THE ROLE OF THE NATURAL ANTIOXIDANTS IN THE OXIHAEMOGLOBIN OXIDATION AND THE DIMINUTION OF NITRITE CONCENTRATION

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Abstract: The paper includes the study of the inhibition of the process of methemoglobinization at oxidation with nitrites in the presence of sodium dihydroxyfumarate (DFH_3Na) and resveratrol (3,4',5-trihydroxystilben). The experimental study was carried out by treatment of the erythrocyte mass by hemolysis and exposure to nitrite. The kinetic investigations were carried out in following conditions: [Resv] = ($5 \cdot 10^{-5} - 1 \cdot 10^{-3}$) mol/l, [$DFH_3Na$] = $1 \cdot 10^{-6} - 5 \cdot 10^{-6}$ mol/l; [HbO_2]=$1 \cdot 10^{-3}$ mol/l; pH 7,1; t = 37^0C. The rate of transformation of HbO_2 in the presence of resveratrol and DFH_3Na was calculated from kinetic curves of consumption of the substrate and formation of MetHb obtained spectrophotometrically (λ_{max} = 540 nm for HbO_2 and λ_{max}=630 for MetHb). It has been found out that the introduction of resveratrol and DFH_3Na in the system $HbO_2 - NO_2^-$ causes the decrease of the autooxidation factor φ_{DFH3Na} approximately by 1.1 – 2.5 times and $\varphi_{resveratrol}$ by 1.1 – 1.7 times. The time of achievement of the maximum rate of oxidation of HbO_2 $d\zeta/d\tau$ (where ζ is the rate of transformation of HbO_2 in MetHb) increases while the phase of fast oxidation of HbO_2 decreases with increase of content of inhibitors. The process of interaction of nitrites with reducers (such as DFH_4, DFH_3Na, resveratrol and (+)-catechine) was carried out as well. It has been established that degree of diminishing of the concentration of nitrites in the system $RedH_2$-NO_2^- decreases as follows: DFH_4<DFH_3Na<Resv<(+)Catechol.

Keywords: nitrite, haemoglobin, inhibitor of methemoglobinization.

1. INTRODUCTION

In the specialized literature there are described groups of compounds that lead to MetHb formation (among them, different xenobiotics). The nitrates, the nitrites and nitric oxides play an important role in the MetHb formation; they penetrate in the human body via various ways: during the ingestion (food and water) and inhalation. The nitrates and the nitrites are indispensably joined with each other in the natural circuit of the nitrogen, but due to the chemical activity of the nitrite ion, their concentration in the environment is insignificant. Thus, the first stage of intoxication with nitrates is their reduction in nitrites. In all living beings, simultaneously with the common process of O_2 elimination, there occurs the self oxidation of HbO_2 in MetHb:

$$HbO_2 \rightarrow MetHb + O_2^{\bullet} \quad (1)$$

From the above reaction, we can observe that the radical of super oxide anion, with pragmatical properties is generated ($O_2^{\bullet}$). This radical was detected in HbO_2-$NaNO_2$ system, via the RMN method [1].

During the process of HbO_2 oxidation with NO_2^-, the oxygen from the haemoglobin molecule that appears as an auto catalyst in this process plays a great role. The authors of this work [2] suppose that after the process of oxide-reduction at the interaction of NO_2^- with $O_2^{\bullet}$ takes place the generation of nitrate oxide. Such a direction of the process is assured by the oxide-reducing potentials of NO_2^- and $O_2^{\bullet}$, accordingly +0, 99 and -0, 33 V. The $O_2^{\bullet}$ radical is not a direct oxidant of HbO_2, but it is compulsory in the process of autocatalysis.

Another important factor in the HbO_2 oxidation mechanism is the formation of $NO_2^{\bullet}$ at the initial stage of the oxidation process. The formation of this radical in the system was established by the use of amines (aniline), which imbibes the process of HbO_2 oxidation, due to the formation of N-nitrosamines with NO_2^- [3].

An important intermediary of the HbO_2 oxidation process with NO_2^- is H_2O_2, which forms as a result of $O_2^{\bullet}$ dismutation [2]. We established that H_2O_2 influence upon the HbO_2 oxidation reaction with NO_2^- depends on the initial concentrations of $[NO_2^-]_0$ and $[H_2O_2]_0$.

Knowing the mechanisms of the reactions between haemoglobin and different methemoglobinisants helps to elaborate methods of inhibition against haemoglobin oxidation.

HbO_2 oxidation with nitrites is being studied as a 2-stage process [4]: the slow process (lag period) and the fast process, (auto catalyst). The kinetic curves have a peculiar S-shape. There should be mentioned that the process of oxidation of the oxygenated form of HbO_2 and Hb with nitrites is different.

In order to decrease the degree of HbO_2 oxidation and diminish the concentration of oxidative particles that form in various systems it is necessary to use different antioxidants, especially natural ones.

The antioxidant activity of polyphenols

The polyphenols are characterized by their antioxidant activity and they can be used as well to reduce the oxidational degree of different substances.

The flavonoids constitute a large class of compounds present in plants, which contain a certain number of hydroxyl phenolic groups attached to the annular structure, endowing reducing properties. The antioxidant activity of phenols is determined by the presence of hydroxyl groups in B ring, in the positions 3' and 4' and in a lesser degree, by the hydroxyl group from B ring, in the position 4'. The phenols, especially the catehine, quercitin, kaemferol and their glycosides, are constituents of the green and black tea [5] and red wine [5]. The diets rich in fruits, vegetables and grapes are recommended against heart diseases accompanying various forms of cancer [7,8], methemoglobinemy, have anti-inflammatory and antimutagene effects [9] etc. These protecting effects have been attributed to the present antioxidants that include flavonoids, carotids and the vitamins C and B.

The researches performed *in vitro* established the antioxidant potential of the polyphenols as the parameter that determined the capture capacity of the free radicals, such as super oxide radicals, the singlet oxygen, hydroxyl radicals, peroxyl radicals, nitric monoxide and the peroxinitrite (they cause different pathologies). The chemical structures that contribute to the antioxidant activity of the polyphenols, including the neighbouring dihidroxi- or trihidroxi-structure, can cellar the ions o metal through the formation of complex and prevent the generation of free radicals. This structure also allows delocalizing the electrons, conferring high reactivity for destruction of free radicals.

The majority of polyphenolic constituents from the food products (flavonols – such as quercitin and kaempherol, flavones – such as luteolin, flavonols – such as catechin, antocynidins for instance, cyanidin and malvidin and their glycosides) present major efficiency, in comparison with the nutrient antioxidants: vitamins C, E, β-carotene, that are easily absorbed in the intestine [10].

Antocynidins and catechines have been tested *in vitro* for their inhibitional influence upon the cyclooxigenaze enzymes (COX) that provoke the multiplication of cancerogen cells and also upon the proliferation of cancer cells in human beings [5]. We established that cyanidine has the strongest inhibitory effect of the COX enzymes and it has hydroxyl groups 3',4' in B ring. The inhibitory activity decreased in the case of delphinidine and pelargonidine, that have 3', 4', 5'-trihidroxilic and 4'-hidroxilic groups in B ring, accordingly. From the point of view of the liaison between the structure and the activity, the number and the position of hydroxyl groups in auto cyanides B ring influence the inhibitory activity of these compounds. For catechine cis-, trans-isomery, epimerisation did not influence greatly the inhibitory activity on COX enzyme, but the presence of galloyl groups in catechine structure influenced more their inhibitory activity on the COX enzymes. Based on the obtained results during the inhibition of proliferation of cancer cells under the action of antocyanidins and catechines, we established that the degree of inhibition is higher for the galloyl derivatives of catechines [5] (gallocatechine – 95%, epigallocatechine – 100% and gallocatechingalate – 97%), but for antocyanidins, it represents almost 75%.

The degree of polymerisation has a greater influence on the inhibitory properties of the polyphenols and it augments together with the galloylation. The polyphenolic fractions extracted from the grapes with a different degree of polymerisation had a different antioxidant/antiradical and antiproliferative effect [6]. The polyphenolic solutions extracted from the grapes have been divided into 2 fractions having a different degree of polymerisation, with RP-HPLC. The antioxidant/antiradical activity determined via the DPPH test for the polyphenolic fraction from the grapes, composed of small, was higher than the fraction which included procyanide flavonols and oligomers with a greater molecular mass. Catherine A Rice-Evans et alt. [10] have studied the total antioxidant activity (TAA) and the antioxidant activity equal to Trolox (AAET) for the polyphenols that are contained in the green tea and red wine. AAET measures the concentration of the Trolox solution (mM) with a potential antioxidant equivalent at a standard concentration of the compound subjected to the research. The authors came to the conclusion that the antioxidant activity of the polyphenolic constituents of the green tea (Fig.1) in correlation with their content, according to their order of antioxidant activity, is: epigallocatechine (34%) ≈ epigallocatechingalate (32%) >> epicatehingallate (7%) ≈ epicatechine (6%) > catechine (1%) [10].

Through radio lithium generation of oxygen species in presence of different catechines, that are active constituents of the tea, we established that the DNA harm caused by these reactive particles, decreases at least in the presence of EGCG [11]. Thus, 66% from the antioxidant activity of the green tea is determined by epigallocatechine and epigallocatechine-gallate, which corresponds to the content of these compounds in the green tea (20,44% from 26,71% of the total number of polyphenols).

Based on the study of the total average antioxidant activity of the red wine, we established that 54,76% is determined by catechine and epicatechine contribution that represent almost 63,54% from the phenol constituents (191 and 82 mg/l accordingly) [10].

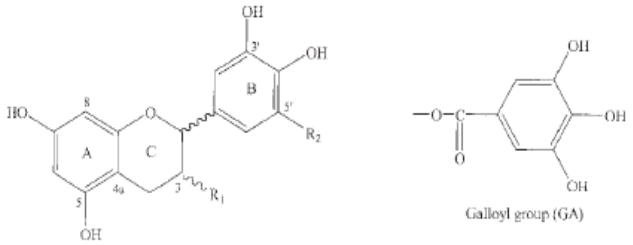


Fig. 1. The chemical structure of the catechines present in tea and in wine [5]. Catechine-(R_1 - OH, R_2 - H), gallocatechine-(R_1 - OH, R_2 - OH), catechingalate (R_1 - GA, R_2 - H), gallocatechingalate (R_1 - GA, R_2 - OH).

In various systems, the free radicals can form as a result of peroxide decomposition in presence of different metals: Fe^{2+}, Cu^{2+}, Cr^{3+} etc. As a result of H_2O_2 reduction through the Fenton reaction in presence of Cr^{3+} (which is toxic and causes genotoxicity), the $OH^{\bullet}$ radical is being formed:

$$Cr(III)+H_2O_2 \rightarrow Cr(IV)+OH^{\bullet}+OH^- \quad (2)$$

The hydroxyl radical formed *in vivo* produces oxidative harm to DNA, with the formation of 8-hydroxy-2-deoxyguanosine (8-OH-dG) that appears in this process as a biomarker [12]. Based on the study results, Silvia Lopez-Burillo et al. [12] established that the antioxidants inhibit the oxidative processes of DNA. Among the polyphenols studied in [12], the highest inhibitory effect is presented by (-)-epigallcatechine-3-gallate (EGCG) in concentration of 1 μM or more, which reduces the 8-OH-dG formation. Tea catechines can form stable compounds with Cu(II) and Cr(III) and as a result, $OH^{\bullet}$ radicals are generated [13]. But it was established that in the case of EGCG $OH^{\bullet}$ radicals are split up by the gallate group present in the complex and thus, the prooxidant effect of this compound is not manifested [14].

The green and black tea can inhibit lipoproteins' oxidation induced by Cu^{2+} [13], thus, it contributes to the prevention of arteriosclerosis and other heart diseases. The inhibition of this very process is determined by the fact that polyphenols can cellar the metals and decrease the concentration of active forms of the oxygen, which in its turn takes part at the protein oxidation.

2. MATERIALS AND METHODS

An inhibitor that was used in the, HbO_2–NO_2^- system is sodium dihydroxifumarole (DFH_3Na). In order to establish the degree of inhibition, we studied the kinetic of the process of HbO_2 (λ_{max} = 540 nm) consumption and MetHb (λ_{max} = 630 nm) accumulation [15]. We counted the degree of transformation (η) after HbO_2 consumption, using the relation $\eta=D_0-D_\tau/D_0-D_\infty$ and the degree of MetHb formation according to the relation $\eta=D_\tau/D_\infty$. In concordance with the kinetic curves under the S-shape we measured the speed of the reaction as a derivative of the degree of conversion in time ($d\eta/d\tau$) [16].

The interaction of the sodium dihydroxifumarole (DFH_3Na) and polyphenols with the nitrite ion was studied according to the variation of the nitrite concentrations in system with the use of Griess reagent [17]. We studied the influence of the reducer concentration on the speed of nitrite transformation reaction. DFH_3Na concentration has been varied within the following interval: 0 - $1\cdot10^{-3}$ M (C^1_{DFHNa} = 0 M, C^2_{DFHNa} = $1\cdot10^{-4}$ M, C^3_{DFHNa} = $5\cdot10^{-4}$ M, C^4_{DFHNa} = $1\cdot10^{-3}$ M). The reaction took place in the citrat-phosphate solution buffer with pH 2,6 at t = 37°C. the other reducers have been studied in the same interval of concentrations.

During the experiments, we used various reagents: (+) catechine (Fluka, 98%, HPLC), DFH_3Na, DFH_4 (Aldrich, 98%), resveratrol (Fluka, 98%, HPLC). There had been used the erythrocytes mass, collected from healthy donors, afterwards subjected to haemolysis during 18-20 hours.

3. RESULTS OF THE EXPERIENCES

The influence of sodium dihydroxifumarole on the HbO_2 oxidation with NO_2^-

The formation of the MetHb in HbO_2 – NO_2^- system in presence of DFH3Na at t = 20°C, pH 7.2 (phosphate solution buffer), $[HbO_2]_0 = 5\cdot10^{-5}$ mol/l depending on various concentrations of DFH_3Na is presented in Fig.2. From the data of the experiments shown in Fig.2., at the variation of $[DFH_3Na]_0$ ($C^1_{DFH_3Na}$ = 0; $C^2_{DFH_3Na}$ = $2\cdot10^{-6}$ mol/l; $C^3_{DFH_3Na}$ = $3\cdot10^{-6}$ mol/l; $C^4_{DFH_3Na}$ = $4\cdot10^{-6}$ mol/l; $C^5_{DFH_3Na}$ = $5\cdot10^{-6}$ mol/l) the maximum concentration of MetHb is reached within t=30 min for $C^1_{DFH_3Na}$ and increases till t ≈ 60 min for$C^5_{DFH_3Na}$.

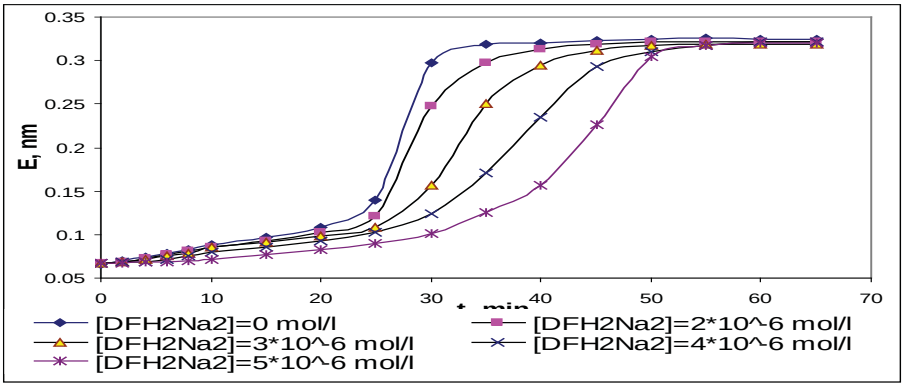


Fig. 2. The kinetic curves of MetHb formation in the HbO_2–NO_2^-–DFH_2Na_2 system, at the variation of DFH_2Na_2 concentration, phosphate solution buffer, pH = 7,2, t = 21ºC, λ = 630 nm, $[HbO_2]$ = 5*10^ -5 mol/l, $[NO_2^-]$ = 5*10^ -4 mol/l.

At the oxidation of HbO_2 with NO_2^- in presence of AAs the speed of the process after the curvature point in $f([AAs]_0)$ varies in a lesser degree in comparison with the oxidation of HbO_2 with NO_2^- in presence of DFH_3Na, depending on its concentration. The influence upon the period of induction is peculiar for AAs. Contrary to this, the duration period of induction for the system with DFH_3Na is shorter, depending on its concentration, but the speed suffers a lot of changes after the curvature point of the autocatalytic process (Fig.2.)

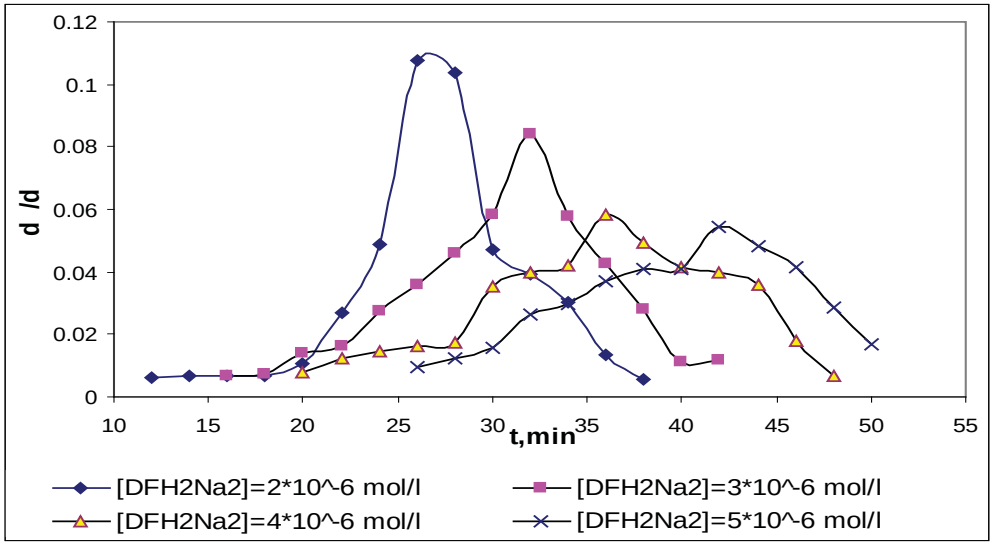


Fig.3. Speed variation (dη/dτ) depending on the time HbO_2–NO_2^- –DFH_2Na_2 system at variation of DFH_2Na_2 concentration, phosphate solution buffer, pH = 7,2, t = 21ºC, λ = 540 nm, $[NO_2^-]$ = 5*10^-4 mol/l, $[HbO_2]$ = 5*10^-5 mol/l.

Speed variation (dη/dτ) depending on time, measured on the basis of data obtained at λ=540nm and λ = 630 nm, depends on $[DFH_3Na]_0$. The maximum variation of the speed (dη/dτ) of MetHb formation in f(η) with the increase of $[DFH_3Na]_0$, reduces. We established that the position of the maximum of kinetic curve dη/dτ changes insignificantly depending on η and corresponds to the interval η = (0,5-0,7).

The change of maximum dη/dτ = f(η) towards the direction of smaller values of η for smaller concentrations of DFH_3Na, is determined by the presence in the reaction medium of $NO_2^{\bullet}$ radicals, with a higher concentration, which assures a greater contribution in the stage of process division, with the participation of this radical and with the reaching of the maximum speed. We observed that dη/dτ size depends on $[DFH_3Na]_0$ and increases when $[DFH_3Na]_0$ decreases. At $C^5_{DFH_3Na}$ we obtain dη/dτ almost twice smaller than in the case of $C^2_{DFH_3Na}$.

The angular coefficient of √η dependence on τ (factor of de auto acceleration φ = tgα) decreases when the concentration of the inhibitor increases. We can suppose that the catalyst concentration (H_2O_2), in the autocatalytic process diminishes when the DFH_3Na concentration increases, because the acceleration factor φ reduces, when DFH_3Na augments. Thus, we established that this inhibitor influence upon the division of the chain in HbO_2 oxidation, decreasing the speed of the auto acceleration process in the reactant medium.

The introduction in the $HbO_2 – NO_2^-$ - DFH_3Na system of H_2O_2 leads to the decrease of the inducing period from 25 min to ≈ 7 min [18]. If we compare the data of the experiment concerning the speed variation in the systems $HbO_2 – NO_2^-$ - DFH_3Na (Fig.3) and $HbO_2 – NO_2^-$ - $DFH_3Na – H_2O_2$, we will observe that the maximum variation of the speed in the system without H_2O_2 occurs at $\tau = 25$ min, for $[DFH_3Na] = 2 \cdot 10^{-6}$ mol/l, and in presence of $[H_2O_2] = 5 \cdot 10^{-5}$ mol/l, fro the same concentration of the reducer– at $\tau = 7$ min. The peroxide can interact with NO_2^- (3 reaction) and thus, there is formed the $NO_2^{\bullet}$ radical that keeps carrying the process.

$$H_2O_2 + NO_2^- + H^+ \rightarrow NO_2^{\bullet} + H_2O + HO^{\bullet} \quad (3)$$

Simultaneously with $NO_2^{\bullet}$ radical there forms $HO^{\bullet}$ radical that further participates in carrying of the reaction and in augmentation of the $NO_2^{\bullet}$ radical:

$$HO^{\bullet} + NO_2^- \rightarrow NO_2^{\bullet} + OH^- \quad (4)$$

In presence of the inhibitor, together with the increase of its concentration, the speed of HbO_2 oxidation reduces. The maximum variation of $d\eta/d\tau = 0{,}18$ speed for $[DFH_3Na]_0 = 2 \cdot 10^{-6}$ mol/l and it decreases up to 0,06 for $[DFH_3Na] = 5 \cdot 10^{-6}$ mol/l (Fig. 3). The speed of MetHb formation increases in presence of H_2O_2 due to the augmentation of the concentration of $NO_2^{\bullet}$ radical, that forms additionally to the interaction of H_2O_2 with NO_2^- and which is an oxidation agent of Fe^{2+} in Fe^{3+} from haem:

$$HbO_2 + NO_2^{\bullet} \rightarrow MetHb + O_2NOO^- \quad (5)$$

The action mechanism of the inhibitor is based on its interaction with $OH^{\bullet}$ radical that produces after the reaction (3) of more stable particles production [19]. The effect of inhibition will depend on the report of speed constants k_1/k_2, in which k_1 is the speed constant at the interaction of $OH^{\bullet}$- radicals with the inhibitor, while k_2 – speed constant of the reaction between $OH^{\bullet}$ and the nitrite ion (4th reaction). In the case when $k_1/k_2 > 1$, we obtain the effect of inhibition, i.e. the speed of the process that leads to Fe^{2+} oxidation in Fe^{3+} is lower than the speed generating radical oxidative particles, because these particles further interact with the inhibitor, not with the substrate. Based on the results presented in Fig.2., we consider that DFH_3Na can interact with both $OH^{\bullet}$ radical and $HO_2^{\bullet}$ radical, which form at the initial stage. Greater the concentration of the inhibitor, lower is the acceleration speed, as in this process the concentration of the peroxide has the role of catalyst and $[HO_2^{\bullet}]$ decreases. The speed of HbO_2 oxidation process decreases due to the reducing of $[NO_2^{\bullet}]$ after the reaction (4).

The effect of nitrite reduction with DFH_3Na and DFH_4

From the obtained data (Tab.1) we established that DFH_3Na the nitrate concentration decreases in the system and the speed of nitrate consumption is greater when DFH3Na concentration augments.

Table 1

The effect of nitrite reduction with DFH_3Na at pH 2,6; t = 37°C, $[NO_2^-]_0 = 1 \cdot 10^{-4}$ M

Nr.	$[DFH_3Na]_0$, 10^{-4}, M	W_{init}, 10^{-7} mol/l·s	NO_2^- reduction, % 30 min	$[NO_2^-]$, 10^{-5} mol/l 30 min
1	0,0	0,83	55,55	4,1
2	1,0	3,17	87,97	1,3
3	5,0	11,0	99,16	0,08
4	10,0	13,83	100,0	0

Based on the numeric curves, we calculated the speed constant (Fig. 4).

In Fig.4., there are presented the kinetic curves of nitrate consumption, depending on the DFH_3Na concentration. The nitrite concentration reduces during 1 min from 100 μM to 95 μM, 84 μM, 28 μM, 8 μM, corresponding for C_1, C_2, C_3, C_4 concentrations of DFH_3Na.

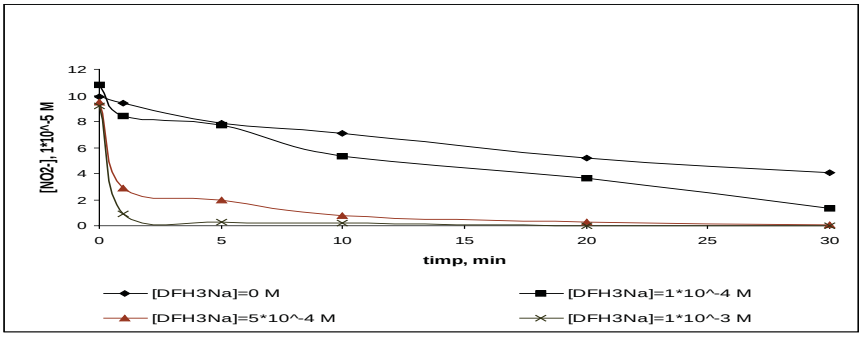


Fig.4. The kinetic curves of nitrite consumption, depending on $[DFH_3Na]_0$, $[NO_2^-]_0 = 1 \cdot 10^{-4}$ mol/l, pH 2.6, t = 37°C.

If we increase DFH_3Na concentration from 100 μM to 1000 μM, NO_2^- concentration decreases from 95 μM to 8 μM, thus, we obtain the following: the augmentation of reducer concentration (10 times) leads to the decrease of NO_2^- concentration (almost 10 times). For DFH_3Na concentration of 100 μM the diminution of the nitrite content is slow, but when the $[DFH_3Na]$ increases the initial speed suddenly augments. The nitrite concentration reduces almost to 0 in 10 min for C^3_{DFHNa} and in 1 min for C^4_{DFHNa} (the rapport $[DFH_3Na]_0 : [NO_2^-]_0$ for C^3_{DFHNa} is 5, while for C^4_{DFHNa} it is 10). The higher the rapport, the greater the speed of the reaction. In the case when $[DFH_3Na]_0 = 0$, at pH 2,6 (citrate phosphate solution buffer), in the system there takes place the nitrite transformation into other forms (HNO_2, NO, N_2O_3, N_2O_4, NO_3^-), thus $[NO_2^-]$ reduces, which was determined by Griess method (kinetic curve for $C^1_{DFHNa} = 0$).

We also studied the kinetic of nitrite consumption in presence of dihydroxifumarole acid (DFH_4). The experimental conditions have been the same as in the case of usage as a DFH_3Na reducer. The data concerning the nitrite consumption, obtained for different DFH_4 concentrations have been inserted in Tab.2.

Table 2

The effect of nitrite reduction with DFH4 at pH 2,6; t = 37°C, $[NO_2^-]_0 = 1 \cdot 10^{-4}$ mol/l

Nr.	$[DFH_4]_0$, 10^{-4}, M	W_{init}, 10^{-7} mol/l·s	NO_2^- reduction, % 30 min	$[NO_2^-]$, 10^{-5} mol/l 30 min
1	0,0	0,83	55,55	4,1
2	1,0	10,25	89,74	1,0
3	5,0	14,17	100,0	0
4	10,0	14,65	100,0	0

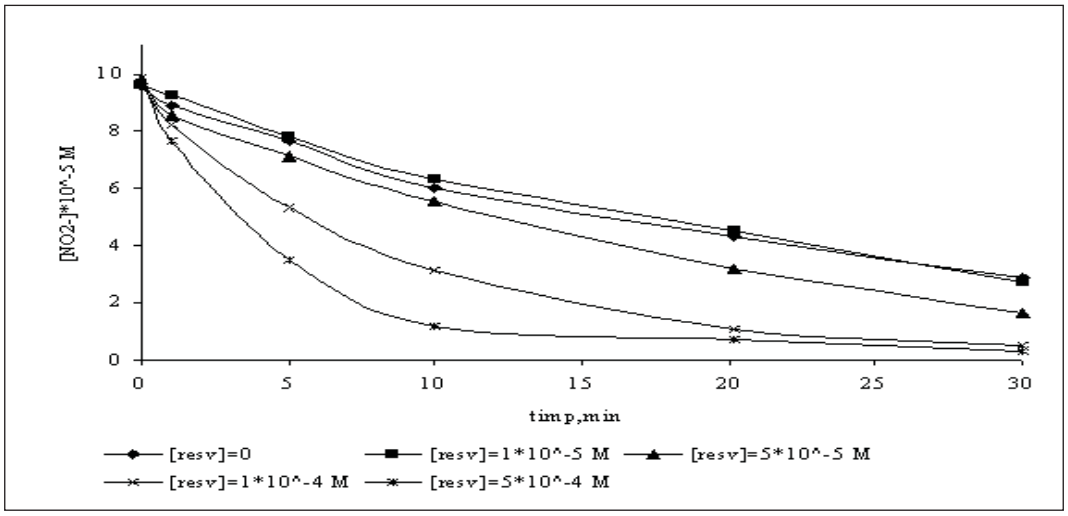


Fig.5. The kinetic curves of nitrite consumption depending on $[Resv]_0$, $[NO_2^-]_0 = 1 \cdot 10^{-4}$ mol/l, pH 2.6, t= 37°C.

In presence of DFH_4, the nitrite concentration reduces with a higher speed, in comparison with DFH_3Na. From table 2 we can observe that at $[DFH_4]_0 = 5 \cdot 10^{-4}$mol/l, the degree of NO_2^- transformation constitutes 100%.

Nitrite reduction with polyphenols

In the present study we researched the influence of rezveratrol and (+) catechine upon the process of nitrite reduction in the pattern sample.

One of the polyphenols, resveratrol (trans-3,5,4'-trihydroxystilben) (Resv), is a phytoalexine synthesized in certain plants, such as eucalyptus, spruce fir, lily. It is also tyo be found inmulberries and ground nuts, but the main natural source of Resv is *Vitis Vinifera*. It is synthesized as a response to fungi development in the grape-vine. The content of resveratrol in ground nuts varies from 0,02 to 1,79 μg/g, and the peel of fresh grapes contains almost 50-200 μg/g. A glass of red wine reaches between 600-700 μg of Resv and has a beneficent effect upon the prophylaxis of heart diseases, leukaemia and cancer.

In the present work we studied the variation of nitrite concentration depending on the Resv concentration (C^1_{Rezv}= 0 M, $C^2_{Rezv} = 5 \cdot 10^{-5}$ M, $C^3_{Rezv} = 1 \cdot 10^{-4}$ M, $C^4_{Rezv} = 5 \cdot 10^{-4}$ M, $C^5_{Rezv} = 1 \cdot 10^{-3}$ M) la pH 2,6 (citrate phosphate solution buffer

in the NO_2^-–Resv system). From the data presented in Fig.5, we observe that during 30 min the nitrite concentration reduces from 100 μM to 0 for C^3_{Rezv}, C^4_{Rezv} and C^5_{Rezv}. In the case of C^2_{Rezv} the nitrite concentration does not reduce up to 0, because the reducer concentration is lower in comparison with the nitrite concentration (50 μM and 100 μM accordingly). Thus, in the system we have a remnant concentration of nitrite after the whole Resv was consummated. In this system, the initial speed of transformation of nitrite (W_{NO2-}) is lower than in the case of DFH_3Na or DFH_4 (Fig.6.) and it is comparable with W_{NO2-} in (+)Ct presence.

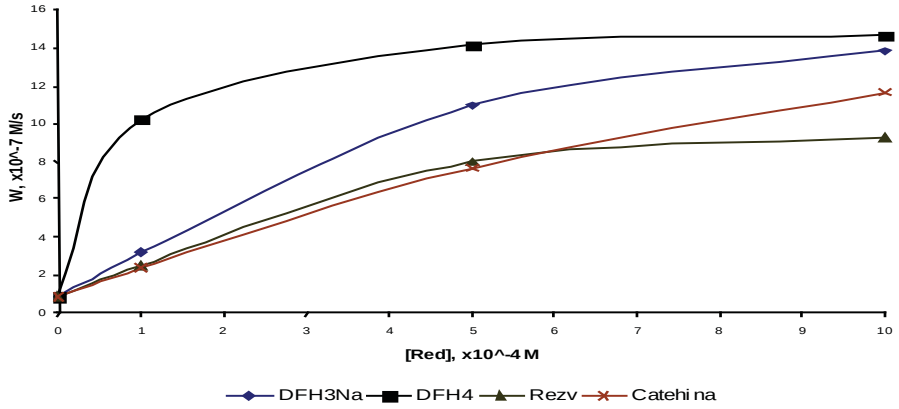


Fig.6. W_{NO2-} dependence based on $[RedH_2]_0$ at pH 2,6 (citrate-phosphate solution buffer), t = 37°C, $[NO_2^-]_0 = 1 \cdot 10^{-4}$ mol/l

PH of the reaction medium plays a great importance in the process of interaction of Resv with NO_2^-. This process was studied on different pH (1.0, 2.6, 3.0, 3.6, 4.0, 6.0) for $[NO_2^-]_0$ and $[Resv]_0$ of $1 \cdot 10^{-4}$ mol/l, in citrate phosphate solution buffer. We established that pH decrease augments the speed of reaction between Resv and nitrite.

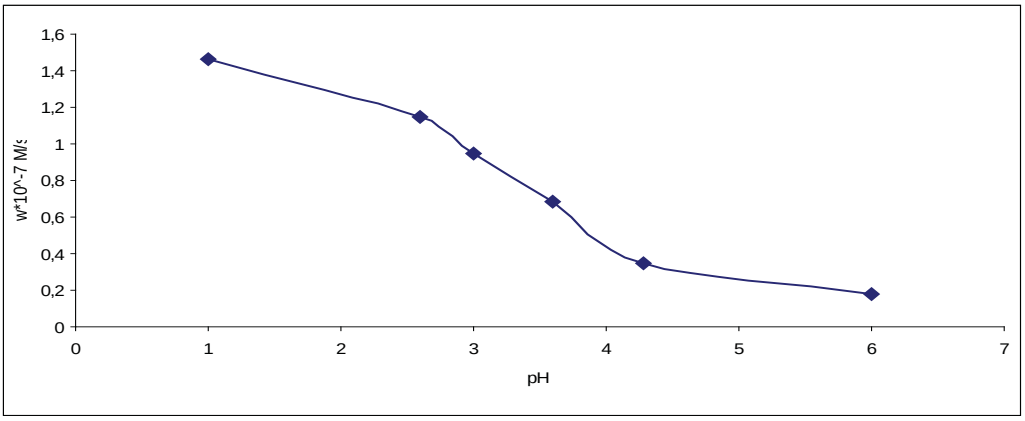


Fig.7. The initial variation speed of nitrites, depending on the pH for $[NO_2^-]_0 = 1 \cdot 10^{-4}$ mol/l, $[Rezv]_0 = 1 \cdot 10^{-4}$ mol/l and t = 37°

The nitrite concentration reduces for pH 1.0 from $1 \cdot 10^{-4}$ mol/l to ~ $0{,}77 \cdot 10^{-4}$M (in 5 min), i.e. with 53 μM, while at pH 3.0 with ~ 23 μM (the same period of time). Thus, less nitrite is used in the reaction, in the case of an increased pH. This may indicate that Resv is a less efficient inhibitor for an increased pH, because when the pH augments, a bigger quantity of nitrites remains in the system. We measured the initial variation speed of nitrites, depending on pH and we established that $W_{NO_2^-}$ decreases when pH increases (Fig.7).

Another inhibitor that was studied is (+) catechine ((+)Ct). The data obtained from the experiences on the reduction of NO_2^- with (+)Ct are presented in Tab. 3.

Table 3

The effect of nitrites reduction with (+) catechine, pH 2.6, t = 37°C, $[NO_2^-]_0 = 1 \cdot 10^{-4}$ mol/l

Nr.	$[(+)Ct]_0$, 10^{-4}, M	$W_{init,}$ 10^{-7} mol/l·s	Effect of reduction NO_2^-, % 30 min	$[NO_2^-]$, 10^{-5} mol/l 30 min
1	0,0	0,83	55,55	4,1
2	1,0	2,33	98,33	0,15
3	5,0	7,66	97,83	0,2
4	10,0	11,67	99,5	0,05

From the data presented in the table 3, we observe that the reduction speed of NO_2^- increases simultaneously with the (+)Ct concentration. For $[(+)Ct]=1 \cdot 10^{-3}$mol/l, NO_2^- concentration reduces with 99,5%. Thus, we can mark that (+)Ct reduces $[NO_2^-]$ when it interacts with NO_2^- in the reactive medium.

4. DISCUSSION

The variation of the concentration of nitrites in acid medium

In the system nitrite – citrate phosphate buffer (pH 2.6), the nitrite concentration decreases ($[RedH_2] = 0$ M). In this case, the quota of nitrites determined via Griess method, is almost 55%. The decrease of the $NO2^-$content in acid medium occurs in 2 ways: oxidation up to nitrates and the transformation into various volatile forms, agents of nitrosation. These forms are produced at the interaction of nitrite ions with the proton (H^+, H_3O^+), leading to the formation of nitrous acid. It has been demonstrated that the diminution of nitrite concentration is accelerated by pH reduction, and the effect of pH decrease leads to increase of HNO_2 concentration from the system, thus, the equilibrium emerges to the augmentation of NO, NO_2 and N_2O_4 concentrations [20]:

$$NO_2^- + H^+ \xleftrightarrow{k_1} HNO_2 \quad (6)$$

$$2HNO_2 \leftrightarrow N_2O_3 + H_2O \quad (7)$$

$$N_2O_3 \leftrightarrow NO + NO_2 \quad (8)$$

$$2NO_2 \leftrightarrow N_2O_4 \quad (9)$$

$$N_2O_4 + H_2O \rightarrow 2H^+ + NO_2^- + NO_3^- \quad (10)$$

Aerobian conditions:

$$2NO + O_2 \rightarrow N_2O_4 \quad (11)$$

Open system:

$$NO\ (aq) \leftrightarrow NO\ (g) \quad (12)$$

$$NO_2\ (aq) \leftrightarrow NO_2\ (g) \quad (13)$$

$$O_2\ (aq) \leftrightarrow O_2\ (g) \quad (14)$$

The main agents of nitrosation that form are N_2O_3, N_2O_4, and NO_2. At moderate acidity (pH 2 -5) all these agents are present in the reaction medium and are detected by [21], through spectrophotometric methods. At pH< 2 the closest agent is H_2O^+NO, which predominates in system ($HNO_2 + H^+ \leftrightarrow H_2O^+NO$). The speed for nitrite transformation depends on the initial concentration of NO_2^-, while the speed for its decreasing is determined by the equation [20]:

$$W = 1,1k_1a[NO] + 0,9k_La[NO_2^-] + k_5[N_2O_4]$$

At the introduction of SCN^- and Cl^- ions into the system, the speed of nitrite variation augments, because new formed species direct the equilibrium towards right:

$$HNO_2 + H^+ \leftrightarrow NO^+ + H_2O \quad (15)$$

$$NO^+ + SCN^- \leftrightarrow NOSCN \quad (16)$$

$$NO^+ + Cl^- \leftrightarrow NOCl \quad (17)$$

In citrate-phosphate solution buffer, the concentration of the nitrites reduces from $1\cdot10^{-4}$ mol/l to $1.5\cdot10^{-4}$ mol/l, while in presence of SCN^- and Cl^- ions, the variation of nitrite concentration reduces from $0.4\cdot10^{-4}$ mol/l to $0.3\cdot10^{-4}$ mol/l and ....accordingly.

The speed for nitrite transformation is greater in presence of SCN^- ions. The speed constant for the reaction (16) ($k_{10.2} = 3{,}4\cdot10^9\ M^{-1}s^{-1}$) is pretty high and thus, the speed of NO_2^- concentration variation is greater than in presence of Cl^- ions with NOCl formation (reaction 17).

The influence of SCN^- ions upon the speed of NO_2^- transformation is determined by pH of the medium. The concentration of NOSCN complex decreases when pH (the interval 1.0-3.5) increases, because of the simultaneous reduction of $[NO^+]$.

The interaction of the reducers with the nitrites

The substrate interact cu nitric trioxide (III), formed from the nitrous acid obtained after the reaction (7). The reaction speed is of 2nd order after the concentration of the nitrite and of 1st order after the substrate concentration:

$$N_2O_3 + S \xrightarrow{k_R} S^+\text{-}NO + NO_2^- \quad (18)$$

$$W = k_{18}[N_2O_3][S] = k_{18}k_6[HNO_2]^2[S]$$

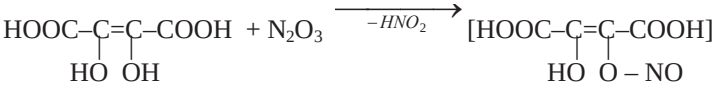
In the case when there are huge concentrations of substrate, the speed of the reaction can be represented via 2nd order after the nitrite and zero after the substrate:

$$W = k'[HNO_2]^2$$

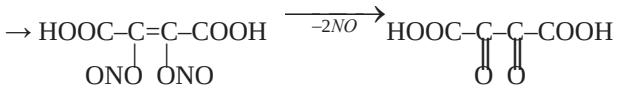
In the system of reaction, DFH_4 acid and DFH_3Na interact cu the formed species (at pH of 2,6, N_2O_3 predominates) and thus, there takes place the reduction of concentration of nitrosation agents.

$$RedH_2 + H^+ + NO_2^- \rightarrow RedH^\bullet + H_2O + NO \quad (19)$$

$$RedH^\bullet + H_2O + NO + H^+ + NO_2^- \rightarrow Red + 2NO + 2H_2O \quad (20)$$



(21)



(22)

The nitrite reducers indicate that the nitrite concentration in the system reduces when their concentration increases. We used (+) catechine and resveratrol to perform the researches (Fig.8.)

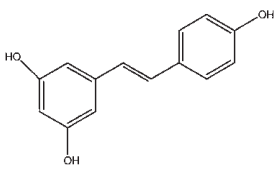


Fig. 8. The chemical structure of the resveratrol

In presence of SCN^- and Cl^- ions, the reducers interact with the nitrosyl NOSCN and NOCl species formed during the reaction (15) and (16), or with other species:

$$RedH_2+NOX \rightarrow HNO_2+Red+2NO+HX+H_2O \quad (23)$$

In the equation (23), NOX represents N_2O_3, NO^+, NOSCN, or NOCl. In presence of nitrosyl species, due to the catalytic effect, the nitrite consumption is greater, if compared with the system where there is only ClO_4^- or citrate-phosphate solution buffer.

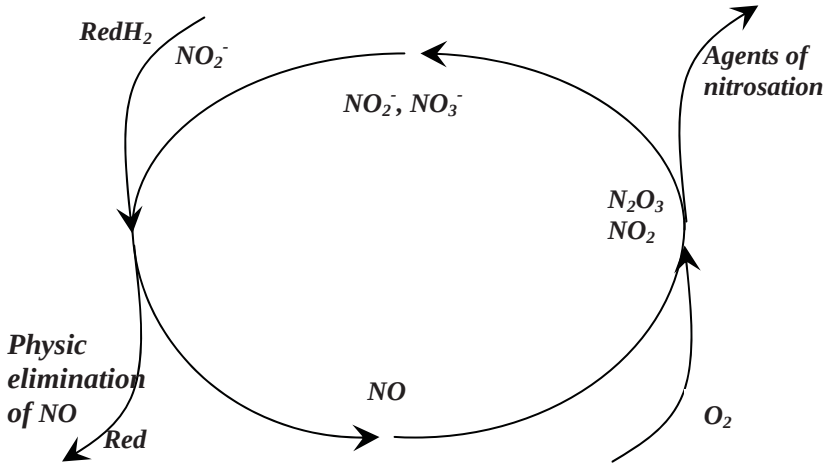


Fig. 9. Cycle of transformation of the nitrites in presence of $RedH_2$ (aerobian conditions).

This indicates that in presence of SCN^-, the reducers can become less effective inhibitors in various processes of nitrosation. The SCN^- ion is a stronger catalyst than Cl^-, this could be explained by the different equilibrium constants of the reaction (16) and (17).

As a result of the interaction of the reducers with different agents of nitrosation, the nitrite oxidation state changes, by reducing up to NO or oxidising up to NO_3^- [20]. The reduction of the nitrite concentration in the system during the reaction under the DFH_3Na or DFH_4 action is determined by the speed of transformation of NO_2^- into NO which further, in the gas phase, is removed. The nitric oxide, formed in aerobian conditions, can be recycled into agent of nitrosation or can be removed via mass transfer. The reduced speed of the mass transfer of NO reduces the inhibitory efficacy of the reducers, because in this case, due to the formation of a cycle, more $RedH_2$ inhibitor is consumed (Fig. 9).

In aerobian conditions, the reaction (11), that leads to the formation of Na_2O_4, will not take place, thus, the NO_2^- concentration, that transforms after the reactions (6) and (7) into nitrosation agents, will decrease.

As a result of the study of the process of interaction between NO_2^- and DFH_4 in aerobian and anaerobic conditions we established that the speed for NO_2^- diminution is greater, in absence of the oxygen. In this case, less DFH_4 is being consumed for the reduction of nitrite at their initial concentration and in the system; the concentration of nitrite reduces considerably.

The increase of the reaction speed simultaneously with pH reduction from 6,0 to 1,0 in Rezv-NO_2^- system is determined by the deviations of the equilibrium, depending on pH in equations (6) and (7) more towards right, thus, in the system, NO_2^-concentration decreases. But in this case, the stoichiometric rapport of the reducers consumed for the nitrite removal will increase.

From fig. 5 we can observe that at pH 1.0 the speed of NO_2^-d concentration is maximum and decreases while pH increases, in the established interval (almost 10 times) In aerobian conditions, the quantity of reducers that will be spent to remove the nitrite will be determined on one hand, by the rivalry between NO recycling and its transformation into nitrosation agent, and the removal of NO via mass transfer, on the other hand.

In conclusions, we may state that the degree of nitrite diminution in the $RedH_2$ – NO_2^- system decreases in the following manner: $DFH_4 < DFH_3Na <$ Resv < (+)Ct.

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