	296	5.5	37.16
Fe	203.6	248.6	7.6	61.14
Cu	42.5	37.2	0.82	44.08
Zn	31.3	38.4	0.39	20.31
Ag	0.6	0.5	0.13	
Pb	3.8	3	0.6	
Co	3.1	2.7	0.25	
Cr	1.5	1.5	-	
Ni	15.8	16.0	-	

Comparing micro-element content in pine buds and in the pine extract, we considered it necessary to express the yield value to underline the extraction capacity in the solvent used.

Taking into account the concentration values of the micro-elements in glycerol-alcoholic extractive solutions and in the spirit of homeopathic medicine we consider these preparations as natural decimal hahnemannian D4-D6 homeopathic dilutions or as centesimal hahnemannian C8-C12 dilutions.

Foliar buds from the two different pine species have similar mineral concentrations.

Foliar bud samples under study show no heavy metal traces. Plant products can be successfully used as raw matter for the food industry and for the pharmaceutics industry.

Literature data concerning the leaves harvested from the urban area and from the high-way area show the lowest heavy metal concentrations. The high value of Pb concentration makes it improper as raw matter for the pharmaceutics sector.

Pine foliar buds have a significant Ni concentration, similar to that of the leaves harvested from the high-way area. Zn and Cu concentrations are superior to that of pine leaves. Foliar buds tend to concentrate some heavy metals such as Zn, Cu, and Ni. This is why pine buds could be detector-type bio-indicator products.

CONCLUSIONS

This study completes the phyto-chemical picture of foliar buds to produce some complex plant extracts with a high content of mineral substances and specific organic substances.

From the point of view of micro-element concentration, we consider these preparations natural decimal homeopathic hahnemannian D4-D6 dilutions or natural centesimal homeopathic hahnemannian C8-C12 dilutions.

The results we obtained contribute to modern scientific funding of therapeutic directions for gemmo-preparations.

Foliar buds from the two different pine species have similar mineral concentrations.

Foliar bud samples under study have no heavy metal traces. Plant products can be successfully used as raw matter in the food industry and in the pharmaceutical industry. Harvesting buds for therapeutic practices can be done only in unpolluted areas after rigorously checking the plant products destined for pharmaceutical use.

Pine foliar buds tend to concentrate and accumulate such heavy metals as Zn, Cu, and Ni, which makes them possible detector-type bio-indicators.

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CHANGES IN MAGNESIUM CONTENT OF THIGH MUSCLE, TIBIA, UTERUS AND EGG INDUCED BY DIET MAGNESIUMM OXIDE SUPPLEMENTATION OF LAYING HENS

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ABSTRACT

The effect of magnesium oxide supplementation of laying hens diet on magnesium content of tibia, thigh muscle, uterus and eggs was investigated. 30 weeks old laying hens, RossoSL hybrid, were fed combined standard diet supplemented with MgO in doses of 1g/kg feed and 3g/kg feed, over a period of 41 days. Samples of uterus, thigh muscles and also tibia were collected at the end of the experiment. 10 eggs of each hen were collected too. Magnesium content was determined by atomic absorption spectrometry. The obtained results emphasized that an increase of magnesium diet content is associated with a decrease of magnesium in bones. Thigh muscles presented a higher content of magnesium when supplemented with 3g MgO/kg feed, while in uterus magnesium content was higher for 1gMgO/kg feed supplementation. Both doses resulted in magnesium enriched eggs production.

Key words: magnesium oxide, magnesium enriched eggs and muscle

INTRODUCTION

One of the most important essential macroelements due to the large array of biological activities in which it is involved is magnesium. Participation in the most important metabolic pathways, mainly as enzymes cofactor (more than 300), the role in transport of potassium and calcium, cell proliferation and signal transduction, as well as its association with stress alleviation, allergies and other implications in defence processes (Szmitz et al., 2007) stimulate the interest in magnesium food resources and supplements. It is generally accepted that the inorganic forms of magnesium supplementation present a limited bioavailabilty, and phytates, phosphates or oxalates decrease magnesium absorption from the small intestine (Torsten, 2008). There is an opened debate on the forms of magnesium sources that have the best bioavailabilty, either organic or inorganic, or of vegetable or animal origin. Previous results of our group demonstrated that MgO reaches a significant absorption in poultry (Pop et al., 2006). The aim of the present approach was to

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evaluate the supplemented magnesium distribution in different muscle and bones and secretion via uterus in eggs (white and yolk).

MATERIALS AND METHODS

Laying hens 30 weeks old, RossoSL hybrid, housed in individual cages, were fed combined standard diet supplemented with MgO in two different doses, 1g/kg feed and 3g/kg feed, over a period of 41 days. 30 hens from the same poultry farm were divided in three groups, control (C) and experimental 1 and 2 (E1, E2), each of 10 individuals. At the end of the experiment all animals were slaughtered. Samples of uterus, thigh muscles and also tibia were collected. 10 eggs of each hen were collected. Magnesium content was determined by atomic absorption spectrometry (Perkin Elmer spectrometer, with graphite furnace, calibrated with standard solutions). This assay method determines the total magnesium concentration, both ionic and protein bound.

RESULTS AND DISCUSSIONS

Results obtained after magnesium oxide diet supplementation confirmed previous results (Pop et al. 2006) for thigh bone and muscle (fig. 1 and fig. 2). Data resulted from our experiments showed that an increase of magnesium diet content is associated with a decrease of magnesium in bones. These results are opposite to those reported by Hess and Britton (1997), who found that a dietary excess of magnesium increased tibia magnesium.

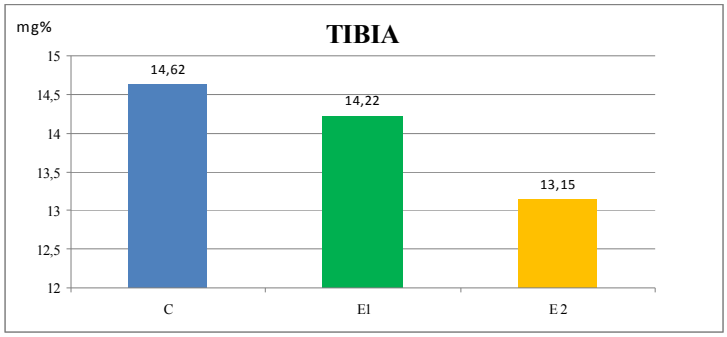

Fig. 1. Magnesium content of thigh bone (mean values)

Thigh muscle presented a lower magnesium content for the hens that received 1g MgO/kg feed as compared with the control group, while 3g MgO/kg feed supplementation induced an increased magnesium content.

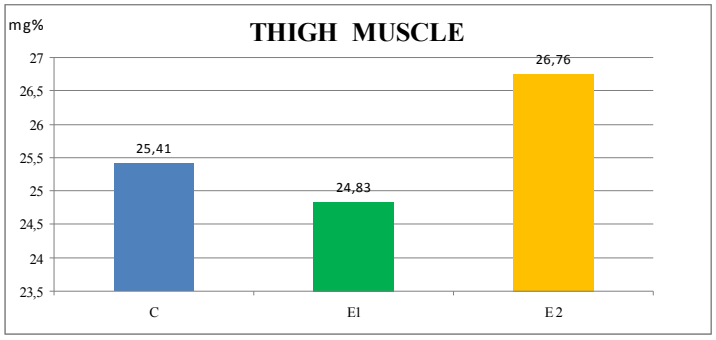

Fig. 2. Magnesium content of thigh muscle (mean values)

Uterus (shell gland) is responsible for egg shell formation. Ogawa et al. (2004) reported that during the stay in uterus, magnesium content of the shell increases. Shell gland is an important reservoir of minerals for the egg shell. Both doses of supplemented magnesium resulted in higher concentrations of magnesium in uterus (figure 3), 1g MgO/kg feed seemed to determine a better accumulation of magnesium than 3g MgO/kg feed.

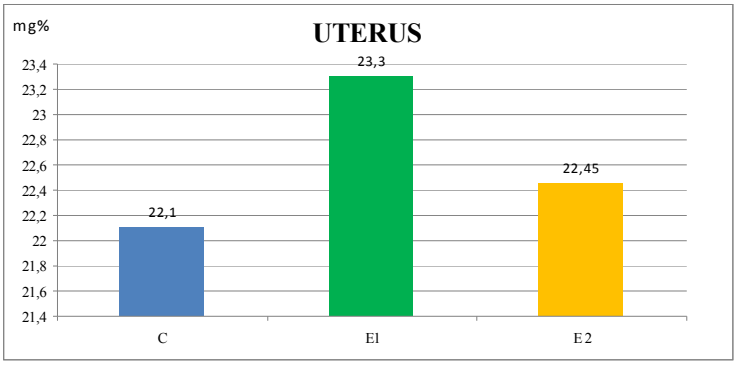

Fig. 3. Magnesium content of uterus (mean values)

An increase of magnesium content of eggs (whites and yolks) is also noticed (figure 4). The total magnesium content of the eatable egg increased with 16.86% in eggs collected from the hens included in E1, and with 34.5% for those produced by hens of E2. It may be assumed that diet manipulation resulted in the obtaining of enriched magnesium eggs

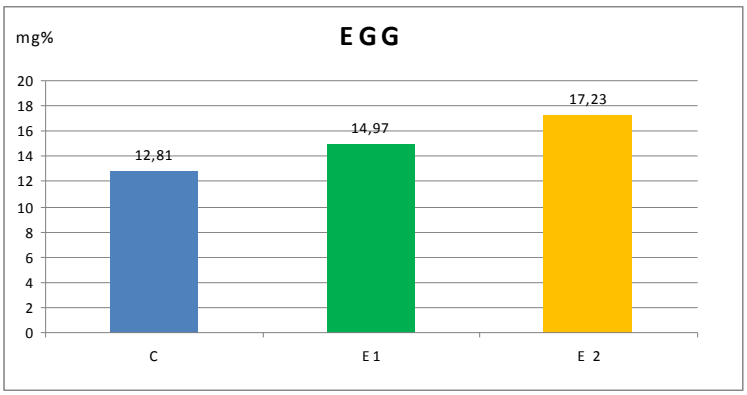

Fig. 4. Magnesium content of eggs (mean values)

Magnesium status is important for many diseases in humans, like osteoporosis, nervous system, cardiovascular and digestive disorders. Martin et al., (2008) emphasized that a long-term dietary intake of magnesium induced beneficial effects on oxidative stress, apoptosis and ageing of rat liver. Obtaining of magnesium enriched eggs could be of importance for human nutrition, because, unlike other food products that lose a great part of the minerals during cooking, eggs retain almost all its components.

CONCLUSIONS

1. MgO diet supplementation with 3g/kg feed induced an increase of magnesium content of laying hens thigh muscle, accompanied by a decrease of this mineral content in tibia.
2. Feed magnesium supplementation resulted in magnesium enriched eggs produced by laying hens of both experimental groups.

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TESTING PHYTOREMEDIATION METHODS FOR THE ZLATNA (ROUMANIA) TAILING DAMS

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ABSTRACT

Conventional remediation methods such as covering contaminated areas with cleaner soil give good results in short term, but they are costly and problematic in terms of logistics and may fail in the long run. Customized methods adapted to a specific type of site should give better long term results with less of an initial impact on the area.

We performed a small scale pot experiment using mine tailing substrate from the Mica Valley, Zlatna, with a mixture of Festuca rubra L. (an excluder species) and Melilotus albus L. (an acidophilic species). Experimental variants were unpolluted soil as positive reference, contaminated soil from the small tailing dam, contaminated soil from the small tailing dam inoculated with mychorrizal fungi; contaminated soil from the large tailing dam amended with top soil, contaminated soil from the large tailing dam,amended with green manure (Rhizobia). The inoculations had positive effects on plant parameters, especially on biomass but also on oxidative stress. Regarding metal concentrations in plants, the particular effect of the inoculation is metal specific. In some cases we found a decrease in the concentration of metal in plants which could be considered another positive effect and seems to be due to reducing of the metals availability as a result of amendments. The more pronounced effects of green manure and top soil were a decrease of the oxidative stress and an increase of biomass production.

Key words: heavy metals; Melilotus albus, Festuca rubra, tailing dam, bioremediation

INTRODUCTION

Mining in the western part of the Romanian Carpathians has taken place since antiquity, with gold and copper as the most important extracted metals (Şerban, 2004). The Zlatna area, in the southern part of the Apuseni mountains, is recognized as one of the most contaminated in Romania regarding heavy metals (Lăcătuşu 1999). Copper mining, which began in the seventies, has left the Zlatna area with two tailing dams and one tailing dump that continue to leach out dangerous pollutants even after the processing plant was shut down. Leachates from tailing dams infiltrate local river systems, exposing the population to health endangering concentrations of heavy metals (Şerban 2004, Lăcătuşu 2008). Although the area is currently under

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remediation, the engineering approach used is very resource consuming and has a high risk of failure if further management is not put into the area after the main remediation works are over. Covering the entire surface of dams with soil from the immediate vicinity and introducing grasses and trees requires further irrigation to maintain plant cover and prevent drought stress, that can lead to loss of vegetation and erosion of the topsoil (Turnau et. al. 2008). Phytoremediation comes as an alternative to conventional methods and aims to establish a durable plant cover by less disruptive means, using the interactions between plants an microorganisms to remove the detrimental effect of polluted areas on surrounding ecosystems and human populations (Pilon-Smiths 2005, 2006, Vangronsveld 2009). This alternative receives higher public acceptance, can be cheaper, and is flexible, with the ability to be directed either towards extraction or towards stabilization of metals in the soil (Neagoe et. al. 2006). Using the right plant species and soil amendments growth can be stimulated and maintained in the tailing substrate even in adverse conditions such as high toxic element concentrations and drought stress. In the experiment reported here we tested the hypotheses that a mixture of plant species with different pH preferences would compensate one another in terms of biomass when grown in substrates with different pHs, thus potentially providing a solution for tailing dams with large heterogeneity in the distribution of the metals on their surface.

MATERIALS AND METHODS

Experimental setting. Substrate for the pot experiment was collected from the Valea Mică tailing dams, 6 kilometers from Zlatna, resulted through dumping the refuse coming from the primary flotation of ores. The dams are mostly barren and arid, with a small wet zone in the upper part that supports some reeds. Heavy metal contents vary: Cu 75 -13806 mg/kg; Zn 238 – 13434 mg/kg and Pb 101 – 4046 mg/kg (Jianu 2008). Metal distributions are quite heterogenous (Figure 1).

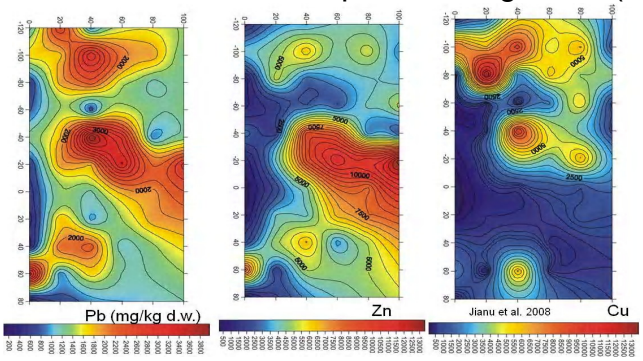

Fig. 1. Heavy metal heterogeneity on the Zlatna tailing dams, after Jianu (2008).

We used 5 experimental variants (4 replicates each): uncontaminated reference soil (R), substrate from the small tailing dam amended with 10% expanded clay to have the same structure as if amended with fungi (im + A), small tailing dam substrate amended with 10% *Glomus intraradices* mycorrhizal inoculum in expanded clay containing around 200 propagules per clay particle (im+F), substrate from the large tailing dam amended with unpolluted soil (IM+R) and substrate from the large tailing dam amended with 10% unpolluted soil and 10% Rhizobium in the form clover roots (IM+Rhi).

Plants chosen for the experiment were *Festuca rubra*, an excluder species, and *Melilotus albus*, an acidophilic plant. The two were cultivated together in 400g pots and

kept in a growth chamber for 72 days in a light/dark regime of 8/8 hours, at 16˚C during the dark phase and 22˚C during the light phase (5000lx), with 40% relative humidity.

Measured parameters for substrate were, elements: As, Ba, Be, Ca, Co, Cd, Cr, Cu, Li, Mn, Mg, Na, Ni, Pb, Rb, Sr, U, V, Zn and, pH, EC, soil moisture, soil respiration, $N\text{-}NH_3$, $N\text{-}NO_3$, $N\text{-}NO_2$, $P\text{-}PO_4$. Plant parameters were biomass; chlorophyll and carotene, lipid peroxides and the same elements as in the substrate.

Soil moisture was calculated after drying soil samples at 105ºC until constant weight. PH was measured in a soil water mixture (1:2.5). Soil samples were kept at 4ºC and processed within 24 hours from prelevation. For nitrogen compounds, 20 g of soil were extracted with 100ml 0.2M KCl solution and for phosphate 5 g with 100ml 0,5 M $NaHCO_3$. Samples were analyzed through colorimetric methods: ammonium by Na nitroprussid, nitrate by sulphosalycilic method, nitrite N1 naphtyletylendiamine and sulphanilimide and phosphate with molid-ammonium and malachite green (Neagoe et al. 2005). Elements were analyzed on an Elan DRC-e ICP-MS from Perkin Elmer after digestion with aqua regia using an Anton Paar microwave oven.

For the chlorophyll and carotenoid assay, 50 mg of dry plant matter was homogenized in a buffer containing 80% acetone, 15% water and 5% NH_3 25% solution. Samples were then centrifuged to remove solids and spectrophotometrically measured at 480, 638, 645, 647, 663 and 664 nm and chlorophyll a, chlorophyll b and carotenoids were measured as described by Schöpfer (1989).

Lipid peroxide tests were performed acording to Heath and Packer (1968): 20 mg of dry biomass was homogenized with 4ml TBA buffer containing10% trichloloacetic acid and 0.25% thiobarbituric acid in ultra-pure water, heated for 30 min at 95º C, cooled for 15 min at room temperature, centrifuged and spectrophotometrically measured at 532 and 600nm.

The Mann-Whitney statistic non-parametric test was used to compare averages between experimental variants and reveal statistically significant differences.

RESULTS AND DISCUSSIONS

Relative abundance. By coupling biomass results with previously published results we obtained a representation showing that pH variation dictates the abundance of the two species: *F. rubra* fully outcompetes *M. albus* at a very acid pH, while at pH slightly larger than neutral *M. albus* dominates over *Festuca* (Figure 2).

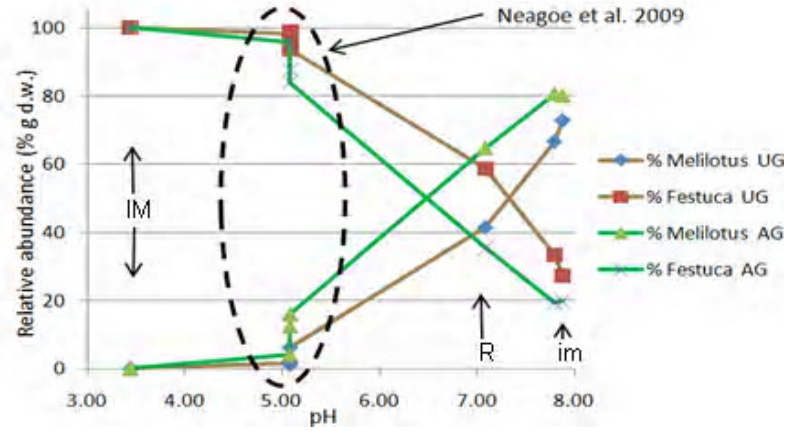

Fig. 2. Relative abundance of *Melilotus* and *Festuca* in relationship to pH and the accordance of our experimental variants. IM = pots with substrate from the large tailing dam, R =

unpolluted reference soil, im = substrate from the small tailing dam, AG = above ground, UG = below ground.

Substrate characteristics

We compared soil parameters before (Table1) and after the experiment (Table 2) to show the influence of the different treatments.

For the unpolluted reference, there was a decrease in humidity as plants took up more water and a decrease in total nitrogen and phosphorous, consumed by plants. For the small tailing dam substrate the pH increased in both variants but more so in the one amended with fungi, probably because of root exudates stimulated by mycorrhization. Mineral nitrogen and phosphorous also decreased.

For the pots with large tailing dams substrate, the variations were more spectacular. The pH increased from 2.98 to 3.44 when amended with clean soil and to 3.35 when amended with clean soil and *Rhizobium*. Mineral nitrogen almost doubled in the variants with unpolluted soil and grew almost 4 times in those with *Rhizobium*. Microbial activity increased significantly when adding mycorrhizal fungi inoculum.

Table 1. Substrate parameters before the experiment. IM = pots with substrate from the large tailing dam, R = unpolluted reference soil, im = substrate from the small tailing dam.

	Humidity	pH	EC	$N\text{-}NH_4$	$N\text{-}NO_3$	$N\text{-}NO_2$	$P\text{-}PO_4$
Substrate	%		µS/cm	µg/g DW	µg/g DW	µg/g DW	µg/g DW
R	12.1	7.05	120	40.12	31.13	4.5	65.27
im	14.75	7.45	284	22.13	11.15	1.18	12.14
IM	7.32	2.98	755	7.08	5.19	4.11	7.55

Table 2. Substrate parameters before the experiment. IM = pots with substrate from the large tailing dam, R = unpolluted reference soil, im = substrate from the small tailing dam, A = expanded clay, F = *G. intraradices* inoculums, R = unpolluted soil, Rhi = *Rhizobium*

	Sample code	H	pH	EC	$N\text{-}NH_4$	$N\text{-}NO_3$	$N\text{-}NO_2$	$P\text{-}PO_4$
		%		µS/cm	µg/g DW			
Average	R	8.38	7.08	136.0	35.7	9.91	0.16	52.95
SD		*0.24*	*0.03*	*9.27*	*6.91*	*3.82*	*0.10*	*1.99*
Average	im + A	5.34	7.79	267.0	11.8	3.39	0.07	4.79
SD		*0.82*	*0.03*	*37.83*	*2.12*	*0.47*	*0.02*	*0.55*
Average	im + F	6.12	7.88	265.3	9.18	6.31	0.08	5.45
SD		*1.49*	*0.06*	*12.09*	*2.32*	*2.31*	*0.03*	*0.47*
Average	IM +R	5.95	3.44	870.5	24.1	3.85	0.02	6.41
SD		*0.24*	*0.07*	*75.03*	*9.32*	*0.30*	*0.02*	*0.83*
Average	IM + R + Rhi	9.17	3.45	963.0	51.5	6.76	0.01	9.01
SD		*2.53*	*0.02*	*79.05*	*4.13*	*2.33*	*0.01*	*1.70*

Biomass

The biomass increased significantly in pots with substrate from the small tailing pond amended with fungi. In pots from the large tailing dam *Festuca* completely replaced *Melilotus* and had more biomass when amended with *Rhizobium* (Table 3).

Table 3. Biomass (g d.w.) and results of statistic test. Significant differences bolded.

Treatment	Code	Estim.	*Melilotus*			*Festuca*			Total
			Underg.	Aboveg.	Total	Underg.	Aboveg.	Total	
R	1	Av	0.690	2.545	3.235	0.975	1.390	2.365	5.600
		SD	*0.246*	*0.656*	*0.892*	*0.396*	*0.387*	*0.631*	*0.666*
im_Exp_Clay	2	Av	0.160	0.480	0.640	0.080	0.115	0.195	0.835
		SD	*0.107*	*0.060*	*0.144*	*0.030*	*0.032*	*0.061*	*0.173*
im_Fungi	3	Av	0.375	**1.070**	1.445	0.140	0.265	0.405	**1.850**
		SD	*0.152*	*0.262*	*0.399*	*0.086*	*0.223*	*0.305*	*0.572*
IM_R	4	Av	0	0	0	0.095	0.210	0.305	0.305
		SD	*0*	*0*	*0*	*0.077*	*0.169*	*0.243*	*0.243*
IM_Rhi	5	Av	0	0	0	**0.255**	0.410	0.665	0.665
		SD	*0*	*0*	*0*	*0.013*	*0.017*	*0.029*	*0.029*
Mann-Whitney test, p	A1	1 vs 2	NS	0.021	0.021	0.021	0.020	0.021	0.020
		1 vs 3	NS	0.021	0.043	0.020	0.020	0.021	0.021
		2 vs 3	NS	0.021	NS	NS	NS	NS	0.020
	A2	1 vs 4	0.014	0.014	0.014	0.021	0.020	0.021	0.021
		1 vs 5	0.014	0.014	0.014	0.020	0.020	0.021	0.021
		4 vs 5	NS	NS	NS	0.020	NS	NS	NS

Pigments and lipid peroxides

The larger biomass of *Melilotus* in the variant with fungi is associated to significantly large concentrations of chlorophils and carotens (Table 4).

Table 4. Assimilating pigments, lipid peroxides and results of statistic test. Significant differences bolded.

Treatment	Code	Estim.	*Melilotus*			*Festuca*		
			Chl. mg/g s.u.	Car. mg/g s.u.	LP TBA r.m. µmoli/g s.u.	Chl. mg/g s.u.	Car. mg/g s.u.	LP TBA r.m. µmoli/g s.u.
R	1	Av	8.991	0.320	0.328	6.922	0.239	0.358
		SD	*0.515*	*0.012*	*0.067*	*0.660*	*0.016*	*0.077*
im_Exp_Clay	2	Av	4.159	0.185	0.284	5.563	0.215	0.277
		SD	*0.427*	*0.017*	*0.063*	average sample		
im_Fungi	3	Av	**5.153**	**0.225**	0.319	6.148	0.204	0.313
		SD	*0.427*	*0.004*	*0.084*	*0.566*	*0.018*	*0.012*
IM_R	4	Av	no biomass			4.572	0.222	0.2115
		SD				*0.398*	*0.030*	*0.0897*
IM_Rhi	5	Av	no biomass			5.057	0.202	0.2466
		SD				*0.701*	*0.025*	*0.0417*
Mann-Whitney test, p	A1	1 vs 2	0.021	0.021		*0.014*	*0.014*	
		1 vs 3	0.021	0.021		0.034		
		2 vs 3	0.021	0.021				*0.019*
	A2	1 vs 4				0.034	0.034	
		1 vs 5				0.021		
		4 vs 5						

Metal concentrations

Metals in *Melilotus* in contaminated substrate with amendments are significanty different than when grown on reference soil. The concentrations are for some metals larger, for other ones smaller. Excepting for vanadium (whose concentrations are not larger on the talings than in the reference soil) the inoculation

lead to a protective decrease in the concentration of metals in roots (underground biomass). In the aboveground biomass this situation holds only for Pb.

For *Festuca* the picture is somehow different: there are many differences compared to reference soil variant, but the inoculation lead in some cases to the increase in the concentration of elements like Cr, Cu and Zn. If we compare the variant with clover roots with that with mixture of reference soil (A2-4 with A2-5) we remark a significant increase in the concentrations of many elements in roots, although in the aboveground part there are also several cases with significant decreases (Table 5).

Table 5. Metal concentrations (µg/g d.w). Metals written in light grey have much larger concentrations in talings than in reference soil, metals in black are of comparable concentrations in tailings and in reference soil. The effect of the inoculation is marked with grey in the cell, when there is a significant decrease or increase resulting from inoculation.

Melilotus underground

Variant	R	im + A	im + F
Estimator	Av	Av	Av
As	0.615	50.89	34.79
Cd	0.463	4.959	1.726
Co	4.838	20.88	4.685
Cr	1.807	106.4	67.29
Cu	51.53	327.1	159.3
Mn	209.4	273.5	190.0
Ni	3.252	137.3	109.0
Pb	9.789	60.63	28.92
V	1.186	1.219	3.514
Zn	86.80	513.2	407.8

Festuca underground

Variant	R	im + A	im + F	IM+R	+Rhi
Estimator	Av	Av	Av	Av	Av
As	1.310	10.196	7.501	11.67	11.00
Cd	0.668	8.906	5.918	7.862	12.99
Co	3.763	3.267	2.493	30.38	51.73
Cr	4.320	90.89	111.5	98.25	129.5
Cu	25.67	135.0	103.8	42.05	111.9
Mn	33.10	300.8	404.3	374.8	1621
Ni	12.26	103.6	77.78	78.41	98.15
Pb	13.78	24.84	20.30	11.44	32.72
V	2.865	0.776	1.246	0.601	2.480
Zn	55.68	408.7	473.4	434.9	439.7

Melilotus aboveground

Variant	R	im + A	im + F
Estimator	Av	Av	Av
As	1.130	239.2	198.1
Cd	1.276	12.70	10.05
Co	17.34	57.58	36.99
Cr	10.34	241.0	230.0
Cu	78.40	2671	2385
Mn	248.4	1154	886.2
Ni	28.18	293.3	208.1
Pb	20.79	354.8	286.2
V	22.05	27.41	17.87
Zn	192.19	1531	1835

Festuca aboveground

Variant	R	im + A	im + F	IM+R	+Rhi
Estimator	Av	Av	Av	Av	Av
As	4.926	33.54	22.62	30.36	27.29
Cd	1.036	17.50	10.18	11.80	15.49
Co	28.65	47.80	18.78	81.68	35.72
Cr	13.67	239.0	173.3	83.73	103.9
Cu	129.0	1203	1951	1379	1092
Mn	64.84	1098	669.6	3842	1314
Ni	18.26	274.7	183.7	246.8	242.4
Pb	9.778	159.3	167.6	62.51	142.5
V	23.49	11.91	8.153	15.21	19.83
Zn	189.8	898.9	1024	958.7	790.6

Bioaccumulation

The bioaccumulation factor (computed as ratio of concentration in plants / concentration in soil after plant removal) one can see that the inoculation lead to a significant decrease of the bioaccumulation in aboveground parts of *Melilotus*. The situation is different for *Festuca*: the inoculation lead to a decrease of bioaccumulation only in several cases in roots, and there is no significant effect in this respect in aboveground parts.

The amendment with *Rhizobium* (clover roots) lead to a significant increase in the bioaccumulation of Mn and Pb in aboveground of *Festuca* (Table 6).

Stocks of metals

For *Melilotus* there are many significant effects compared to reference (in most cases and increase in the stocks), but almost no significant effects as a results of the inoculation, because the decrease in bioaccumulation was compensated by an increase in plant biomass leading to comparable stocks with the variant with expanded clay.

In the case of *Festuca* the number of significant differences compared to reference are much fewer, but the effect of inoculation with fungi lead to a significant increase in the stock of metals in several cases, both in roots and aboveground parts.

Table 6. Bioaccumulation factors. Metals written in light grey have much larger concentrations in talings than in reference soil, metals in black are of comparable concentrations in tailings and in reference soil. The effect of the inoculation is marked with grey in the cell, when there is a significant decrease or increase resulting from inoculation.

Melilotus underground

Variant	R	im + A	im + F
Estimator	Av	Av	Av
As	0.109	0.615	0.552
Cd	0.105	0.164	0.142
Co	30.64	38.67	12.51
Cr	0.119	0.513	0.478
Cu	1.708	1.024	0.977
Mn	0.044	0.188	0.155
Ni	1.024	5.108	3.114
Pb	0.618	0.549	0.331
V	0.307	0.498	0.354
Zn	1.320	0.630	0.746

Festuca underground

Variant	R	im + A	im + F	IM+R	+Rhi
Estimator	Av	Av	Av	Av	Av
As	0.472	0.085	0.063	0.105	0.097
Cd	0.085	0.228	0.143	2.318	1.809
Co	50.74	30.74	6.391	167.7	94.66
Cr	0.157	0.497	0.366	0.438	0.912
Cu	2.799	0.461	0.795	0.614	0.397
Mn	0.011	0.180	0.117	1.827	0.715
Ni	0.654	4.704	2.882	9.791	11.77
Pb	0.292	0.248	0.268	0.136	0.230
V	0.327	0.220	0.160	0.426	0.583
Zn	1.333	0.370	0.417	0.566	0.444

Melilotus aboveground

Variant	R	im + A	im + F
Estimator	Av	Av	Av
As	0.058	0.130	0.097
Cd	0.038	0.065	0.024
Co	8.430	13.392	1.600
Cr	0.021	0.224	0.142
Cu	1.118	0.126	0.065
Mn	0.037	0.045	0.033
Ni	0.120	2.342	1.701
Pb	0.293	0.094	0.046
V	0.016	0.022	0.070
Zn	0.635	0.209	0.166

Festuca aboveground

Variant	R	im + A	im + F	IM+R	+Rhi
Estimator	Av	Av	Av	Av	Av
As	0.125	0.026	0.021	0.041	0.038
Cd	0.055	0.116	0.083	1.442	1.512
Co	6.893	2.184	0.848	59.86	164.9
Cr	0.050	0.192	0.235	0.527	1.130
Cu	0.559	0.052	0.042	0.020	0.041
Mn	0.006	0.049	0.070	0.177	0.881
Ni	0.444	1.784	1.193	2.920	4.546
Pb	0.414	0.039	0.032	0.025	0.053
V	0.040	0.014	0.024	0.017	0.073
Zn	0.415	0.169	0.192	0.257	0.253

The effect of the amendment with *Rhizobium* (clover roots) compared to reference soil as amendment lead to an important increase in the stock of metals as a result of large biomass coupled with a lack of decrease in the bioaccumulation (Table 7).

Table 7. Metal stocks (µg). Metals written in light grey have much larger concentrations in talings than in reference soil, metals in black are of comparable concentrations in tailings and in reference soil. The effect of the inoculation is marked with grey in the cell, when there is a significant decrease or increase resulting from inoculation.

Melilotus underground

Variant	R	im + A	im + F
Estimator	Av	Av	Av
As	0.491	58.14	63.50
Cd	0.623	3.034	3.106
Co	8.414	14.17	11.51
Cr	5.264	56.15	68.77
Cu	38.55	641.7	754.5
Mn	123	278.4	284.0
Ni	13.45	68.59	64.34
Pb	9.813	85.56	65.49
V	10.958	6.586	5.582
Zn	102.8	371.6	569.9

Festuca underground

Variant	R	im + A	im + F	IM+R	+Rhi
Estimator	Av	Av	Av	Av	Av
As	4.391	2.854	4.313	4.632	7.173
Cd	0.949	1.460	2.007	1.964	4.087
Co	27.14	3.302	3.522	12.82	9.457
Cr	13.22	19.02	32.94	13.03	27.26
Cu	116.1	85.58	381.6	218.8	286.1
Mn	58.67	77.96	139.6	581.4	346.2
Ni	16.76	22.51	36.40	40.70	63.54
Pb	8.862	11.00	32.38	9.939	37.42
V	21.86	0.860	1.637	2.411	5.209
Zn	175.8	73.92	193.8	146.3	203.3

Melilotus aboveground

Variant	R	im + A	im + F
Estimator	Av	Av	Av
As	1.387	24.65	31.33
Cd	0.904	2.473	1.504
Co	10.33	10.24	4.280
Cr	3.378	52.26	62.03
Cu	104.6	162.1	146.7
Mn	414.2	135.4	165.0
Ni	6.221	67.81	98.03
Pb	19.916	30.01	27.05
V	2.483	0.608	3.055
Zn	175.6	251.8	360.3

Festuca aboveground

Variant	R	im + A	im + F	IM+R	+Rhi
Estimator	Av	Av	Av	Av	Av
As	2.143	1.236	2.980	3.733	4.597
Cd	1.097	1.024	2.086	2.756	5.432
Co	6.311	0.348	0.804	9.829	21.31
Cr	6.960	10.76	39.54	33.22	54.16
Cu	41.29	14.38	37.01	16.181	46.52
Mn	53.64	32.23	144.3	122.7	675.5
Ni	20.33	12.66	25.37	25.32	40.14
Pb	21.75	2.652	7.139	4.060	13.54
V	4.704	0.087	0.707	0.162	1.014
Zn	93.73	49.61	185.1	142.0	183.5

CONCLUSIONS

The relative abundance of *Melilotus* and *Festuca* is reversed as the pH of the substrate goes from 3 to more than 7.

The total biomass was larger as a result of inoculation with fungi, and was larger also in the variant with *Rhizobium* (clover roots) amendments compared with reference soil amendement.

The larger biomass of *Melilotus* in the variant with fungi was associated to significantly larger concentrations of chlorophils and carotens.

The stocks of metals in *Melilotus* did not increase significantly in the inoculated variant, because the increase in biomass was associated with a decrease in bioaccumulation factor, but the stock of metals in *Festuca* increase because there was not a decrease in bioaccumulation. This strong increase in the stocks of metals effect in the case of *Festuca* occurred also in the variant with clover roots.

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EFFECTS OF ANTIOXIDANTS IN CISPLATIN TOXICOLOGY

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ABSTRACT

A review is given of the pro-oxidant effects demonstrated in vivo and in vitro by the anti-cancer drug, cisplatin, with emphasis on situations where antioxidants were shown to reduce the level of oxidative stress – including beneficial clinical outcomes.

+Key words: cisplatin, toxicity, oxidative stress, antioxidant

INTRODUCTION

Cisplatin (cis-diamminedichloroplatinum II, CP) is currently one of the most important cytostatic agents in treatment of a wide range of solid tumors. It has been known that this compound exerts its main therapeutic effects via interaction with DNA. However, the clinical usefulness of this drug is limited by nephrotoxicity, ototoxicity and a wide range of other side-effect (such as nausea, progressive peripheral sensory neuropathy, fatigue, vomiting, alopecia, hematological suppression (Sherman and Lippard, 1987; Santos et al., 2007, Chirino et al., 2009).

Serious side effects of chemotherapy such as cisplatin-induced toxicity are, in part, the result of the formation of free radical such as superoxide anion and hydroxyl radical. These highly reactive oxygen species can cause extensive tissue damage through reactions with all biological molecules - lipids, proteins and nucleic acids, leading to the formation of oxidized substances such as the membrane lipid peroxidation product malondialdehyde. Also, free radicals may deplete GSH levels and inhibit the activity of antioxidant enzymes (Meyer, Madias, 1994; De Forni, Armand, 1994).

Enzymatic and molecular defense mechanism are present in the cell to prevent the integrity of biological membranes from oxidative processes caused by free radicals. One example is represented by glutathione (GSH), a cysteine-containing tripeptide with intracellular concentrations as high as 10 mM and multiple protective roles. It has been known that cisplatin can bind to glutathione, and administration of GSH alongside cisplatin was found to have beneficial effects in increasing the efficienty of anti-cancer treatment probably due to the antioxidant effects of this peptide (Wang and Guo, 2007; Prasad et al., 2006; Hagrman et al., 2003).

Thioredoxin is an example of enzyme which can protects against oxidative stress; increased TRX levels in certain types of tumors accompany cisplatin

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resistance phenomena (Yamada et al., 1997; Yokomizo et al., 1995; Witte et al., 2005).

It was shown that ~one day after cisplatin administration, 65-98% of the total platinum was bound to blood proteins and especially to albumin. This binding can modify the redox state of albumin with consequences on its physiological functions but does not limit the cytotoxicity of the platinum - it only does limit its urinary excretion. In fact, administration of albumin together with cisplatin was found to limit nephrotoxicity (Wang and Guo, 2007).

The administration of antioxidants such as Vitamin E, Vitamin C, selenium and carotenoids, before or after treatment with CP has been used to protect or ameliorate against nephrotoxicity in human and animals, without compromising the anti-tumor activity (Clements et al., 1990; Wanger, 1992; Chorvatovicova, 1991).

Plasma contains a large number of antioxidants, some of which prevent the initiation of the process of oxidation while others inhibit the further progression of the cascade of reaction. Vitamin E is the main lipid-soluble, chain-breaking antioxidant in membrane and in plasma. Vitamin C is a major, extremely versatile antioxidant of human plasma. It can scavenge a wide variety of free radicals in plasma or cytosol and is the main reductant of oxidized vitamin E. It is capable of preventing initiation of lipid peroxidation, while other water-soluble antioxidants such as beta-caroten, bilirubin, uric acid, and thiol compounds are only effective in decreasing the rate of lipid peroxidation. Ceruloplasmin acts as a preventive antioxidant in plasma, by binding the plasma copper and inhibiting iron-dependent lipid peroxidation and hydroxyl radical formation. Selenium is an essential part of glutathione peroxidase, an important intracellular antioxidant (Weijl et al., 1998).

It was observed that cisplatin chemotherapy induces acute and more gradually a decrease in several major plasma antioxidants. This phenomenon is probably determined by more than one mechanism, namely oxidative stress-induced consumption of antioxidants and renal loss of water-soluble low molecular weight antioxidants do to hyperfiltration in combination with a specific cisplatin-related renal tubular defect. This is an undesirable situation as it may lead to diminished protection from chemotherapy-induced oxidative stress and increased oxidative damage to normal tissues such as renal tubular cells. Some studies show that supplementation of antioxidants nutrients may protect against cisplatin-induced oxidative damage while retaining the antitumor efficacy (Weijl et al., 1998).

However, it is possible that antioxidants may play a role as prooxidants, as has been suggested for vitamin C. Which antioxidants and the amount to ingest to obtain a preventive effect - this remains to be investigated. The benefit of antioxidant ingestion after cancer has also yet to be demonstrated (Noda and Wakasugi, 2001).

The antioxidant effect of aminoguanidine was investigated and compared with the effect of well-known antioxidant vitamin C and E combination. In both cases the capacity of this compound to prevent tubular damage and perivascular inflammation observed in kidney samples of the cisplatin-administrated group were demonstrated. Administration of this antioxidant with cisplatin decreases malondialdehyde levels and prevents the decrease in liver glutathione level and the increase in serum urea levels caused by cisplatin (Atasayar et al., 2009).

Resveratrol, a natural molecule with antioxidant, antifungal, anti-inflammatory, antiplatelet and anticancer action exerts a powerful antioxidant effect on generation of reactive oxygen species and lipid peroxidation in blood platelets induced by platinum compounds. This beneficial effect was observed by the production of thiobarbituric acid reactive substances (TBARS), the level of conjugated diene and

the generation of superoxide anion radicals and other reactive oxygen species (Olas and Wachowicz, 2004). Moreover, indicators of renal injury such as increased serum creatinine levels, urinary volume and urinary protein caused by the administration of cisplatin, was also significantly reduced with resveratrol (Amaral et al., 2008).

Carnosine, a biological dipeptide predominating in long lived tissues such as skeletal muscles and brain, was shown to exhibit a protective effect on cisplatin-induced nephrotoxicity in mice. The effects were evaluated by plasma creatinine, urea, malondialdehyde, nitrate, superoxide dismutase and catalase activities (Noori and Mahbood, 2010).

Caffeic acid phenethyl ester, a plant derived phenolic compound and an active component of propolis from honeybee hives, has a strong antimicrobial, anti-inflamator, antioxidant and antineoplastic activities. At a concentration of 10 μM, caffeic acid completely blocks the production of ROS in human neutrophils and in the xantine/XO system and other enzymes (such as catalase, superoxide dismutase, glutathione peroxidase, myeloperoxidase, xantine oxidase, etc). It has been reported that caffeic acid suppresses lipid peroxidation, displays antioxidants activity and inhibits lipoxygenase activities (Iraz et al., 2006).

Apocynin, which is used as a specific NADPH oxidase inhibitor, was able to ameliorate the renal histological damage and the increase in blood urea nitrogen, serum creatinine, and urinary excretion of total protein, N-acetyl-β-D-glucosamidase and glutathione-S/transferase induced by cisplatin (Chirino et al., 2008).

Lycopene a naturally occurring carotenoid as tomatoes has attracted considerable attention as a potential chemopreventive agent in rats. It is a highly efficient antioxidant and has a singlet-oxygen and free radical scavenging capacity and have a protective effect against cisplatin-induced nephrotoxicity and oxidative stress (Atessahin et al., 2005).

Figure 1 and 2 show preliminary results obtained by our group in demonstrating direct detection of free radicals in a biological sample (blood serum, in this case). Small but well-measurable changes are seen as a result of cisplatin treatment, in the region corresponding to free radicals, after the serum has been challenged with hydrogen peroxide. While previous studies, discussed above, did demonstrate oxidative stress as a result of cisplatin, direct detection of free radicals has previously not been described.

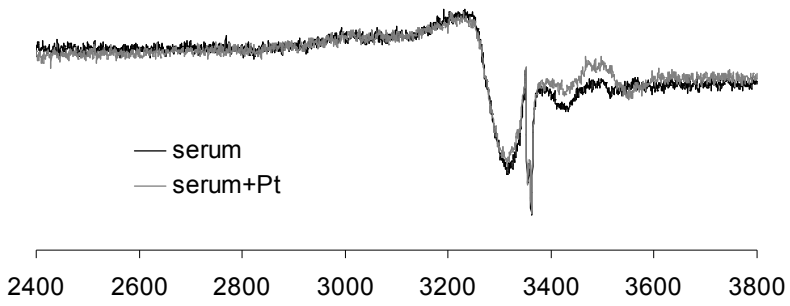

Fig. 1. EPR spectra of blood serum treated with cisplatin

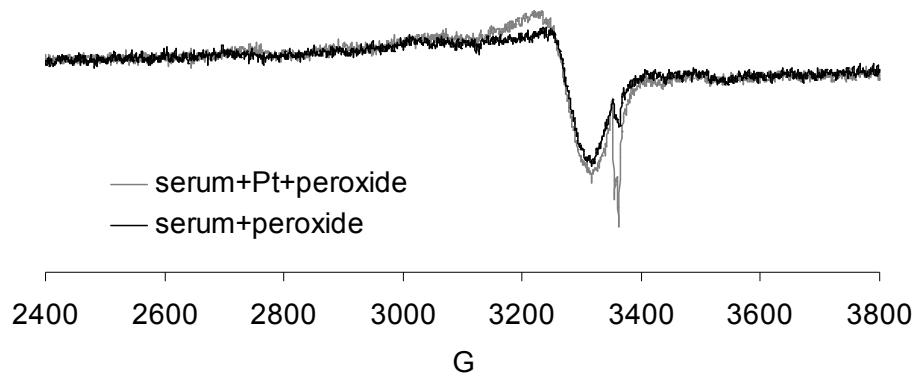

Fig. 2. EPR spectra of blood serum treated with hydrogen peroxide

Figure 3 shows another illustration of our ongoing efforts to understand mechanisms of cisplatin-induced oxidative stress at the molecular level, where oxidation of oxyhemoglobin appears affected by cisplatin.

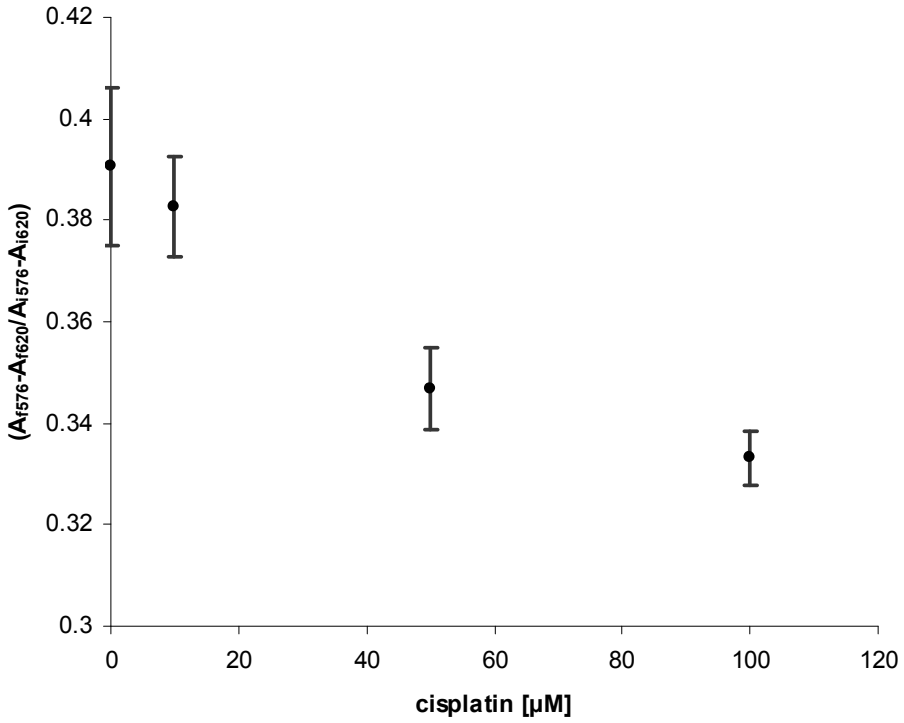

Fig. 3. Oxidation of oxyhemoglobin as monitored in UV-vis spectroscopy (at wavelengths indicated on the y-axis) in the presence of therapeutically-relevant cisplatin concentrations

CONCLUDING REMARKS

It appears that the pro-oxidant effects demonstrated in vivo and in vitro by the anti-cancer drug, cisplatin, may be reduced by selected antioxidants, although the mechanisms of these processes have not been understood to date. Our current

efforts are aimed precisely at understanding these mechanisms at molecular level, as illustrated by the preliminary results shown here.

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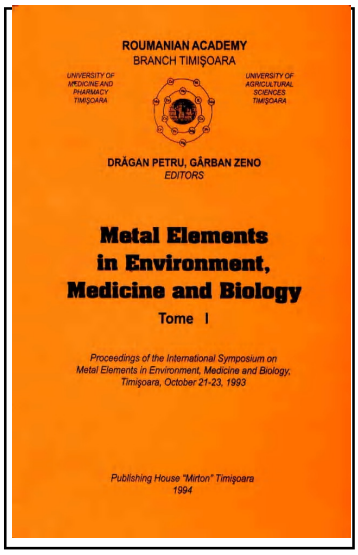

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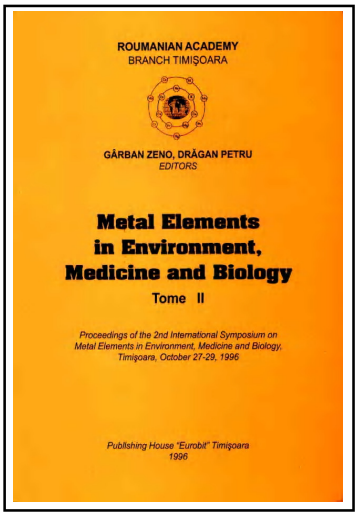

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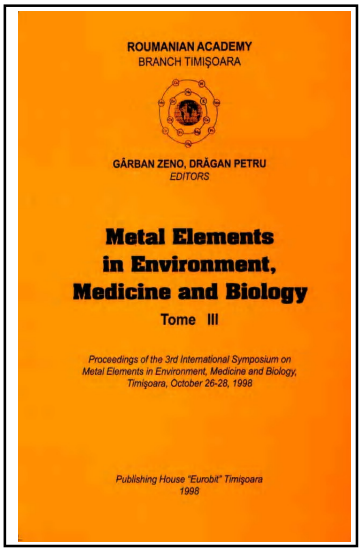

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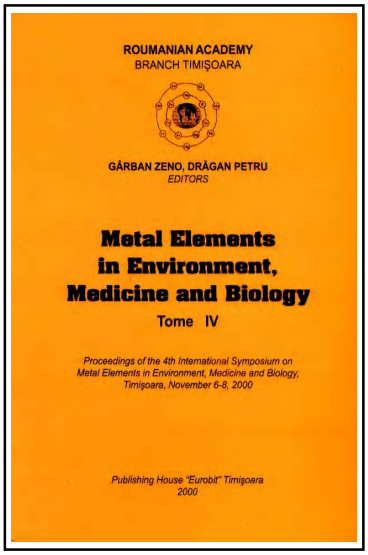

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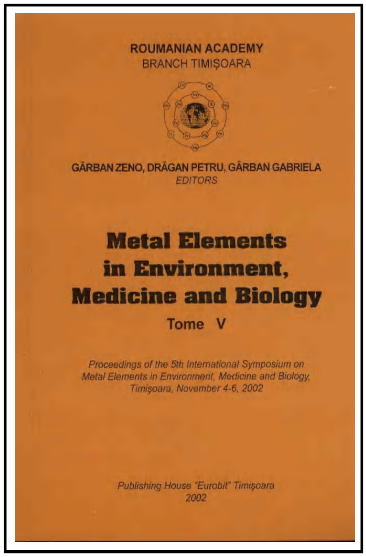

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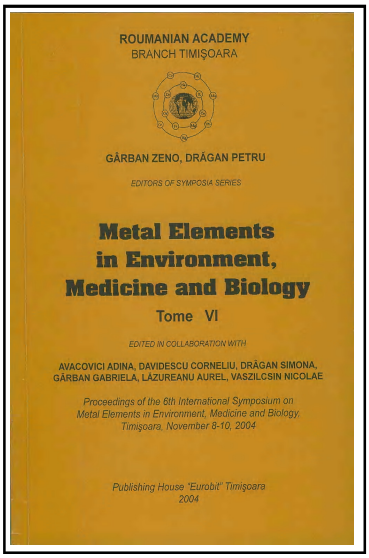

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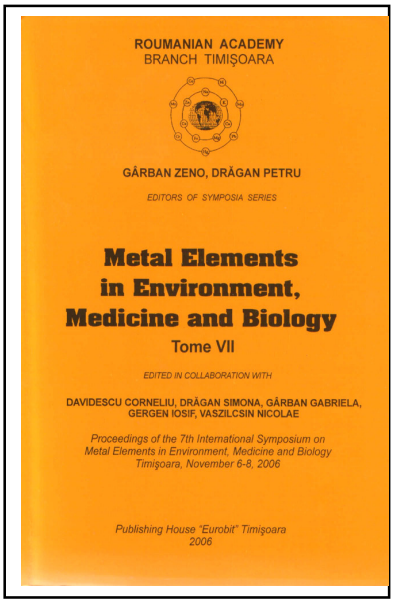

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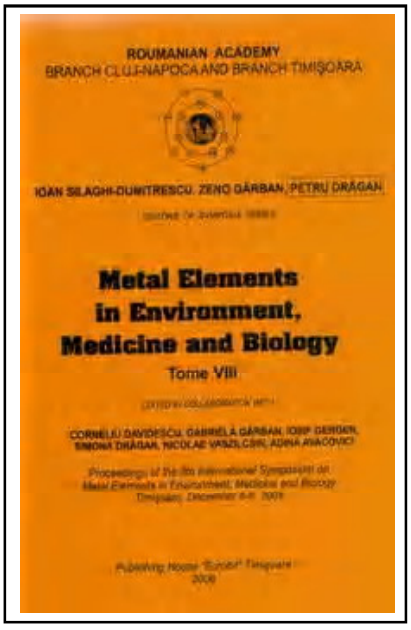

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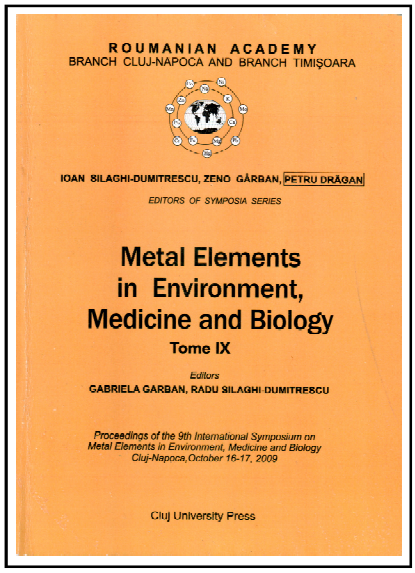

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Politehnica University Timişoara - November 11, 2010

Celebrating the 90th anniversary (November 11, 1920 - November 11, 2010) of Politehnica University Timişoara foundation, by the Royal Decree 4.822/11.11.1920 signed by His Majesty Ferdinand I - king of Roumania, we wish further successes in its prestigious activity, emblematic for Banat region, Roumania and Europe.

We express our gratitude to those personalities from Politehnica University who sustained the development of the Symposia Series "Metal Elements in Environment, Medicine and Biology" (M.E.E.M.B.)

Acad. Toma Dordea - ex president of the Roumanian Academy, Timişoara Branch
Prof. Alexandru Nichici, PhD - Rector (period 1992-1996)
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and to all professors who contributed to Symposia Series

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Timişoara, November 10, 2010

Zeno Garban
Founding member of „Working Group"
for M.E.E.M.B. (1979)

IN MEMORIAM

PROF. IOAN SILAGHI-DUMITRESCU, PhD

Corresponding Member of the Roumanian Academy

June 1, 1950 - December 25, 2009

On December 25th passed away Prof. Ioan Silaghi-Dumitrescu – corresponding member of the Roumanian Academy and distinguished personality with prodigious and prestigious activity in chemical sciences.

Graduated in 1974 from the Inorganic Chemistry of the Department of Chemistry of the Babes-Bolyai University at Cluj-Napoca, Romania, he obtained his PhD from the same university in 1981. After three years spent in the industry at the Sanex Enterprise for Fine Ceramics in Construction (1974-1977), he joined the faculty at the Department of Chemistry and Chemical Engineering of the Babes-Bolyai University, where he eventually became a full professor in 1994; here, he served as head of the Inorganic Chemistry Chair (1994-2007) and as Dean of the Department (2008-2009).

Professor Ioan Silaghi-Dumitrescu's research contributions were in the fields of inorganic and organometallic chemistry, with synthesis and structural characterization of transition metal compounds as well as of compounds involving elements from main groups 13, 14 and 15 (e.g., cumulene and heterocumulene systems with heavy elements, biologically-active compounds). He was among the first researchers in Romania to approach chemistry with computational techniques, starting very early on from force constant calculations and spectrum simulations to offer general principles for assigning coordination modes with thiophosphoric ligands at a time when structural analysis with X-ray diffraction was not available in Romania. He then evolved towards quantum chemistry, where he provided notable contributions with molecular modeling studies. His results on the structure of clusters involving main group (post-transition) elements, organometallic clusters, cumulenic and heterocumulenic systems, nanotubes and calixarenes, were reported in journals among the most prestigious in the field of chemistry. Much of his research was aimed not solely at obtaining structural information, but also at understanding and guiding experiments performed in his own research group as well as by collaborators from various countries, thereby allowing for establishing strategies in synthesis and for explanation and prediction of properties for a wide range of inorganic, organometallic and organic compounds. His results have been reported in 182 scientific articles in relevant journals across the world (December 2009).

The Center for Molecular Modeling and Quantum Chemistry founded by him in 2007 (developed from the Laboratory for Structure and Molecular Modeling established in 1996), featuring an internationally-competitive computational infrastructure, has consolidated the school of theoretical chemistry in Cluj and has provided a sound basis for high-level collaborations with prominent researchers from the United States of America and from China.

He was a visiting professor at the National Autonomous University in Mexico (UNAM) in the Theoretical Chemistry Group at the Institute of Chemistry (1995-1996) and at the University of Georgia, Athens, Georgia (USA) (2000-2008, for 1-2 months each year). He also had research-related stays at the University of Nottingham (1992) and Heidelberg University (1993-1994), and was engaged in active collaborations with groups from the Universities in Toulouse, Rouen, Lille, Leipzig, Braunschweig, Köln, Budapest, Pecs, Beijing, Guanjou, Moskow (Idaho).

He received the *"Gheorghe Spacu*" prize from the Romanian Academy for his "Research in structure and molecular topology" in 1989 and the *"Gheorghe Spacu*" Medal and "Diploma de Onoare" from the Romanian Society of Chemistry (2009).

As leader of the Working Group for Metal Research from Cluj-Napoca, Prof. Ioan Silaghi-Dumitrescu, PhD – corresponding member of the Roumanian Academy extended, since 2008, the co-operation with the members of the Working Group for Metal Research from Timişoara in the domain of metallomics. As part of co-operation the Symposium "Metal Elements in Environment, Medicine and Biology" (M.E.E.M.B.) enlarged its organizing and scientific framework taking place in uneven years at the Branch Cluj-Napoca of the Roumanian Academy and in even years at the Branch Timişoara of the Roumanian Academy.

Passing into eternity of Prof. Ioan Silaghi-Dumitrescu, PhD afflicted the scientific community not only from Roumania but also from abroad and oblige us to continuu studies in metallomics in his memory and for the progress of this domain.

The Editorial Board of
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