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Detection of Biological Efficiency of Fe(III) Complexes with Heterocyclic N-Donor Ligand Nicotinamide (nia) Using Algal Assay

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Past studies of aquatic pollutants have focused on either metals or organic contaminants. However, under natural conditions, many metals react with organics (alkyl, aryl, cyclopentadienyl or related groups, chelates and surfactants) or inorganic compounds to produce toxic metallic complexes (Florence et al. 1992). Some metals can be transformed into metallic complexes by the actions of microorganisms (metylation of mercuric compounds) (Chau and Wong 1977). There is also a number of man-made toxic metallic compounds, such as organo-Pb used in gasoline, organo-Sn used in antifouling paints and organo-As used in pesticides (Thayer 1984). Whether man-made or natural, these compounds have been found in rain, snow, lakes and rivers. They accumulate in plankton and invertebrates, and are poisonous to fish (Radojevic and Harrison 1987). Industrial and pharmaceutical wastewaters contain a variety of waste by-products which can combine with non-toxic or slightly toxic elements (e.g. germanium, iron) to produce toxic organic or inorganic derivatives (Thayer 1984). On the other hand, it is known that the toxicity of some compounds can be expressive lowered after formation of coordination compounds. Because many of these complexes have unknown chemical structures, no conventional methods are available for the determination of their presence and toxicity. However, lipophilic compounds bioaccumulate in lipid bodies of alga cells (Gnassia-Barelli and Romeo 1993), and energy dispersive X-ray microanalyses have been used to detect metals and metal complexes that have accumulated in cell organelles (Jensen et al. 1982; Fargašová 1998). It is therefore possible to determine the presence of toxic metallic complexes in wastewaters if their toxicity and bioavailability to living cells can be verified, using tests such as algal assays and X-ray fluorescence analysis (RXFA).

The objectives of this study were to assess the toxicity of a selected number of new prepared Fe(III) complexes with N-donor ligand (nia) for pharmaceutical industry (for using as possible fillers or components of some drugs) and to identify the presence of iron from this complexes in alga cells. Here presented results are only a part of experiments in which also Cd(II), Zn(II) and Ni(II) complexes with nicotinamide are tested or as possible drug components (Zn(II) complexes) or as intermediate products which could appeared after metal complexation in biological systems.

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MATERIALS AND METHODS

During the tests the suspension cultures of the green alga *Scenedesmus quadricauda* (TURP.) BRÉB., strain Greifswald 15 were used. Algae grew in a liquid calcium-depleted modified Knopp solution (pH=7.2) 16 days (Fargašová 1993). During the tests the algae were kept in non-agitated Erlenmeyer flasks under constant temperature (25 ± 1° C) and permanent light conditions (2,000 cd/m^2). The growth rate (expressed through suspension density) was determined each two days by using spectrophotometer (λ=665 nm) in presence of tested compounds as well as in control. The EC_{50} values (50 % growth rate inhibition) and their 95 % confidence intervals were estimated by probit analysis (Fargašová 1994) and expressed chronic effect of tested compounds. All tested complexes were for growth rate inhibition tests used in 10 various concentrations in the range 200-1,000 mg/L and $FeCl_3$ was tested in the range 50-500 mg/L. Chlorophyll *a* and chlorophyll *b* content was determined by using spectrophotometric method (Fargašová 1996). Chlorophyll was determined in 95 % ethanol extract measuring absorbance at 665 and 649 nm and its amount was calculated under the following equations:

$$\text{chlorophyll } a \text{ (chl}a\text{)} = 13.70(A_{665}) - 5.76(A_{649})$$

$$\text{chlorophyll } b \text{ (chl}b\text{)} = 25.80(A_{649}) - 7.69(A_{665})$$

in μg/mL culture. The uptake of Fe ions from tested complexes as well as from $FeCl_3$ compound was determined by X-ray fluorescence analysis (RXFA) using an energy dispersive multichannel X-ray fluorescent analyzer (model Canberra 8100. U.S.A) equipped with a digital data recorder (Fargašová 1998). Iron accumulation in alga cells was determined after their washing by distilled water to eliminate iron remains partially at the surface of the algae. For chlorophyll content determination and iron uptake the tested compounds were used in concentrations equal to calculated EC_{50} values for alga growth (mg/L): A 480; B 730; C 850; D 700; E 580 and $FeCl_3$ 160. All experiments were set up in a completely randomized design with 3 replicates. The suspension density at the beginning of all experiments was about 5x10^6 coenobia (four cells connected in one unit) and algae were in the exponential phase of the growth. For statistical evaluation of the results ADSTAT 2.0 program has been used.

The following iron compounds were used during the tests: $FeCl_3(nia)_3$ (A); $Fe(ClO_4)_3(nia).2H_2O$ (B); $Fe_2O(ac)_2(nia)_6Cl_2.3H_2O$ (C); $Fe(NO_3)_3(nia)_3.3H_2O$ (D); $Fe(Cl_2ac)_3(nia)_3$ (E); $FeCl_3$. All used complexes were prepared at the Department of Inorganic Chemistry, Faculty of Chemical Technology, Slovak University of Technology, Bratislava, Slovak Republic as completely new synthesized compounds which could be used in pharmacy. They were characterized as water soluble and stable under the test conditions and during the whole experimental period (Ondrejkovičová et al. 1995; Melník et al. 1997).

RESULTS AND DISCUSSION

Results presented in this work are directed to the specification of differences in biological effectiveness of Fe(III) complexes. By the help of alga growth inhibition, photosynthetic pigments production and the accumulation of iron into

Table 1. Inhibitory effect of iron (mg Fe/L) bounded in Fe(III) complexes and $FeCl_3$ expressed as EC_{50} values and their 95% confidence intervals (CI) for growth rate of alga *S. quadricauda* after 16 days cultivation

Comp.	A	B	C	D	E	$FeCl_3$
EC_{50}	50.7	54.0	154.8	59.2	40.3	72.0
95 % CI	(44.5-52-7)	(49.7-57.1)	(149.0-157.2)	(54.0-61.8)	(37.1-43.3)	(70.2-76.8)

the alga cells the effectiveness of Fe bounded in the Fe(III) complexes and $FeCl_3$ has been expressed. At the first part of this study the inhibitory effect of iron from tested compounds was determined and calculated as EC_{50} values (Table 1.). On the base of these values and their statistical evaluation, compounds can be arranged in the rank order of inhibition as follows: $E>A\geq B\geq D>FeCl_3>>C$. From this rank order it is evident that Fe bounded in Fe(III) complexes with heterocyclic N-donor ligand (nia), except complex C, indicated the strongest inhibitory effect than Fe in compound $FeCl_3$. Binding of Fe with nicotinamide, which belongs to the group of vitamins and it is commonly presented as the PP factor (pelagra preventive factor), in majority of tested complexes increased iron unfavorable effect on alga growth. Only complex C had opposite effect on alga growth and it decreased Fe unfavorable effect. This Fe(III) complex was the single from complexes which had two Fe atoms bounded in complex. This indicates that Fe reduced unfavorable effect of bounded ligand (Fargašová et al. 1999) and the intensity of this reduction depends on iron content in complex. The EC_{50} value for complex C was about 2 times higher than that for $FeCl_3$ and 2.7-3.8 times higher than those for other tested complexes. The unfavorable effect of complex C was two times lower than that of $FeCl_3$. Iron as an essential trace element is mostly presented as having a small toxicity to all living organisms (Khangarot and Ray 1989; Doyle and Otte 1997; Fargašová 1999). Its EC_{50} values in dependence of tested aquatic organisms and chemical compounds are arranged between 7.2 and 110.0 mg Fe/L (Khangarot and Ray 1989; Doyle and Otte 1997; Fargašová 1999) and this is in good agreement with the order of magnitude for *S. quadricauda* and all tested compounds.

The composition and contents of photosynthetic pigments appear to be important for taxonomical classification as well as for determination of physiological characteristics of algae (Rowan 1989). Photosynthetic pigments production in *S. quadricauda* coenobia was determined after 16 days cultivation as in the media supplemented with tested compounds as in the control medium. The chlorophyll *a*

Table 2. Effect of iron from Fe(III) complexes and $FeCl_3$ on chlorophyll contents (μg/mL) in alga *S. quadricauda* after 16 days cultivation in media with compound concentrations equal to EC_{50} values for growth inhibition

Chlorophyll *a*							
Comp.	**Contr.**	**$FeCl_3$**	**A**	**B**	**C**	**D**	**E**
Amount (μg/mL)	3.09 (0.009)	1.41 (0.035)	2.91 (0.064)	4.17 (0.085)	2.73 (0.063)	1.95 (0.052)	1.8 (0.042)
$I_{Contr.}$ (%)		54.4**	5.8*	+35.0**	11.7**	36.9**	41.7**
I_{FeCl3} (%)			+106.4^{xx}	+195.7^{xx}	+93.6^{xx}	+38.3^{xx}	+10.6^{xx}

Chlorophyll *b*							
Comp.	**Contr.**	**$FeCl_3$**	**A**	**B**	**C**	**D**	**E**
Amount (μg/mL)	1.08 (0.022)	0.48 (0.013)	1.05 (0.024)	1.38 (0.028)	0.78 (0.019)	0.51 (0.010)	0.81 (0.012)
$I_{Contr.}$ (%)		55.5**	2.7*	+27.8**	27.8**	52.8**	25.0**
I_{FeCl3} (%)			+118.8^{xx}	+187.5^{xx}	+62.5^{xx}	+6.3^{x}	+68.8^{xx}

(values in brackets mean standard deviations)
+ increase in pigment production = stimulation; * no significant difference in comparison with control ($P>0.05$); ** significant difference in comparison with control ($P<0.05$); ^{x} no significant difference in comparison with $FeCl_3$ ($P>0.05$); ^{xx} significant difference in comparison with $FeCl_3$ ($P<0.05$); $I_{Cont.}$ – inhibition/stimulation in comparison with control; I_{FeCl3} – inhibition/stimulation in comparison with $FeCl_3$

and chlorophyll *b* content in the algae treated with Fe(III) complexes and $FeCl_3$ is shown in Table 2. Results indicate, in comparison with control, for major cases statistically significant decrease in production of both chlorophyll types. The exception was observed only for Fe bounded in Fe(III) complex A for which the decrease in chlorophylls production was not significant ($P>0.05$). Reversal Fe from complex B increased, in comparison with control, as chlorophyll *a* as chlorophyll *b* amount about 35.0 % and 27.8 %, respectively. When comparison was done to $FeCl_3$ iron from all Fe(III) complexes increased chlorophylls amount (Table 2.). This increase was not significant only for chlorophyll *b* content in algae treated with complex D. Results introduced in the literature (Sinha et al. 1994; Fargašová 1999) indicate an increase in chlorophyll content with increase of Fe concentration in the background. In our laboratory tests binding of Fe in complexes with N-donor ligand, in general, decreased unfavorable effect of iron. In comparison with the control the intensity of inhibitory effect of Fe from complexes as well as from $FeCl_3$ compound on chlorophylls production was as follows: chlorophyll *a*: $FeCl_3>E\geq D>C>A>>B$; chlorophyll *b*: $FeCl_3\geq D>C\geq E>>A>>B$. From these inhibitory rank orders it is evident that the strongest inhibitory effect on both chlorophyll production had Fe bounded in $FeCl_3$ and the lowest had Fe from complexes B and A. Decrease in cell

chlorophyll content after treatment with Fe bounded as in $FeCl_3$ as in Fe(III) complexes C, D and E indicated as described Rai and Chandra (1992) destruction of photosynthetic pigments. De Filippis and Pallaghy (1994) suppose that this destruction can be involved in the toxic effects of metals.

Concentrations of iron determined by the RXFA method in alga cells were very different in accordance with a tested compound (Table 3.). Differences in the concentration of iron accumulated in cells of alga were significant between the control and all tested compounds, as well as between $FeCl_3$ and Fe(III) complexes. The highest concentration of iron has been found in cells of algae,

Table 3. Mean concentrations of iron in alga cells of *S. quadricauda* and their statistical evaluation after 16 days cultivation in treated and control media

Compound	Contr.	$FeCl_3$	A	B	C	D	E
µgFe/mg DW	0.175 (0.004)	0.55 (0.001)	1.55 (0.006)	3.33 (0.006)	10.78 (0.008)	4.05 (0.005)	3.29 (0.004)
$Fe_{comp}/Fe_{contr.}$		3.14**	8.57**	19.0**	61.6**	23.14**	18.8**
Fe_{comp}/Fe_{FeCl3}			0.49^{xx}	1.06^{xx}	3.43^{xx}	1.29^{xx}	1.05^{xx}

(values in brackets mean standard deviations)
DW – dry weight; ** significant difference in comparison with control ($P<0.05$);
^{xx} significant difference in comparison with $FeCl_3$ ($P<0.05$)

which were growing 16 days in the presence of the complex C with two Fe atoms bounded in complex. The lowest concentration was found in cells, which were growing in the medium with the complex A. Accumulated amounts of iron were for all tested complexes significantly higher than that for $FeCl_3$. Gradual succession for accumulation of Fe by alga *S. quadricauda* is as follows: C>>D>B=E>A>$FeCl_3$. From the above-mentioned results it can be concluded, that iron was accumulated in alga cells in high amount. In comparison with other metals (Mn, Zn, Cu) also literature describes high accumulation of Fe in the plants as well as in phytoplankton (Doyle and Otte 1997; Fargašová 1998). However, the iron amount accumulated from $FeCl_3$ was in comparison with iron amount accumulated from Fe(III) complexes very low its efficiency to other observed parameters (growth, chlorophyll production) was high. The highest accumulation of Fe has been confirmed for the complex C, which had the lowest inhibitory effect on alga growth. Iron from the complex C has been accumulated in cells of alga in a very high amount, at least 2.7 times higher than the amount of Fe accumulated from other complexes. Fe bounded in this complex (C) inhibited as well photosynthetic pigments production and strongly influence mainly chlorophyll *b* content in alga cells. Based on the results it can be stated, that Fe bounded on heterocyclic N-donor ligand influences not only the production of oxygen by algae as introduced Fargašová et al. (1999), but also the growth of alga, photosynthetic pigments production and the accumulation of iron in alga

cells. However, not only the Fe(III) ion, but also ligand on which the metal binds is responsible for inhibitory/stimulatory effect as well as for the accumulated effect of the complex (Melník et al. 1997; Fargašová et al. 1999).

The modes of coordination of ligands in the complexes A-E have been investigated by means of infrared absorption spectra. On the base of ring vibration of nia it was confirmed that in all complexes nia is coordinated to Fe atom through the nitrogen atom of its heterocyclic ring (Ondrejkovičová et al. 1995; Melník et al. 1997). The presence of lattice and coordinated water in the complexes B-D was indicated by its characteristic bands. These bands were absent in the spectra of the anhydrons complexes A and E (Ondrejkovičová et al. 1995). Biological effect of the complexes B and D has been confirmed as enough strong mainly on the growth of alga. However, the effect of these complexes on the production of oxygen was introduced by Fargašová et al. (1999) as very stimulative. Alga cells of *S. quadricauda* indicated good penetration of both of these complexes through the cell wall. The result of this good penetration was that Fe(III) complexes B and D have quite well cumulated in alga. Inhibitory effect of the complexes A and E on the growth of alga suspension is mentioned as stronger than the effect of the complexes B and D. Accumulation of Fe in the cells of algae was for these complexes (A, E) lower or equal to complexes B and D. Chlorine atoms in complex A are coordinated to iron atom (Melník et al. 1997). Dichloroacetate groups in E and nitrate groups in D are unidentate coordinated to iron atom through O-atom (Ondrejkovičová et al. 1995). We suppose that the complex C consists of chlorine anions and a complex cation $[Fe_2O(ac)_2(nia)_6]^{2+}$ in which two iron atoms are bridged by two acetate and one oxo groups. IR spectrum of complex C has similar feature to μ-oxo-diiron(III) complexes of the type of hemerythrine (Scarrow et al. 1987). Biological effect of the complex C upon the growth is described as the poorest one from among all tested complexes, but by Fargašová et al. (1999) only this complex was confirmed as inhibitor of oxygen production by alga *S. quadricauda.* Fe from the complex C has been accumulated in alga cells in the highest amount, in comparison with other complexes.

From among biological effects of Fe(III) complexes, up to the present, only their anti-microbial effect upon various strains of bacteria, yeast and filamentous fungi was observed. Effectiveness of these complexes was limited only to filamentous fungi, above all on *Botrytis cinerea* (Melník et al. 1997). For fungi it was confirmed, that the Fe(III) dichloroacetato complex (E) is, generally, biologically more active than similar complexes with anions from mineral acids (Melník et al. 1997). At present this reality for alga *S. quadricauda* has been confirmed as for growth inhibition as for chlorophyll *a* production.

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