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CONTENT

Effect of magnetotherapy in regeneration after extreme physical activity in the Hungarian Home Defense Sector

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Abstract

Every method which can be used easily in operational and tactical fields and is able to shorten the time required for regeneration can improve the numbers of deployable people, and also their personal security. The increase of the levels of lactic acid caused by extreme muscle work is only mildly intensive among athletes, and can be corrected by aerobe-type physical activity. However, in the defense sector the time that could be used for these activities is very limited, and the possibility of hammered in regeneration during relaxation/sleeping is much more promising. The probably faster decrease of the lactate levels gives theoretical proof to the supposed faster regeneration. As expected, the rate of the change in the serum lactate levels caused by the passive relaxation and the placebo treatment is nearly the same. However, light physical activity causes the lactate levels to decrease significantly faster during the first hour, likewise does magnetotherapy. The results of the weapon assembly test meant to prove practical applicability met our expectations. As the effect of the regeneration with magnetotherapy the results were significantly better in all three groups.

Keywords: lactate recovery, magnetotherapy

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Introduction

The personnel of the defense sector are exposed to extreme environmental effects and harms, exotic illnesses during missions, danger of injuries caused by potential explosives or ABV-injuries, rescue missions in special conditions, encumbrance caused by the use of defensive equipment, systematic vaccinations and medications for prevention, and post-traumatic stress. These are only a few examples of the challenges faced by the disaster medicine that require special theoretical knowledge and practical experience. The most effective way of preserving/restoring the health of the personnel often exposed to extreme danger and completing special tasks is proactive prevention based on constant risk analysis. The planning and implementation of the encumbrance and the time required for regeneration provides the soldiers, firefighters, counterterrorists, and the policemen the ability to complete their tasks at the highest possible standards (and higher dimensions of security) according to their training. However, during tactical operations and deployment in areas of operation appropriate periods of time can't always be provided for resting, which risks both the performance of the command and the health of the soldier. Every method that is able to shorten the relaxation time required for regeneration can significantly improve the quality of the professional staff, and also their personal security.

The electromagnetic field can be considered one of the most important fields of military and technical sciences, but the exploitation of the possibilities of its biological effects definitely can't be considered complete yet. The constantly changing cumulative effect of the encumbrance affecting the organisms of the personnel of the defensive sector offsets the inner environmental stability form the optimum zone, and starts compensating mechanisms constantly. Special encumbrances occurring due to the environment and its effects to the inner environment (gas exchange, salt-water balance, food intake, muscle work, heat control, circadian rhythm, psychic and cognitive balance) mean constant driving force for the research of the new solutions for a more stable homeostasis. The biological use of the new military technology opens up opportunities for clinical usage, the examination of superselected, healthy, moreover professional personnel with extreme encumbrance-physiological parameters. The scientific world reports positive effects of the electromagnetic field (EMF), it doesn't mention negative health-risking events among the people examined in laboratories (Sander et al. 1982.; Ruppe et al.; 1995).

The literature of magnetotherapy reports the improvement of the circulatory system and the increase of the circulation in many cases (Rikk et al. 2013). The latter by itself can mean that the

'oxygen debt' after physical encumbrance can be settled faster, and the metabolic differences occurring on the levels of microcirculatory can be compensated easier.

The increase of the levels of lactic acid caused by extreme muscle work is only mildly intensive among athletes, and can be corrected by aerobe-type physical activity (Kohut, 2008). However, in the defense sector the time that could be used for these activities is very limited, and the possibility of hammered in regeneration during relaxation/sleeping is much more promising. The magnetotherapy doesn't require any extra activity from the professional staff, so sleeping/relaxing on magnetotherapic mattresses can be particularly suitable for acquiring higher levels of regeneration during a given period of time. This way the regeneration time, which is so important in the terms of deployment will be shorter using the passive time of our companions for the acceleration of the restitution.

Methods

We determined the anaerobic barrier and the heart rates of the submaximal and the vita maxima encumbrance of the participants with spiroergometry. Every participant will complete the encumbrance test (according to their levels of fitness) four times. The first time we help the regeneration without any equipment, the second time with light physical activity, the third time with magnetotherapy, and the fourth time with placebo therapy after the restoration of the resting heart rate.

The therapy is done with two identical (A, B) devices provided by the manufacturer. The difference between the two devices is that device B does not generate magnetic field, this device is used for the placebo treatment. The usage of the two devices is identical too (choosing programme, timing, etc.), so the person doing the examination does not know if the real therapy is done with the device A or the device B.

This way we aim to guarantee that the pair is blind, the research is placebo-controlled.

Entry and exclusion criterias

The patients are selected from the professional staff, from soldiers with health appropriate for their age, who are available for service, and are healthy, all in all 17 people.

Entry criterias are age (30-40 years), body composition (body fat 13-17%), level of fitness (maxVO2,…) and blood pressure appropriate for age.

Exclusion criterias are any health problem listed in the contradictions, and passing the limits given in the entry criterias.

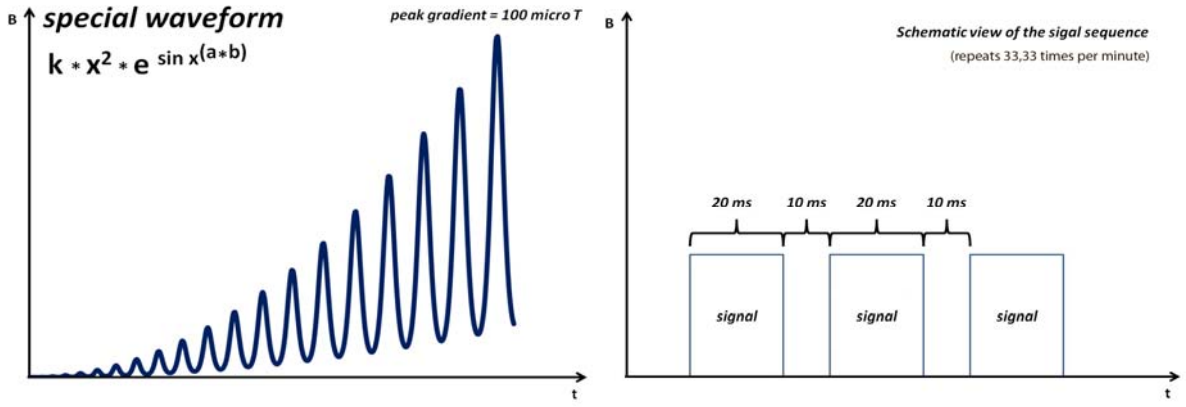
Applied device and the dosage of the treatment

Pulsating signal

The pulsating signal form is used in clinical practice since the 1970s, when the signal sequences follow each other in given intervals, which is a defining characteristic of the signal form. These signal forms were tested for treating many types of illnesses in the past decades. The first therapeutic device approved by the FDA is based on this signal form (Basset et al., 1974).

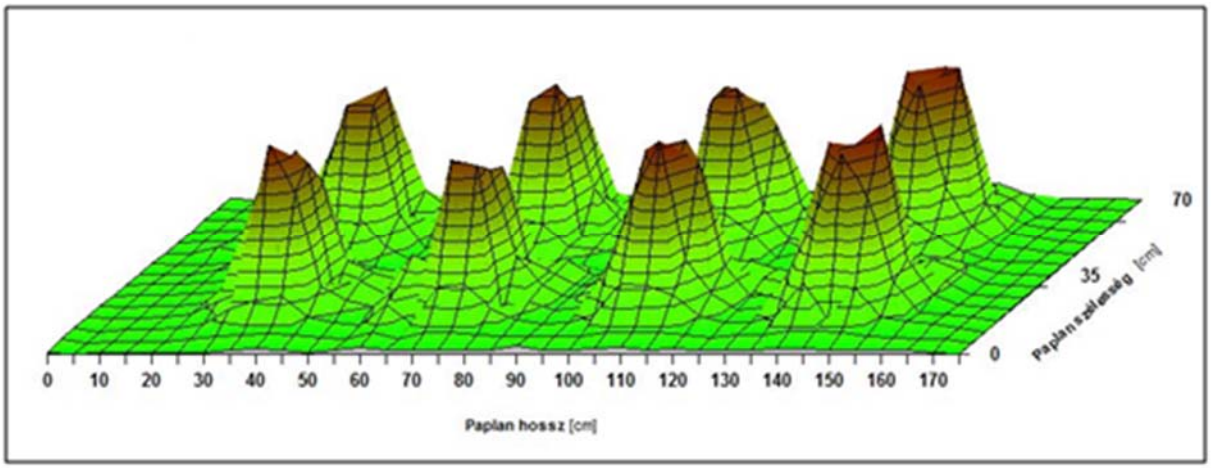
The factors influencing the efficiency of this type of devices are the signal sequences, and the length of the pauses between the signal sequences. Every mentioned device operates in the low frequency territory. The pause between the sign groups provides that the possible increase of heat in the tissue is less than 1 C° during a 30 minute treatment.

The device used for the research operates with a special waveform. It's based on an amplitude (exponentially) modulated sinus wave. Their upper and lower curves are both quadratic equations. Asymmetrical waveform.



The length of the signal sequence is 20 ms, the pause between the sequences is 10 ms. The waveform repeats 33,33 times per minute. The maximum intensity of the signal is 100 microT (the point of measurement is 20 cm above the mattress).

Size of the mattress applicator: 180 x 70 cm, which provides the treatment of the whole body at once. The applicator contains 8 induction spirals.



The applied magneto-therapeutic device is suitable for both clinical and home use.

We used a device with the parameters given above, at maximum intensity for 15 minutes.

Safety considerations

The device used for the examination is certified, it is a II/A class medical device. It has all required permissions. According to the expertise of ÁNTSZ OSSKI the magnetic field generated by the device (as non-ionizing radiation) stays below the exposure limits at all times. The MAUDE an COCHRANE databases don't contain reports of unfavorable events in connection with the device used during the research or its equivalent devices. According to the recommendation of the WHO the maximum of 300 microTesla magnetic fields are not considered harmful for the health. During our previous researches we examined the effect of the expression of the Ku70 gene to the therapy, based on which results we concluded that the device does not cause acute damage in the DNA.

In conclusion, the device can be used safely, without any side effects.

Diagnostic methods, measured parameters, collection of data

Before the start of the research, every participant was examined by an internist-cardiologist; we recorded their actual height, weight, body fat percentage and their cardio-respiratory parameters. The resting blood pressure was recorded before and after the treatment, and the heart rate and peripheral skin surface temperature constantly during the treatments. After every encumbrance

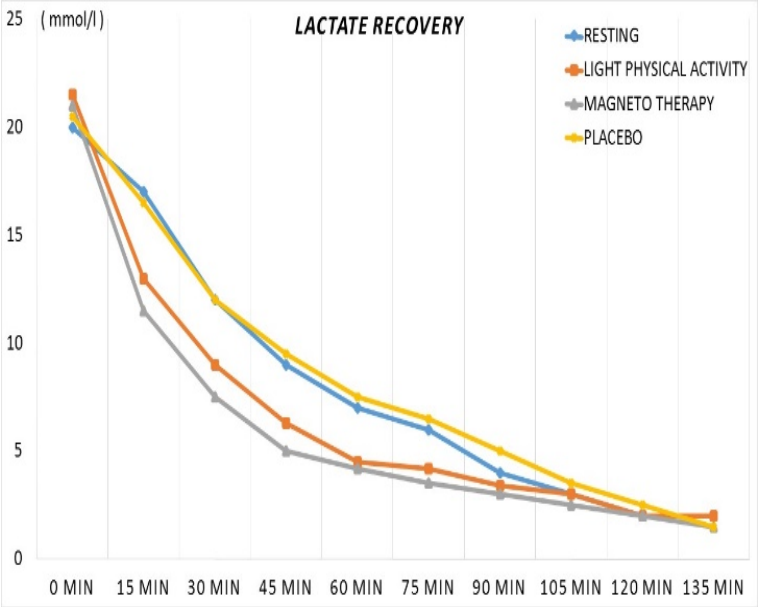
immediately, and then in every 15 minutes for a total of 10 times we measured the level of serum lactate.

The decrease of the lactate levels at an expectable faster rate gives theoretical proof for the supposed faster regeneration. As a proof for the practical usability we made the participants complete a weapon assembly test at the same time after the encumbrance, which was completed significantly faster by the group that regenerated faster. We used the legendary AK 47 Kalasnyikov assault rifle because of its widespread recognition. The record of the dismantling and then reassembling this type of rifle in Hungarian Defense Forces is 18,2 s (Révay Zoltán aircraftman, 1971).

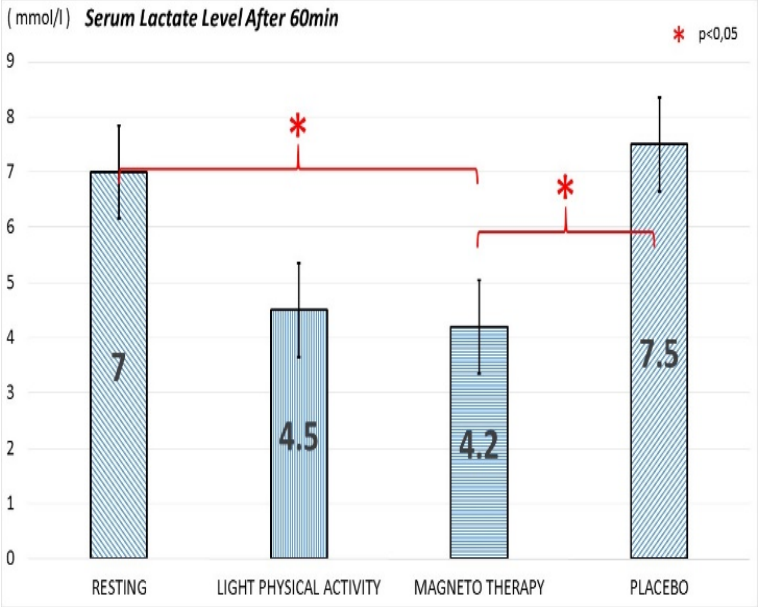
Statistic methods

Using the Statistica for Windows based on the measured parameters we examine the reality of the differences between the groups with variation analysis (ANOVA). We recorded the level of significance at p=0,05.

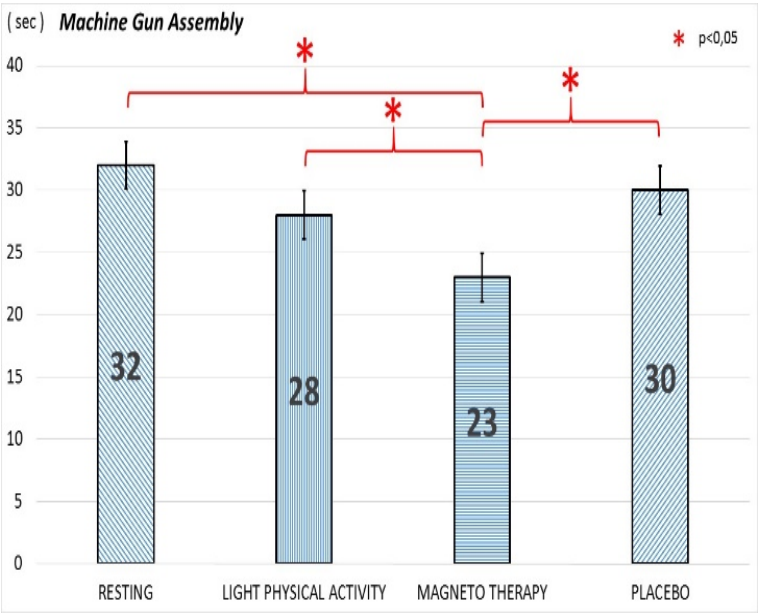
Results



As we expected, the change of the serum lactate levels caused by passive resting and placebo are nearly the same. However, as the effect of the regeneration caused by light physical activity the lactate levels decreased significantly faster during the first hour, likewise as the effect of magnetotherapy. We only measured numeric difference between these two (for the benefit of magnetotherapy).



At the time of the weapon assembly (after 60 minutes of regeneration) there was only numeric difference between the results of the physical activity and the magnetotherapy group (for the benefit of magnetotherapy), but these results were significantly different from the results of the passive resting and the placebo group.



The results of the weapon assembly test met our expectations. As the effect of magnetotherapy treatment we got significantly better results than in the other three groups.

Conclusion

As the effect of intensive effort, such as running, when the energy usage of the body suddenly increases, the lactic acid produces faster in the tissue than it breaks down, thereby the concentration of the lactic acid increases. This is a useful process, because this way the concentration of the NAD+ does not decrease, so the energy investment is sustainable. However, as the effect of extreme encumbrance the pathologically increased levels of lactate are harmful for physical performance. 60% above the maximal encumbrance there is a level, which the body cannot maintain anymore, pyruvate

made from glucose enters the citric-circulatory, without O2 the lactic acid piles up, causing acidosis in the muscles. The increased levels of lactic acid can be decreased in many ways. For example, in muscles with good oxygen supply it transforms into red grape acid, which either goes into the citric-circulatory or in another way with Cory-cycle it transforms into glucose in the liver.

It's a known fact, that the 'oxygen debt' caused by extreme physical encumbrance and the increased lactate levels go back to normal after 6-8 hours, however, if we continue the workout with low intensity, the process will be much faster. Its explanation is that the red muscles participating in physical activity consume large quantities of lactate, thus helping equalizing the 'oxygen debt'.

As the effect of heavy workout the glycogen levels of the muscles decrease. During encumbrances the liver provides the optimal blood sugar levels of the organs with increased catabolism. This causes the decrease of the muscle glycogen, which probably happens because sportsmen can't consume enough calories (carbohydrates) during the regeneration period, which would upload the storages. However, small injuries created during the encumbrance also affect the transport of the glucose to the muscles and also the synthesis of the muscle glycogen (Ebbeling 1989). This is caused by the decrease of the concentration of the glucose receptor protein – the GLUT-4 – in the muscle membrane caused by an injury, or the under-regulation of the mRNS of the GLUT-4 (Richter et al. 1995). Probably the cause of 'heavy legs' is muscle glycogen, which is experienced by many overloaded sportsmen, just like the decreased blood-lactate levels after submaximal and maximal encumbrances (Urhausen 2000). Low glycogen levels, of course, limit the performance.

As the effect of increased oxygen intake caused by workout the ROS production increases, the effects of which are known. It is known, that during the contraction of the muscles ROS also produces in the muscles, and these have a harmful effect for the muscles. During the exhaustion caused by acute workout the malfunction of the sarcoplasmic reticulum and the balance of the calcium is caused by the ROS. The overworking of the muscles cause damage in the muscles and exhaustion, which is partially inducted by the macromolecules damaged by the ROS (Ji 1993). The post-encumbrance ultrastructural damage and the decrease in the integrity of the muscle membrane can be the cause of the other symptoms of over-workout (such as prolonged decrease of muscle strength, muscle pain, damage of the calcium levels' balance, increased damage of the protein).

We must accept the notion that the most important part of today's digital battlefield is the human, because we can't pay the price of the soldier's life in money, but we can do that for any

technical device. We can't put an alterego in our companion's place with the same military career, battle experience, psychological, mental, and physical parameters who could also be inserted to the corps. This sheds light to the true value of any procedure that could increase the deployability, value of battle, and regeneration even with a few percent. In extreme conditions these parameters could decide the fate of the mission or our companions. This work aims to add 1 more percent to the efficiency of the soldiers and thereby the safety of the citizens.

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CARCINOGENIC NITROSAMINES AS FORMALDEHYDE PRECURSORS

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1. INTRODUCTION

Nitrosamines have been found everywhere in the environment. They may to be present in ambient atmospheres, in a large variety of foods, in cigarette smoke condensata, in wheat, in smoked meet, in beverages and in other miscellaneous sources. It has been demonstrated that 1 ppm to 100 ppm levels of nitrosamines are present in the environment and in different consumer products.[1] The nitrosamines are usually formed in food through reaction of an appropriate amine and nitrous acid. The concentration of dimethylnitrosamine (DMNA) especially in fish meal from fish preserved with potassium nitrite has been shown to be proportional to the concentration of dimethylamine and to the square of the concentration of nitrite[2] and the latter is the limiting factor. Nitrates are widely occur in environment particularly in plants and nitrites are readily form from them in the presence of microbes which contains nitrate reductase enzyme systems. The presence of nitrates, nitrites, amines in the upper level of gastrointestinal tract could lead to the in vivo formation of nitrosamines.[3] In addition, many compounds can react as catalyst for nitrosamines formation. Oxidisable phenolic compounds can catalyze the formation of nitrosamines from secondary amines and nitrite at gastric pH.[4] Because, great differences in pH can be found in the biological system the importance of pH in the formation of nitrosamines could play an essential role. The nitrosation of amines can be influenced by the basicity of amine[5] and pH.[6] Experiments were carried out to examine to what extent the different nitrite concentration exist in various diseases of the stomach. In patients with atrophic gastritis the concentration of nitrite and nitrosamines were significantly elevated in comparison with those of all other groups.[7] Nitrosamines are formed during processing and smoking of tobacco products. Protein, agricultural chemicals and alkaloids in tobacco serve as major precursors for volatile, nonvolatile, and tobacco specific nitrosamines. These compounds show the highest concentration of any group of carcinogens in cigarette mainstream smoke.[8]

the other alkaloids. Different drugs containing dimethylamine groups have been shown to react with nitrite producing dimethylnitrosamine. Nitrosamines are present all over in urban atmosphere[9] and could have been washed out of the air into the soil. It appears to be stable for about 30 days in soil before it starts to disappear slowly[10-11].

In this chapter we will discuss the metabolic pathway of dimethylnitrosamine and other nitrosamine, the formation of formaldehyde which may play an important role in the genotoxicity of dimethylnitrosamine and other nitrosamines and the aminoacetonitrile action on the carcinogenesis of these compounds.

2. METABOLIC ACTIVATION OF NITROSAMINES

Over 300 nitrosamine compound have been tested for carcinogenic activity, and majority are carcinogenic. It has been observed by Barnes and Magee[2] than Magee[12] that dimethylnitrosamine could induce malignant liver tumor and must be metabolically activated before it becomes hepatoxic and that metabolite or metabolites may be responsible for carcinogenicity. Magee showed that dimethylnitrosamine was cleared rapidly from the bodies of rats, mice and rabbits with very little being excreted in urine or the feces. Later Magee and Farber[13] showed the formation of 7-methylguanine by DMNA on rat liver DNA and kidney RNA. The investigation of dimethylnitrosamine metabolism showed that it follows the common pathway for the metabolic activations to ultimate carcinogen (Fig. 1.).

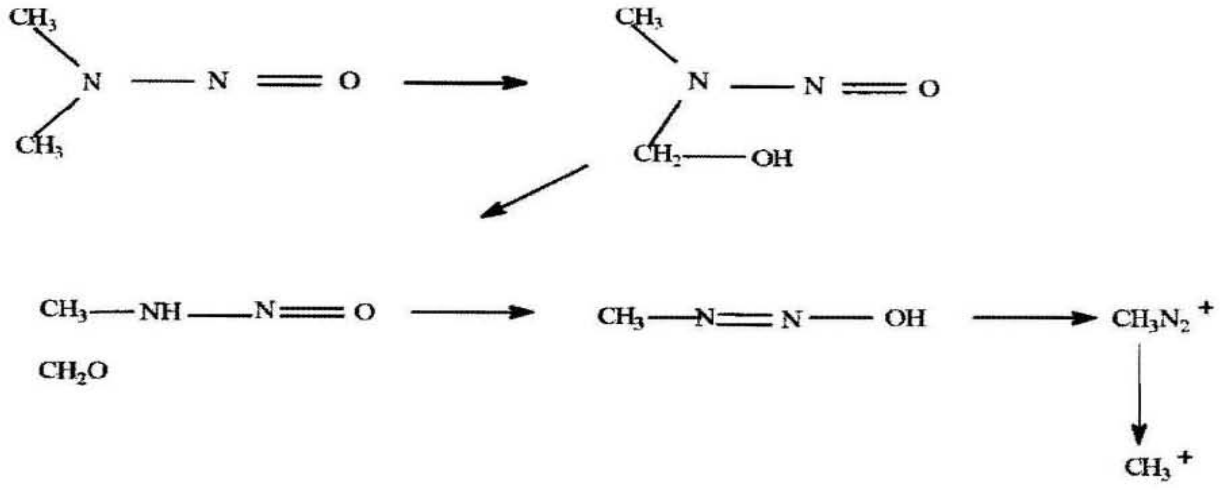


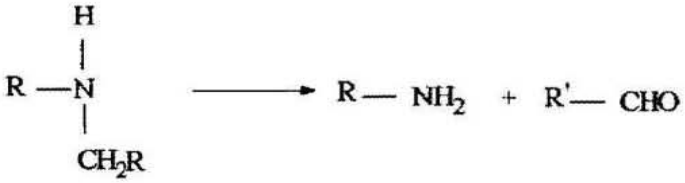
Figure 1. Metabolism of DMNA

The initial step in the metabolism α -hydroxylation which is mainly catalyzed by microsomal cytochrome P450 (CYP) mixed function oxidase. The α -hydroxynitrosamine has a very short life time under physiological conditions and spontaneously cleaves to yield formaldehyde and alkyldiazoniumhydroxide[14]. In this hypothetical scheme the diazohydroxide in the cytosol is in equilibrium with the very

unstable alkyldiazonium ion which can react directly with cellular nucleophyles or lose nitrogen to produce a carbonium ion[15]. The cationic species may then react with water to form alcohols or with cellular nucleophyles to for alkylated products. Investigation by Lijinsky and coworkers[16] with deuterium labelled nitrosamine have shown that alkylation of nucleic acids does not proceed via formation of diazomethane as an intermediate and that a carbonium ion is formed directly from the demethylated moiety of dimethylnitrosamine. The initial CYP enzymatic hydroxylation is a crucial step in the bioactivation of nitrosamines to „ ultimate" carcinogenic form .Lijinsky supposed that CH_3^+ will go intact to DNA guanosine.

3. CYTOCHROMES P450 (CYP) ENZYMES FAMILY

The CYP comprises a vast „superfamilies" of enzymes[17] which contribute to the synthesis of macromolecules and several families in animal species concerned in the oxidative detoxication of drugs and other xenobiotics. Other families also found in animals which are concerned in the oxidative metabolism of endogenous substrates. One part of the P450 family appears to have some unique role in reproduction and development others are concerned with the oxidative detoxication of animals of many man-made chemicals. A great part of CYP enzymes likewise show specificity for different substrate. However, all P450 have the same oxygen activating system enabling these enzymes to insert oxygen into a variety of different compounds. CYP has subsequently been found in many organs and tissues of different animals. Over 230 individual CYPs have been characterized according to their protein sequences and form of these enzymes appears to be present in all biological systems. There is a large number of different CYP with widely varying substrate specifities even between the forms present in the same species, so it is likely that, given innumerable variety of CYP substrates more than one oxygenating intermediate is possible. The major mammalian CYP families, which are very important in the metabolism of nitrosamines, show the following main conversion (Fig 2.)

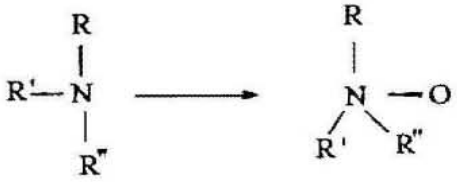


N— dealkylation

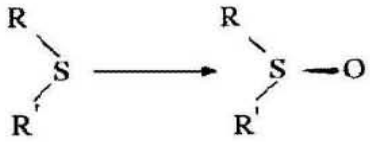
O—dealkylation

aliphatic carbon hydroxylation

S—dealkylation



N— oxidation



S— oxidation

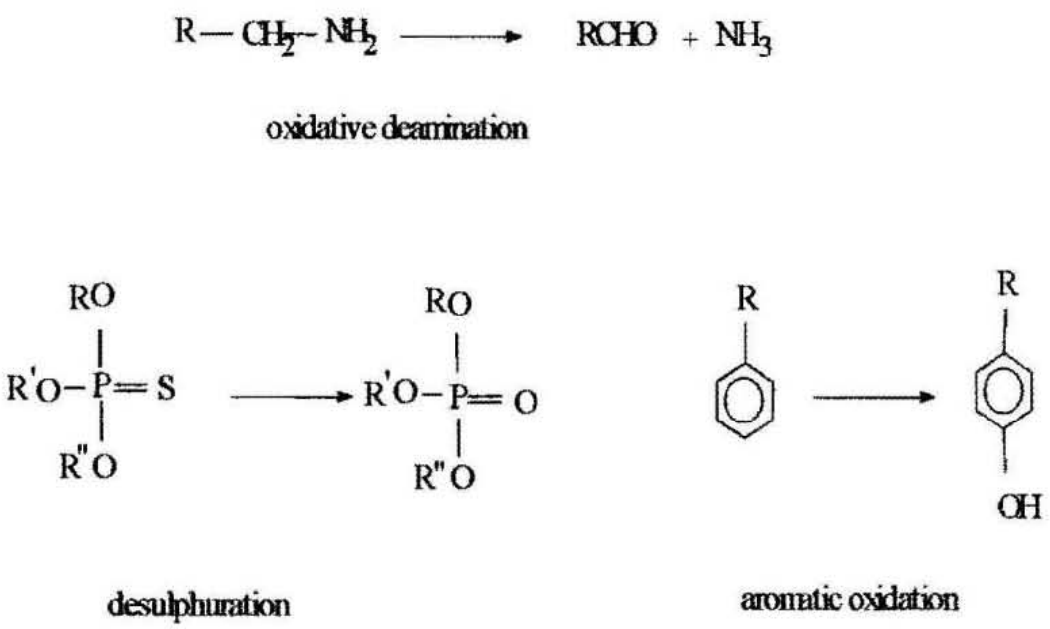


Figure 2. The main CYPs mediated conversions.

More than 60 different varieties of enzymic reaction are known to be catalyze by CYP of whichever 300 genetically distinct isoforms have now been sequenced[18] that are capable of metabolizing hundreds of thousand of different chemicals[19].

Studies on the enzymology of dimethylnitrosamine show that mainly CPY 2E1 subfamily are the most important isozymes in the metabolic transformation.

The CYP 2 family comprises 10 subfamilies A to H, J and K of which the first five are all present in mammalian liver, but in different amount and with different inducibilities.[20-24] These five subfamilies show varied substrate specificities with some degree of overlap, particularly between the CYP 2B and CYP 2C.

Dimethylnitrosamine is CYP 2E1 substrate.[25] CYP 2E1 may be induced by starvation or fasting, low calorie or high fat diet uncontrolled diabetes, excessive alcohol consumption, exposure to halogenated chemicals, anesthetics.. Being predominantly in the high-spin form, CYP 2E1 will activate oxygen in the absence of substrate giving rise to sensitive oxygen species which are themselves potentially toxic, carcinogenic, resulting in oxidative stress, chronic inflammation, malignant transformation. All compounds or events which stimulate CYP 2E1 activity should consequently rigorously avoided. In addition, when CYP 2E1 is highly active because of exposure to ethanol[26] can lead to high losses of other CYPs which are very important in other detoxification mechanism.

4. PRODUCTION OF FORMALDEHYDE IN THE METABOLISM OF NITROSAMINES

Dimethylnitrosamine is the most intensively studied in the large family of carcinogenic nitrosamine. It was established, that formaldehyde is the major product

among the several generated metabolites by the enzymatic reactions[27]Formaldehyde originates not only from dimethylnitrosamine but from other nitrosamines compounds in the microsomal enzymatic processes. In the literature many kinetic studies of oxidative dealkylation of dimethylnitrosamine to formaldehyde have been reported. Czygan et al.[28] studied in the microsomal metabolism of dimethylnitrosamine and the CYP dependence of its activation. Later Lake et al.[29], Chau et al.[30], Jensen et al.[31], Farrelly and Stewart[32] carried out kinetic studies on the formaldehyde production in the metabolism of dimethylnitrosamine and other nitrosamine.

Table 1. shows different nitrosamines which produce formaldehyde during metabolism All these nitrosamines with the exception of nitrosomethylpropylamine exhibited simple Michaelis – Menten kinetics and the chain length of the second alkyl group appeared to have a profound influence on the metabolism on the oxidation of methyl group to formaldehyde [32].

Nitrosamines have been shown to be carcinogenic in 39 species of experimental animals. The similarities in the activation of the compounds by animal and human tissues culture in vitro and the formation of same DNA adducts provide evidence that nitrosamines are also human carcinogens.

Table 1.Formaldehyde producing nitrosamines

Compound	Relative productions rate of formaldehyde
DMNA	1.00
MENA	1.50
PrMNA	3.80
BuMNA	1.60
PeMNA	0.96
HexMNA	0.55
HepMNA	0.40
CyHexMNA	0.51
BzMNA	0.51
PhEMNA	0.81
NeoPeMNA	0.92
TFEMNA	3.80

It is generally accepted that oxidative demethylation of dimethylnitrosamine in mediated via NADPH dependent mixed function oxidase system, and during the oxidation a reactive species is generated which is responsible for mutation and carcinogenesis [33] DNA methylation does occur during this oxidative dealkylation of ($^{14}CH_3$) – dimethylnitrosamine by hamster liver microsomes.[31] Loveless[34] showed the relevance of

O^6 – alkylation of deoxyguanosine to the mutagenecity and carcinogenicity of nitrosamines It was established in mild acid hydrolyzed DNA with HPLC the presence of methylated bases which were identified as $O^6 - CH_3$ – guanine, $7 - CH_3$ – guanine and $3 - CH_3$ – adenine. Similar results have received Magee at al.[33] and Pegg[35] in their experiments with in vivo of DNA by dimethylnitrosamine as well as the in vivo and in vitro alkylation by methylnitrosourea.

The "ultimate" carcinogen derived from nitrosamines is considered to be an alkylating agent and this may be a carbonium ion. Though there is a more extensive alkylation at N – 7 – guanine in DNA, there is a more important correlation between O^6 – alkylguanine formation in carcinogenesis. The presence of O^6 – alkylguanine in DNA is responsible for the initiation of the carcinogenic process by nitrosamines and other nitroso-compounds

5. Inhibition of Dimethylnitrosamine Carcinogenesis by Aminoacetonitrile

In the studies of the inhibition of dimethylnitrosamine carcinogenesis it has already demonstrated that aminoacetonitrile (AAN) prevents the liver lesions induced by dimethylnitrosamine by hindering the enzymatic demethylation of this compound[36-38]. However, one could not exact demonstrate that this protective action of aminoacetonitrile is due solely to this mechanism. Previously showed by Wawzonek et al[39] that aminoacetonitrile inhibit the production of cirrhosis in the liver in rats by chronic administration of carbon tetrachloride. Among the earliest biochemical changes after a dose of dimethylnitrosamine sufficient to cause liver necrosis inhibition of protein synthesis in the liver[40]. Aminoacetonitrile administration was shown to prevent this early inhibition of hepatic protein synthesis[41]. It was found by Fiume and Roffia[37] that the concentration of dimethylnitrosamine in the liver of rats treated with aminoacetonitrile was higher than that when the animals received the nitrosamine alone. This finding supported the hypothesis that metabolism of dimethylnitrosamine was inhibited.

Dimehtylnitrosamine is metabolized in vivo and in vitro through the first step of oxidative demethylation by CYP (subfamily mainly by CYP 2E1) which leads to production of formaldehyde. In spite of the fact that various aldehydes are formed during bioactivation of N— nitrosamines their carcinogenic potency has not been studied extensively. It was demonstrated by Albert et al.[42] that inhalation of formaldehyde which is the metabolic product of dimethylnitrosamine can induce various nasal cavity tumors in rat and mice. Grafstrom et al.[43] have observed DNA protein cross – links, DNMA single – strand breaks and inhibition of the ligation step of DNA repair on the effect of formaldehyde. Quantum chemical calculation that S_{N2} nucleophylic attack on the alkyl diazonium ions formed from DNA is more likely than the involvement of carbocation intermediates[44]. Studies of the methylation of rat liver DNA after i.p. administration of deuterated dimethylnitrosamine have been ruled out the formation of diazomethane as the alkylating intermediate derived from dimethylnitrosamine.[45]

With the aim to established the possible reaction of formaldehyde formed during the metabolism of dimethylnitrosamine and the role of aminoacetonitrile on this reaction we have carried out investigation in guinea pig liver homogenate. Fig.3 shows the reaction between formaldehyde and AAN and Table 2 represents ^{1}H-NMR dates of the reaction mixture.

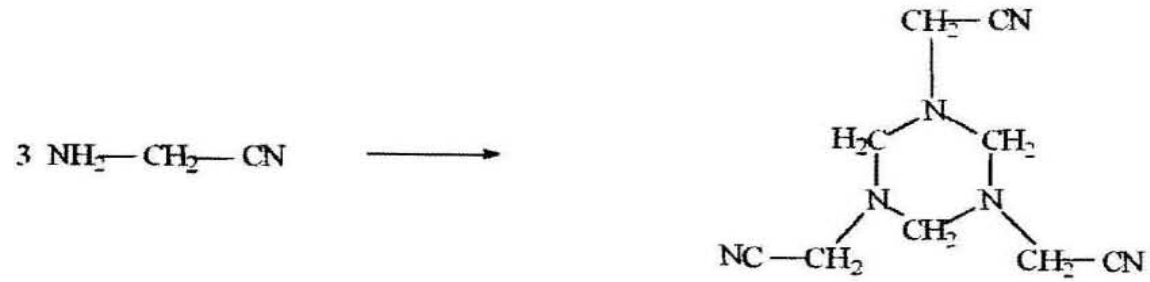


Figure 3. Formation of MAAN in the reaction of formaldehyde with AAN.

1 g guinea pig liver was homogenized by 0,15 mol/ KCl solution and the suspension (3 cm^3) was centrifuged at 10000 g for 10 minutes. 1 cm^3 of the supernatant was diluted with 9 cm^3 PBS (pH: 7.4). Ten µl of N,N – Di(^{14}C)-methylnitrosamine was added to 1.0 cm^3 diluted liver homogenate.(Specific radioactivity: 555 MBq/mmol, 15 mCi/mmol). Radioactive concentration of the solution: 1.85 MBq/cm^3, 50 µCi/cm^3.). To the mixture 500 µl of water solution of aminoacetonitrile. H_2SO_4 was added and then it was incubated at 37 ^{0}C. After each 1 hour 20 µl of the mixture was applied on Fixion 50x8 cationexchange thin—layer sheet and chromatogram was developed in a solution of 50 g citric acid, 30 g NaOH , 7 cm^3 37 % HCl in 500 cm^3 of water. The separated compounds could be identified by ninhydrin reagent solution after drying the thin –layers at 105 ^{0}C temperature. The distribution of radioactivity on the thin layer sheet was determined by LSC. Each 0.5 cm of the sheet were rubbed off the layers and extracted with 4 cm^3 of 50 % EtOH and mixed in Clinisosol™ scintillator (Inst. of Isotopes, Hung. Acad.Sci.) and their radioactivity was measured in Berthold BF 5000 and Packard TriCarb 3390 LSC respectively.

In vitro experiments were also carried out to study of the reaction in water solution between AAN and formaldehyde. 1 mmol of aminoacetonitrile was reacted in 2 cm^3 water with 2 mmol formaldehyde at 37^{0}C for 10 hours. In each 2 hours 20 – 50 µl of the reaction mixture was analyzed on Fixion-50x8 thin layer sheet. The chromatographic analyses were carried out as mentioned above. For the assessment of formaldehyde production, the liver homogenated system was used without AAN. The analyses were carried out by our radiometric method.

Five compound could be identified by TLC analyses of the incubation mixture on Fixion-50x8 sheet glycine, AAN, MAAN, in small quantities methylglycine (sarcosine) and methylaminoacetonitrile(sarcosinnitrile, SAN) The main metabolite was MAAN. Studying the reaction of formaldehyde on AAN in vitro could be established that AAN reacted immediately with formaldehyde while MAAN was formed (Fig. 3). The reaction of formaldehyde on AAN first was studied by Klager[47]. According to NMR investigation MAAN was as a trimer product possessing the cyclic structure not a Schiff base (Fig. 3 and Table 2) and in acidic medium formaldehyde could be released and by dimedone could be determined from the metabolite. After 10 hours 35 % of the total radioactivity could be found in MAAN. At an advanced stage of the reaction of formaldehyde on AAN could be identified SAN respectively. On the TLC glycine be also identified which was originated from the hydrolysis of AAN during the chromatographic separation. The same

metabolites could be discovered in the investigation with liver homogenate as in the examination of the reaction of formaldehyde on AAN in vitro. MAAN was also prepared by the method of Johnson and Rinehardt[48] and it was chromatographed together with the incubation mixture of liver homogenate for the identifying the metabolite by this process also.

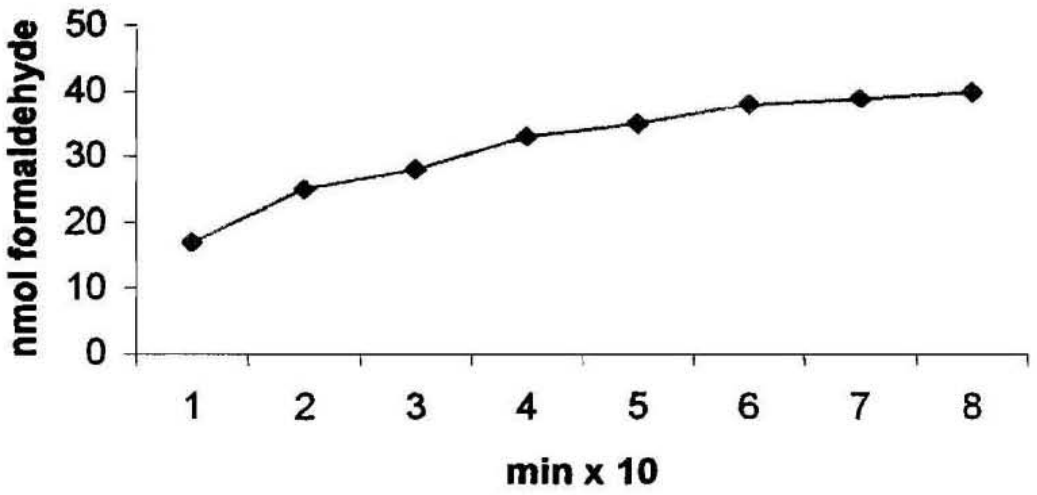


Figure 4. Formation of formaldehyde from DMNA($^{14}CH_3$) in the incubation mixture containing liver homogenate

Table 2. ^1H – NMR data of the reaction mixture of CH_2O on AAN in D_2O. 2 mmol aminoacetonitrile was reacted in 25 cm^3 D_2O with 4 mmol formaldehyde at 37^{0}37 for 10 hours. The reaction mixture was analyzed with JEOL Fx – 100 NMR spectrometer. The NMR spectrum was correlated with standard methylenaminoacetonitrile (MAAN) NMR spectrum.

Compound	^1H – chemical shifts ppm
N–CH_2–C	3.55
N–CH_2–H	3.81

6. Discussion

Investigating the metabolism of DMNA it was concluded that DMNA is not itself hepatotoxic but the methylating intermediate formed during metabolism account for toxicity[49-50] It was proved by Magee[40] already in 1958 that the first biochemical change brought about by DMNA administration is the inhibition of protein synthesis in the liver. Later Fiume[41], then Mager[51] et al demonstrated that AAN is capable of preventing the inhibition of DMNA protein synthesis in such a way that AAN inhibits the metabolism of

DMNA. This statement was based on the fact that higher DMNA concentration was found in the liver of animals treated with AAN than in those not treated. On the other hand it was observed by them that due to DMNA treatment the extent of DNA and RNA methylation of the liver of rats treated with AAN was much lower than that of animals not treated. From this it was again concluded that DMNA metabolism was inhibited by AAN, as a consequence of which the required metabolic activation and during that formation of methylating intermediate occurred only partially. Studying the oxidative demethylation of DMNA by microsomal fraction of liver Jensen et al.[31] established that the formaldehyde produced from DMNA correlated with DNA methylation, the rates of DNA methylation and of formaldehyde production are very similar suggesting a common rate limiting step for both of these processes. In the presence of AAN the relative quantity of 7- methylguanine was practically zero with a minimun of formaldehyde formation (3% of control). Similar results were obtained by them in experiments carried out with diethyldithiocarbamate but in this case no formaldehyde and no 7-Me-guanine could be detected. By CO addition to the incubation mixture the formation of formaldehyde and 7-Me-guanine could also be inhibited. These results were interpreted as evidence that all these substances are potent inhibitors of DMNA metabolism. In our experiments with homogenized liver it was clearly shown that the activated formaldehyde produced in the oxidative demethylation metabolism of DMNA reacted on AAN and mainly trimeric MAAN was formed. Its radioactivity could only originate from $^{14}CH_2O$ produced from DMNA metabolism and not from methyl cation. In the determination of $^{14}CH_2O$ content of MAAN in acidic medium namely formaldehyde could be released and by dimedone could be determined from the metabolite. In a small quantity of formaldehyde also reacts with AAN and sarcosine and methylaminoacetonitrile was formed by direct spontaneous methylation with formaldehyde. The reductive methylation on AAN by formaldehyde cannot exclude in the biological medium.

Earlier the „ultimate" carcinogen derived from nitrosamines is considered to be as an alkylating agent the methyl cation.[52,53,45,54] However studies of the alkylation step in vivo and in vitro are hampered by the short life time of many of the intermediates of nitrosamines and CH_3^+ may not be the alkylating agent forming immediately CH_3OH. Other theoretical consideration suggested that diazonium ions formed in the metabolism of nitrosamines are the most likely alkylating agents.[55, 44, 56]. However, diazonium ions react immediately with the solvent in the biological system forming $CH_3OH_2^+$ which is not as reactive as a methylating agent on nucleotide. Spontaneous methylation and formylation reaction proceeding simultaneously between L – lysine and formaldehyde, glycine and formaldehyde in vitro have been reported by Thihák et al.[57], Trézl et al.[58]. In biological systems N^{ϵ}- methyl- L-lysine can also be formed with formaldehyde derived from formaldehyde precursors from DMNA [59-60]. Significant quantity of ^{14}C – labelled formaldehyde and 1-methylhistidine (80 %), N^{ϵ}-dimethyl – L – lysine /12 %), N^{ϵ}-trimethyl – L – lysine(8 %) were detected by Turbeville and Cradock in the liver of rats injected with ^{14}-C-labelled DMNA already two hours after injection. When they used 14C-methionine as precursors more radioactivity was present in dimethyl-lysine then in methyllysine. It was reverse in histone treated with ^{14}C – DMNA and the incorporation of the radioactivity was much higher in monomethyllysine than dimethyl- and trimethyl-lysine However, when sodium (^{14}C) formate and ^{14}C-DMNA were used as precursors a much grater proportion of the radioactivity of liver histon is recovered as serine than in the case of ^{14}C-methionine labelling. The high radioactive incorporation into serine could

be resulted only from high amount of formaldehyde formed. The high radioactivity in monomethyl-lysine is the consequence of spontaneous methylation by activated formaldehyde. Although it was assumed by the authors that formaldehyde had to play a role in methylation they did not find any evidence to prove it. Due to this they only stated that the released formaldehyde was built into the one carbon pool.

Hundreds of different organic compounds were described by Sawicky and Sawicky [61] as formaldehyde precursors from which a significant quantity of formaldehyde can be released by demethylase enzymes under in vitro and in vivo conditions. Although reactions of formaldehyde with amino acids, proteins, nucleic basis and other organic compounds are also discussed in different papers, but they did not suppose the spontaneous N – methylation reaction.

The results of the spontaneous methylation experiments with formaldehyde support the assumption that formaldehyde cannot be excluded from the methylation reaction of DNA and from the initiation of malignant transformations. Similarly to the others we have studied also formaldehyde production from DMNA in liver homogenate. The analyses were carried out by dimedon method. The measurement data showed that the hydroxylation of α-carbon atom and the formation of formaldehyde approaches to a limit value during 90 minutes which showed a rapid decomposition pathway of DMNA relatively.(Fig. 4.)

It is well known, that CYPs play an important role on metabolism in several aspects of cancer. CYP 2E1 is primarily responsible for the bioactivation of many low molecular weight carcinogens and is involved in the metabolic oxidation of carcinogenic nitroso compounds including nitrosamines.[62] DMNA is a substrate for CYP 2E1 and all likelihood induces CYP 2E1 being predominantly in the high – spin form will activate oxygen giving rise to reactive oxygen species. The kinetics and thermodynamics of various stages in the catalytic cycle have been studied in different microsomal systems and show broad similarities suggestive of a common mechanism.[63-64].

In the catalytic cycle, the first stage is the substrate binding which is rapid and of high affinity. The second stage is the transfer of the first electron from reductase or a redoxin. The latter is in itself made up of three separate stages involving electron transfer from NADPH to reductase from reductase to redoxin and finally from reduxin to CYP. Apparently, the binding of redox component to CYP lowers the redox potential[68] which suggest that there is some cooperation between the binding interaction and electron transport pathway. The next step is the rapid binding of molecular dioxygen to the high – spin iron(II)CYP- substrate complex. The resulting oxyferrous CYP complex autoxidies biphasically in a first-order process forming in this instance peroxide rather than superoxide, although the latter may be a precursor as the dismutation of superoxide to peroxide is well known.[65] There is also evidence for the production of hydrogen peroxide in the breakdown of the reduced oxycitochrome CYP complex especially where substrate oxygenation is unfavorable.

In the 5. stage the mechanism by which the oxygenated CYP substrate complex breaks down to form products. A number of mechanism have been proposed for the breakdown of iron peroxy complex.[67] There is varying support for these mechanism and it is possible that a different one may operate depending on the circumstances, such as type of substrate or CYP isozyme. Studying the nature of the oxygenating species there is some circumstantial evidence for the existence of an iron oxen (Fe=O) intermediate in CYP. It was demonstrated also in model system. The Fenton reagent ($Fe(II)_2$ H_2O_2)

appears to show some of the characteristic of CYP – mediated oxygenation of carbon substrate and organic peracids are also able to reproduce approximately the same reaction products depending on the nature of the chemical. It is thought that the Fenton reaction generates hydroxyl radicals:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

Whereas a hydroperoxy species has been implicated in other model systems. The active oxygens species may differ from one substrate to another or between different CYP systems. As we have seen oxidative demethylation processes of nitrosamines formaldehyde is the major product which can react with peroxide and reactive oxygen species originated from CYP 2E1 reaction building excited formaldehyde molecules.

We have found in our experiments with peroxyde formaldehyde-^{14}C and L-lysine that this excited biradical formaldehyde could rapidly methylate the amino acid and same quantity of methylated L-lysine was produced during 1 minute like in the reaction mixture without hydrogen peroxyde during 24 hours.[68-69] This reaction was accompanied by the emission of chemiluminescence which was measured by LSC. Singlet oxygen is also generated from the reaction system which was proved by Lichszteld and Kruk.[70] They also proposed two hypothetical reaction mechanisms, one of which possibly resulted in excited CH_2O molecules in alcalic medium. (Fig. 5). Trézl and Pipek[71] simulated the liberation of excited formaldehyde in a model reaction similar to that of Lichszteld and Kruk but modified to real biological circumstances. In this case the measured chemiluminescence intensity was very low but adding L-lysine as a third component to the reaction mixture increased the intensity by a factor ca. 65. In the chemiluminescence spectrum measured values agreed fairly with the theoretical calculations for the $^3A_2 \rightarrow {}^1A_1$ transition These data show that at pH 7.4 in the model biological reaction, besides the ground state formaldehyde excited triplet formaldehyde molecules can also be formed in the presence of L-lysine. Trézl and Pipek proposed a transition mechanism (Fig. 6) In this way in the model reaction partly ground state formaldehyde and partly excited triplet formaldehyde molecules are liberated.

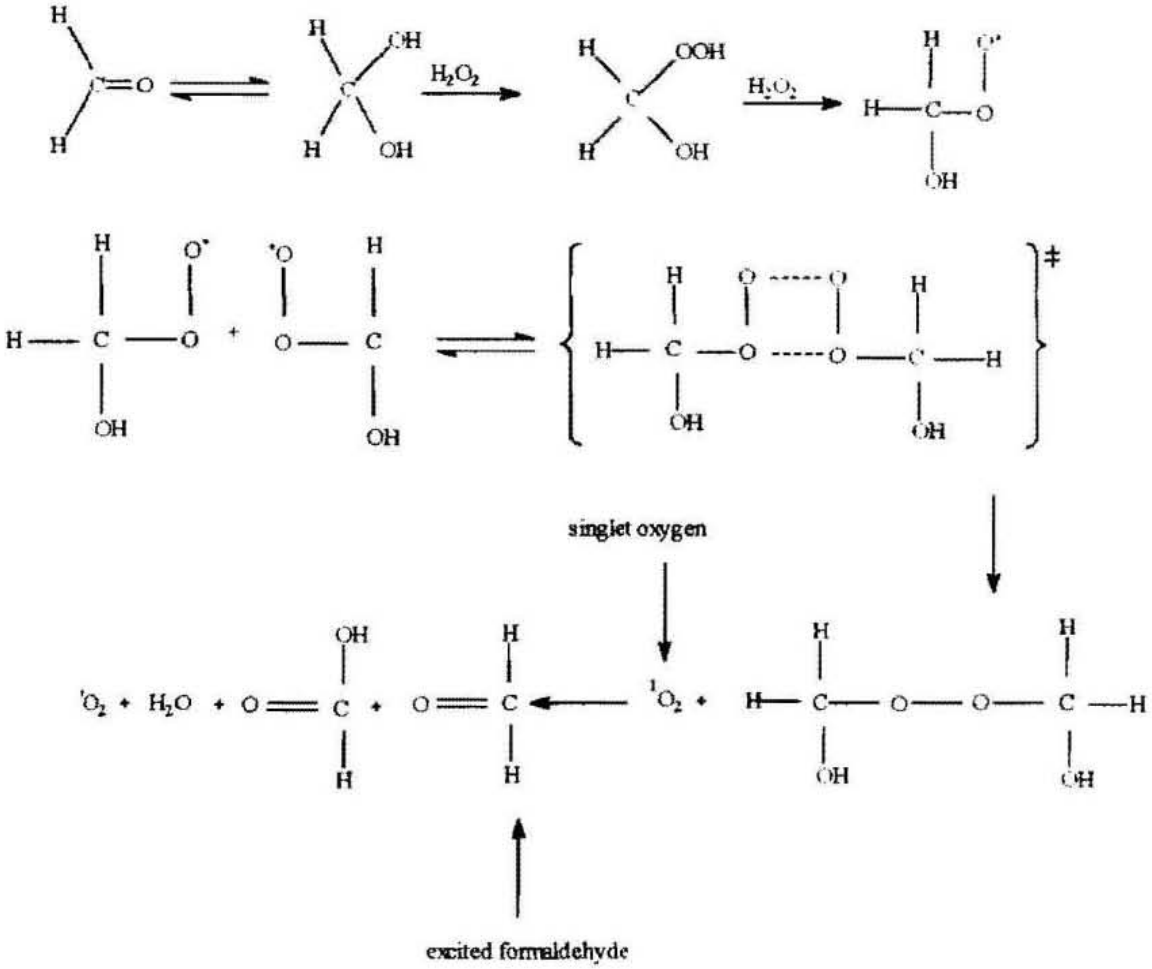


Figure 5. Formation of singlet molecular oxygen and excited formaldehyde during oxidation of formaldehyde by peroxide

Other experiments showed that there is a great difference in the reactivity between the two states of formaldehyde. Johansson and Tjalve[72] injected ^{14}C - formaldehyde and ^{14}C-DMNA intravenously into mice and investigated the radioactivity distribution with whole-body autoradiographyc measurements. It was found that the radioactivity from ^{14}C-DMNA remained in huge quantities in the liver, where excited ^{14}C-CH_2O was liberated, while the non-excited ^{14}C-CH_2O dispersed through the whole body. Since CH_2O is much more reactive in the excited state and can attack the cellular components of tissues (proteins, DNA, RNA, etc.) It was demonstrated also by Duran and Faljoni[73] that singlet oxygen was formed in the demethylation process of nitrosamines and the reaction was accompanied by the emission of chemiluminescence. The methylation process of N^{ε} - L- lysine by excited formaldehyde may confirm the assumption that this activated molecule can take part in the O-methylation of nucleobasis guanine.

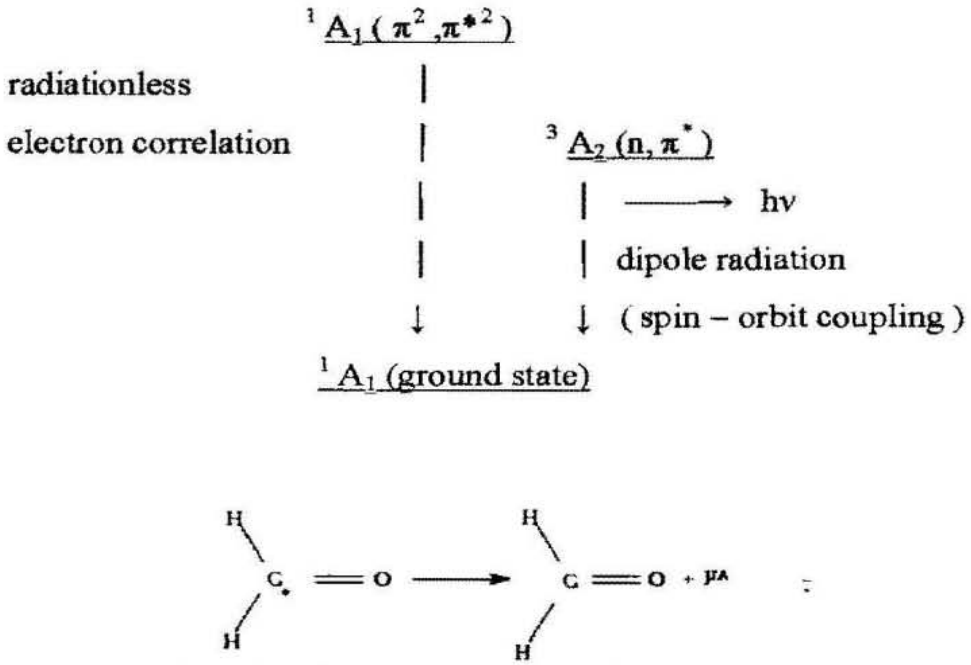


Figure 6. Excited formaldehyde. Proposed transition mechanism of the reaction intermediate of the model reaction.

Our experiment carried out with AAN on DMNA metabolism suggest that DMNA is not inhibited by AAN at least in time as otherwise no metabolites could have been detected on the chromatogram. The appearance of the radioactivity of trimeric MAAN and other metabolites shows that the metabolism of DMNA started during the analysis. AAN as well as DEDTC react rapidly with formaldehyde, consequently - in our opinion – Jensen et al. could not have found formaldehyde in their experiments as in those condition formaldehyde was captured by AAN and DEDTC so it was not possible to detect formaldehyde by dimedone. The low yield of 7 –CH_3-guanine in their experiments was due to the elimination of excited formaldehyde as mehtylating agent by AAN and DEDTC immediately. CO exerts its action through the iron complex . The Fe(II) state exhibits high affinity for oxygen as well as for CO. It is known that hem iron in the Fe(II) state is a good π-donor which facilitates the strong binding of π-acceptor ligands such as oxygen, carbon monoxide and nitric oxide. When the iron atom is out – of – plane as it will be in high – spin – form, one can assume that this affinity for small σ - donor/ π - acceptor ligands is greater.[65] Accordingly, CO is also able to bind rapidly to reduced CYP but at a slightly lower rate ($K= 10^4 – 10^6\ M^{-1}s^{-1}$) than, and about a tenth of the affinity of oxygen. Pegg[74] investigated the inhibition of the alkylation of nucleic acids and of the metabolism of 1,2–dimethylhydrazine by AAN. He demonstrated that pretreatment of rats with AAN inhibited the metabolism of 1,2-dimethylhydrazine-^{14}C to $^{14}CO_2$ and increased the expiration of azomethane- ^{14}C. Our opinion, these data support our results that AAN does not inhibit the oxidative demethylation of 1,2–dimethylhidrazine because azomethane-^{14}C and formaldehyde-methylhydrazone-^{14}C are formed which readily forms formaldehyde^{14}C. The measured decrease of $^{14}CO_2$ resulted due to the reaction of AAN with formaldehyde–^{14}C because the formaldehyde → formate → CO_2 was inhibited.

It should be established, that little attention was paid to the formation of formaldehyde produced in DMNA metabolism and to the possible reactions of formaldehyde, because it was accepted that formaldehyde enter into the normal process of one carbon pool, where formate then carbon dioxide are formed. Only an „ultimate" carcinogenic carbonium ion was considered to be responsible for the initiation of malignant transformations due to mainly the O – methylation. Based on our results, the role of „activated, excited" formaldehyde cannot be neglected in this methylation process.

The Fig. 7. represents the possible mechanistic cycle for aliphatic hydroxylation, formation of formaldehyde and the effect of AAN on the system. AAN has two action on the inhibition of the nitrosamine carcinogenesis. First, it can react rapidly with the formaldehyde and form trimer MAAN. On the other hand AAN eliminates likely the reactive oxygen species generated by induced enzyme CYP 2E1 as we could established Fenton reaction and peroxidase peroxide system.[75] It was also identified in the quantum chemical study of relative reactivities of AAN[76] number of nonbonding electrons as a criteria for correlating the relative nucleophilicity of amine and nitrile nitrogen and the electrophilicity of nitrile, which is important in the inactivation of oxygen species.

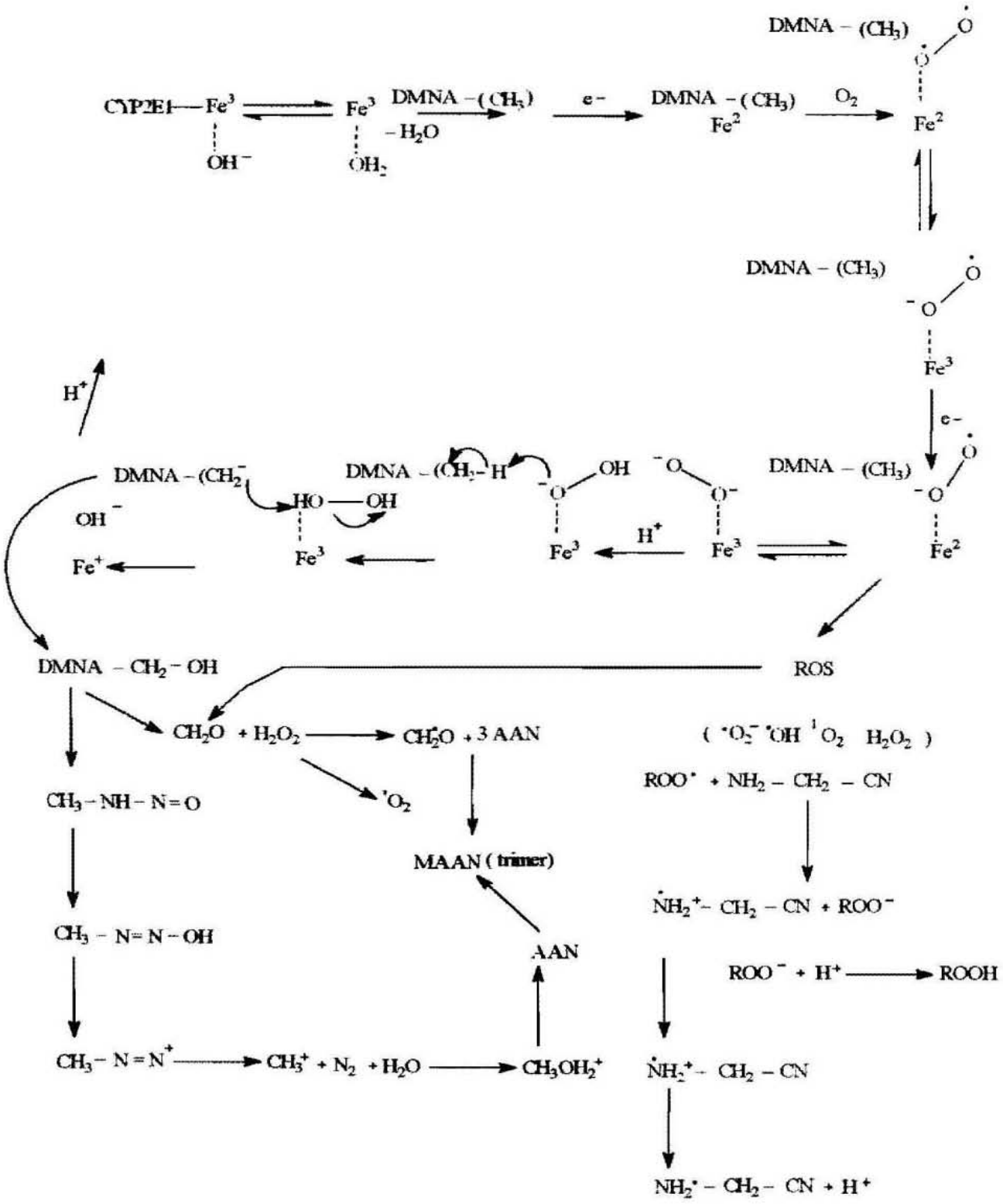


Figure 7. The possible mechanistic cycle for aliphatic hydroxilation, formation of excited formaldehyde, singlet oxygen and the effect of aminoacetonitrile (AAN) on the system.

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POLYPHENOLS IN RED GRAPE, WINE AND GREEN TEA, THEIR BIOLOGICAL ROLE AND SPECIFIC REACTION WITH FORMALDEHYDE

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1. INTRODUCTION

The oxygen mediated energy release favored by most organism and this process has an absolute fundamental importance in human body. Nevertheless, in this energy release short-lifetime free radicals are produced, instable reactive atoms or molecules of witch can react fast with the neighboring molecules. A number of free radicals fulfill physiologically important roles within the body such as superoxide and nitric oxide function as second messengers. However, free radical species in the human body must be carefully controlled as they are highly reactive and can cause tissue destruction and alter the structure, function of lipid, protein or nucleic acids. Medical science now regards oxidation process as a primary cause of degeneration and aging. The human body has a defense mechanism that helps to prevent oxidative damage and fight against free radicals and further helps to repair damage that has already occurred. This mechanism strikes to maintain the balance between the formed and inactivated free radicals. The system comprises preventive antioxidants (caeruloplasmin, metallothionine, albumin, transferrin, ferritin, myoglobin) which prevent the formation of new ROS scavenging antioxidants (enzymes: superoxide dismutase, glutathione peroxidase, glutathione reductase, catalase, metalloenzymes, and repair enzymes: DNA repair enzymes, methionine sulphoxide reductase, small molecules: glutathione, ascorbic acid, tocopherols, uric acid, bilirubin).

Besides of the normal oxidative pathways in the human body other oxidative reactions of different kinds occur due to exposure to environmental harmful compounds. In the drinking water and the atmosphere we are exposed many chemical compounds of different kinds, including different solvents, cleaning detergents, car exhaust vapors,

cigarette smoke etc. In addition, there are a variety of chemicals, additives, artificial colors, flavors, bleaching compounds, synthetic sweeteners in the food, which can contribute to oxidative process in the human body. If the level of the free radicals exceeds the available reducing capacity such toxic oxygen stress will lead to tissue damage. Fortunately our diet contains substantial amount of specific flavonoids as bioactive components. It is well known that numerous species of flavonoids are known to be distributed in the plant families and therefore different natural products still serve a model for therapeutic substances. Beneficial effects of wine - particularly red wine - and green tea has received considerable attention in the last years because of their value in moderation the risk factors mainly for cardiovascular, but also other diseases. Epidemiological studies show that red wine consumption is related to a decrease of mortality in cardiovascular disease.[1] Later Renaud and Lorgesil[2] demonstrated using data of WHO that dairy fat consumption is highly correlated with cardiac heart disease and French people had a lower than expected cardiac heart disease in spite of the fact the very high dairy fat consumption and high serum lipid level. Gronbach et al[3] showed that the mortality associated with moderate intake of wine beer and spirits. These observations have been known as the „French paradox".

It has been hypothesized that the phenolic compounds in red wine are responsible for this intervening effect.[4] In the last time many scientific articles demonstrated the beneficial effects of red grape polyphenols and other several plant polyphenols especially that of green tea. Scientists studied the physiological effect of these substances, and the mechanism of their action in biological systems.

In this chapter the biological effect of red grape and green tea polyphenols and especially the possible reaction of these compound with formaldehyde produced in biological systems will be discussed.

2. RED GRAPE - WINE AND GREEN TEA POLYPHENOLS

Plants are rich natural sources of polyphenols and more than 4000 individual substances found in them. Especially red grape, wine and tea rich in different polyphenols and an extensive research suggests that the presence of polyphenols in red grape, wine and tea may account for the beneficial action.

The classification of polyphenols according to Peri and Pompei[5] is shown in Fig. 1.

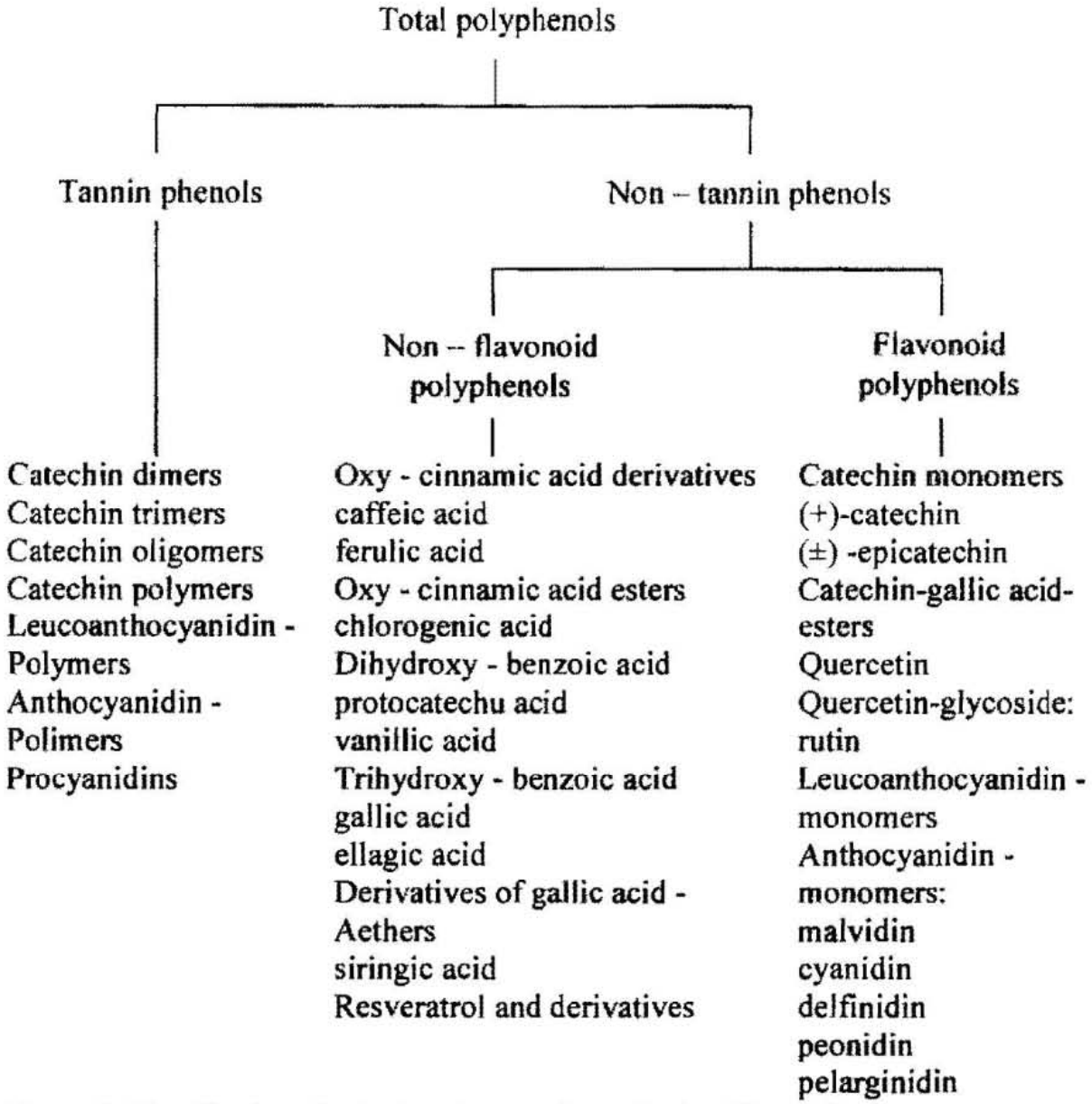
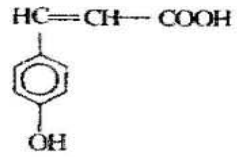


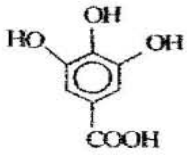
Figure 1. Classification of polyphenols according to Peri and Pompei.

Earlier it has been suggested that tannins, which are synthesized in bulk, are part of the chemical defense system of plants. Published results have also shown that natural antimicrobial compounds polyphenols will be synthesized in grape in response to fungal infections[6-10] which are natural „immun" substances in the defense mechanism of grapes. Now, it seems that polyphenol complexation with proteins may also well be a critical property of polyphenols operative in plant defense it is necessary reappraisal of the earlier hypothesis on the defense mechanism.[11]

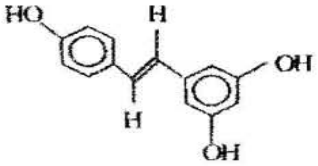
Figure 2. Structure of some grape and green tea polyphenols



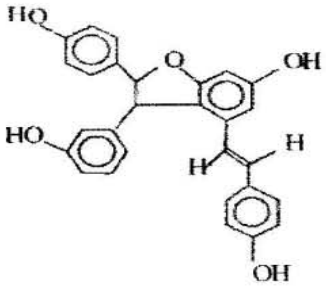
Hydroxy cinnamate



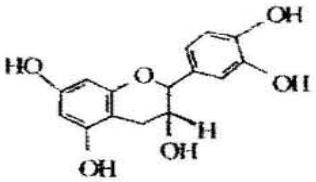
Gallic acid



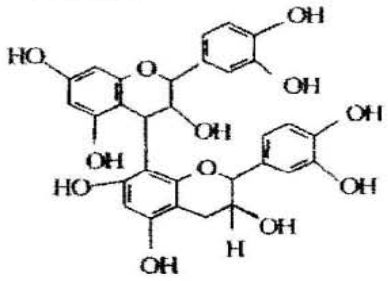
trans – resveratrol



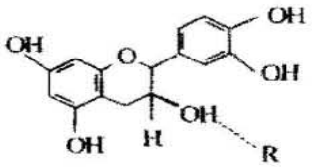
trans – E – viniferin



(+)-catechin

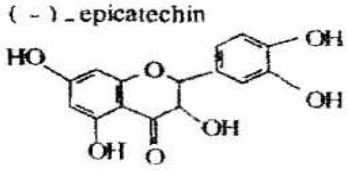


procyanidin

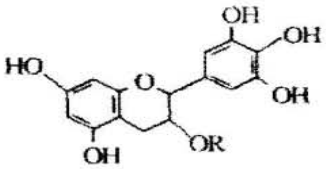


epicatechin gallate
R = gallic acid

(-)-epicatechin



quercetin



epigallocatechin (R = H)
epigallocatechin gallate (R = gallic acid)

Table 1. Oxy radicals and oxygen species

Hydroxy radical	$^{\bullet}OH$
Superoxide radical	$^{\bullet}O_2$
Peroxy radical	$ROO^{\bullet}$
Hydroperoxy radical	$^{\bullet}O_2H$
Hydrogen peroxide	H_2O_2
Singlet oxygen	$^1O_2$
Triplet oxygen	$^3O_2$
Hydroperoxide	ROOH
Peroxide	ROOR
Phenoxy - aroxy radical	$ArO^{\bullet}$
Epoxide	
Nitric oxide	NO

These unstable free radicals are extremely reactive and can react with other compounds which results in the stabilization of the radicals. This stabilizing process can be damaging to normal healthy tissues to biomolecules.

4. ROS AND ATHEROSCLEROSIS

Atherosclerosis is one of the largest cause of mortality in the general population. It is associated with endothelial dysfunction, platelet activation, lipoprotein aggregation, macrophage foam cell formation, inflammation and thrombosis.[15] ROS are thought to initiate atherosclerosis by damaging blood vessel walls. According to our present knowledge, the oxidative modification of LDL has a crucial importance in the genesis and progression of artherosclerosis.

LDL particles contain cholesterol, triglicerides, phospholipides, free cholesterol and fatty acids from which approximately the half are unsaturated and hereby easily oxidisable. Fortunately, they contain also numerous lipophyl antioxidant molecules which interact with the free radicals thereby neutralizing them and can defend LDL from oxidation. When the antioxidant capacity is exceeded by free radicals, ensue the oxidation of LDL in intima. Platelet activation is increased also by oxidative stress and activated platelets can increase LDL oxidizability. LDL oxidation and platelet activation are two key events in atherogenesis which leads to the formation of atherosclerotic lesion. These processes are interrelated in that oxidized LDL can activate platelets and they increase the susceptibility of LDL to oxidation. Macrophages absorb oxidative LDL by scavenger receptors four times faster uncontrollable than native LDL.[16] Foam cells produced by this way are filled with lipid particles[17] and cause „fatty streaks" still on the intact endothel which is the earlier form of atherogen plaque.

3. BIOLOGICAL EFFECT OF GRAPE, WINE AND TEA POLYPHENOLS

Physiological activity of red grape – wine and tea polyphenols can be summarized in the following manner:

- Antioxidant
- Free radical scavengers
- Inhibitors of platelet aggregation
- Metal ion chelators
- Inducers in apoptosis
- Anti-inflammators
- Vasodilatators
- Inhibitors of intestinal cholesterol absorption
- Blocking agents for peptide endothelin-1 synthesis
- Inhibitors in cancer

One of the most important feature of red grape, wine and tea polyphenols is their antioxidant and free radical scavenger activity. Oxidative stress and damage in the living systems is known as one of the reason of cardiovascular diseases, atherosclerosis of the aging process,[12] cancer. Some animal studies have suggested that free radicals contribute to the pancreatic islet cells in the pathogenesis of insulin dependent diabetes mellitus. Free radicals and ROS are effective indirectly as cellular messengers and elicit an inflammatory response. Higher concentration of these species may cause oxidative modification of biological molecules. Elevated ROS and free radicals initiate lipid peroxidation and accumulation of lipid peroxides in the sperm membranes causing a reduction in sperm motility and viability[13] causing infertility. Over-production of free radicals is also an incidence of cataract, in liver, lung diseases and have been implicated in the pathology of a number of neurological disorders (Parkinson disease, Down syndrome, Alzheimer disease).

In the last years, researchers suggested that free radicals, ROS may be involved in the pathogenesis of viral infection, particularly in HIV.

Radicals in free form were recognized by Gronberg[14] who discovered the relatively stable triphenylmethyl radical. Oxyradicals are a class of free radicals with the unpaired electron residing predominantly on an oxygen atom. The main oxyradicals are shown in Table 1.

Oxidative LDL shows cytotoxic and chemotactic property[18, 19] and is responsible for sticking of monocyts and adhesion of thrombocyts. During these processes different specific adhesions membran-proteins[20, 21] are expressed, PDGF (platelet derived growth factors) and the proliferation of smooth muscle cells.
Esterbauer et al[22] showed first that oxidation of LDL can be defend by antioxidant vitamin E.

5. FREE RADICALS AND DIABETES

Low total antioxidant status have been reported in diabetic patients[23] and diabetic mices.[24] In diabetes mellitus toxic amount of ROS are released by endothelial cells and infiltrating the macrophages during islet inflammation. Metabolism of diabetics can be generally characterize by increased oxidative loading in that the antioxidant capacity, the regeneration of antioxidants are limited, namely NADPH is necessary for the regeneration. However, NAPH is used up for the competitive reduction of elevated amount of glucose to sorbitol in the non-insulin dependent tissues. Hereby ascorbic acid metabolism has also implicated in diabetes because ascorbic acid is required for the regeneration of vitamin E and may be oxidized to dehydroascorbic acid which can disrupt cell structures and acts as a neurotoxin. In normal pathway dehydroascorbic acid will be reduced to ascorbic acid in the tissues. Increased amount, higher concentration of dehydroascorbic acid and low level of ascorbic acid results in increased susceptibility of the cell to oxidative damage.[25]

6. FREE RADICALS AND CANCER

In cancer development lower total antioxidant status have been determined in patients.[26] Higher free radical concentration in the human body are thought to act as promoting agents. A number of additional free radical sources coming from the environment --- cosmic rays, ozone, nitrous oxide, auto exhaust emission, heavy metals: lead, mercury, cadmium, cigarette smoke, ionizing radiation—, can cause oxidative stress in the body which stimulates the initiated cell, transforming it to a cancerous cell.
It is well known, that free radicals cause lipid peroxidation, which has also been implicated as a causatory factor in cancer development, ROS can alter gene expression, which activates transcription factors, phosphatases other cellular kinases, inhibit the repair system and damage chromosomes.

7. FREE RADICALS AND AGEING

Aging is a complex process which contribute to reduction of total antioxidant status caused by ROS reactions.[27-29] The free radical theory of ageing is founded upon that different free radical reaction are responsible for the progressiv accumulation of the changes with tissues in normal biological metabolism[28-30]. There are many publication which have studied ROS reaction in connection of ageing process which reactions are enzymatic and non-enzymatic origin.[12, 31, 38] ROS produce higher lipid peroxidation in the cellular membranes, oxidation of proteins and activate proteases, endonucleases,

phospholipases. These chain reactions are particularly elevated in different pathological conditions.

8. EFFECT OF POLYPHENOLS ON THE FREE RADICAL PROCESSES

Red grape, wine and tea polyphenols are inhibitors of initiation of free radical processes as metal-complexing agents that prevent the Fenton-Haber-Weiss reaction and also operate as chain breaking antioxidants.. In Fenton reaction produced $^{\cdot}OH$ are extraordinary reactive and it was show that iron complexation affected the efficience of Fenton reaction.[39] It was also found that metal chelation is a very important protective effect of the polyphenols.[40,41] All the protective polyphenols were active at very low concentration and their effect was observed only under those conditions in which iron chelators also afforded protection. Active polyphenolic compounds effectively chelated iron in an in vitro system. It was also established, that the antioxidant activity of polyphenol tannic acid is mainly due to iron chelation rather than $^{\cdot}OH$ scavenging and is also capable of reducing Fe^{III} ions.[42]

Polyphenols as chain-breaking antioxidants are electron donors and capable for the inactivation of various peroxy radicals.[43] The following scheme shows the reaction of polyphenol antioxidants with peroxy radicals (Fig. 3).

Only the process 4 is detected which may give the false impression that polyphenols are H – atom donors.

Several studies have shown the beneficial effect of polyphenols as antioxidants on the coronary heart diseases and studies have revealed that polyphenols act as antioxidants by scavenging ROS. The structure activity relationship of polyphenols and their antioxidant activity is well documented.[44]

It was also established that epicatechin, epicatechin gallate and quercetin act as antioxidant by lipid peroxidation inhibition, ROS scavenging and metal iron chelating.[45-47] Conjugated metabolites of (-) – epicatechin and quercetin participate in antioxidant defense in blood plasma.[48] Proanthocyanydin rich extract from grape seed attenuates the development of atherosclerosis in cholesterol feed rabbits[49] showing an increase association between the intake of dietary flavonoids and diseases.[50] It was also demonstrated, that polyphenols in red wine act as antioxidants of LDL oxidation in vivo.[51]

The inhibition of the atherosclerosis process follows thereby also, that polyphenols stabilize the free radical protection of the polyunsaturated fatty acids in LDL. Red grape based polyphenols are more powerful protectors against heart disease than α–– tocopherol.[52] Whitehead [53] studied the serum antioxidant capacity to determine the effect of the ingestion of red wine, white wine and high dose of ascorbic acid.

It was shown in an invitro study that a high antioxidant capacity of grape polyphenols in addition to its ability to increase the antioxidant capacity in vivo.

It was also found that catechin oligomers and the procyanidin dimers and trimers extracted and purified from grape seed were more active fractions, and monomer catechin, epicatechin, myrcetin had the highest antioxidant activity.[54]

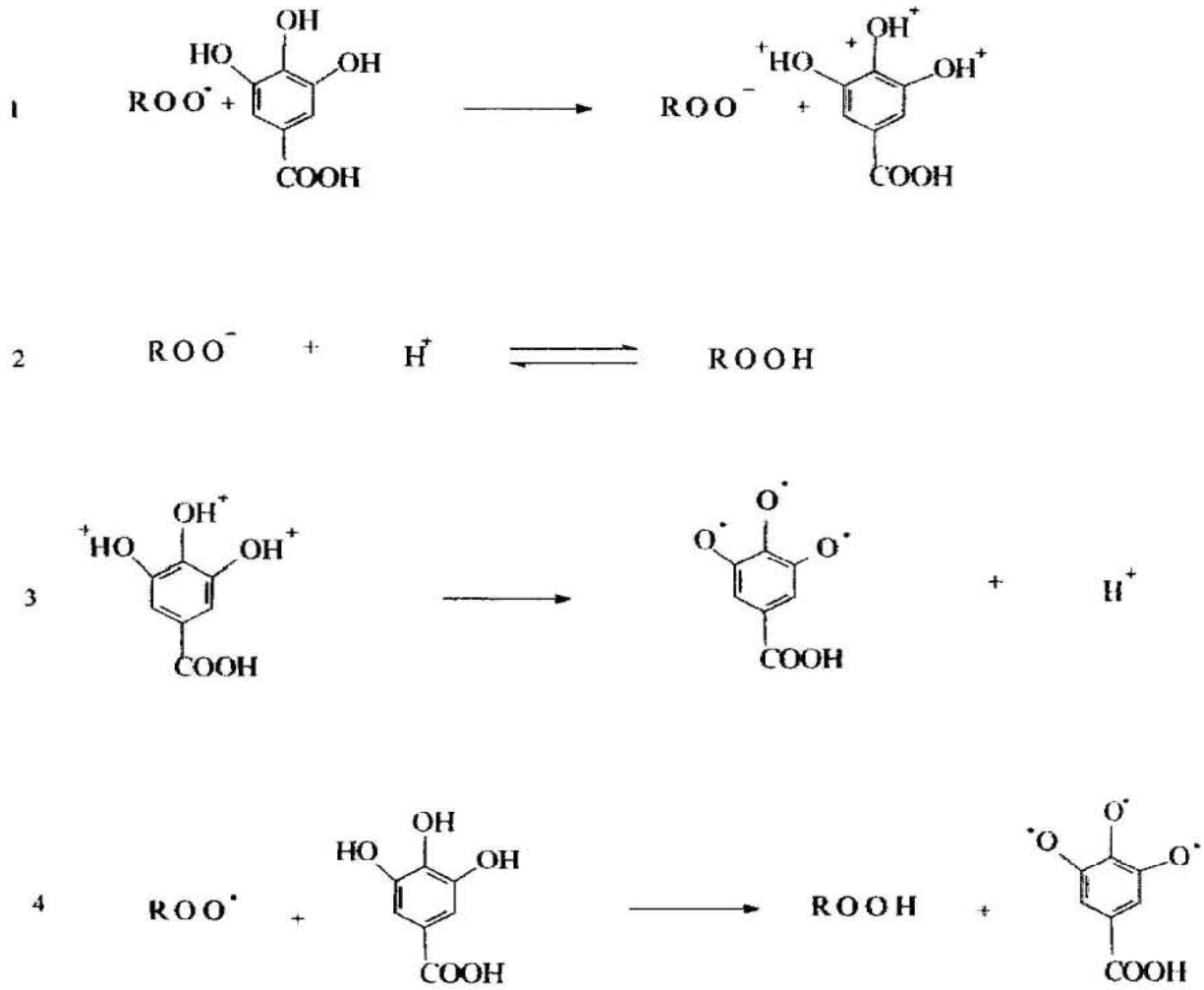


Figure 3. Gallic acid as elctron donor in chain-breaking antioxidant reaction

The polyphenols have been shown to decrease platelet aggregation in vitro [55-58] and cause platelet disaggregation of pre – formed platelet trombi in vitro.[59] Wine and grape polyphenols inhibited platelet activity and thrombosis eliminating the cyclic flow reduction.[60] It was demonstrated by Aldini et al[61] that procyanidins have the ability to protect endothelial cells from peroxynitrite damage.

Quercetin is mostly present as conjugated derivatives in human plasma[62] and essentially exists as glucuronide and sulfate conjugates in the blood circulation. [63, 64]
It is well known that this is the overproduction of endothelin-1 what causes arteries to constrict to harden and clog up. The results of investigation indicate that formation of ROS play an important role in mediating hypertension induced by chronic elevation in endothelin.[65] It was also established that ROS are involved in angiotensin II induced endothelin–1 gene expression within endothelin cells.[66] Earlier it was demonstrated that red wine strongly inhibited the synthesis of endothelin–1.[67] This action was associated with modification in phosphotyrosin staining, indicating that the polyphenols in red wine cause specific modification of tyrosin kinase signalling.[68]
It was established that polyphenols, especially the of large polymeric type or condensed tannins appear to be responsible partly for the reduced glycemic response to carbohydrate

foods and partly for lower blood glucose response to legumes compared with cereal products.[69]

Green tea polyphenols increased glucose tolerance and it was also found a reduction in serum glucose level in alloxan diabetic rats and increased antioxidant potencial.[70] It was also showed that polyphenol – enriched chardonnay white wine is able to induce ethanol – independent in vivo effects in a model of insulin deficient diabetes characterized by a major oxidative stress.[71] Red – wine polyphenolic extract in streptozotocin treated diabetic rats showed antidiabetic activity.[72] Polyphenol – enriched, low-iron available carbohydrate restricted diet can inhibit the progression of diabetic nephropathy.[73] Red wine and tea polyphenols inhibit the digestion and absorption of protein, energy and iron.[74-75]

Iron, hyperinsulinemia and hyperglychemia act in concert to up-regulate free radical reactions[76] and polyphenol enriched diet slowed down progression of diabetic nephropathy by diminishing oxidative stress pathways of tissue damage.

9. ANTICANCER AND APOPTOTIC ACTIVITY OF POLYPHENOLS

Grape – wine and tea polyphenols show anticancer and apoptotic properties. Numerous publications discuss with these effects of resveratrol, epigallocatechin gallate, epicatechin on the different cancer cell lines. The antitumor activity of fresh green tea was also demonstrated epidemiologically and the results showed, that the green tea may play a role in the prevention of cancer formation.[77, 78] Later it was demonstrated, that these polyphenols inhibit the growth of various human tumor cells.[79, 80] Researcher have considered epigallocatechin and epigallocathechingallat the most active components of green tea because are the most abundant catechins. ROS play important role in tumorigenesis altering gene expression, damaging DNA, affecting cell growth, differentiation.[81, 82] Different ROS have been implicated in cancer promotion[83] through two type of promoters which induce, generate free radicals. Type I promoters (e.g. phorbol esters) generate ROS indirectly through effectors such as protein kinase C to produce superoxide as a by-product of the arachinodic cascade. Type II promoters generate ROS directly without the mediation of other effectors.[84, 85] The main antitumorigenic efficiency of polyphenols are believed to be related to their antioxidative properties, (but not entirely). The prooxidant activity of these compounds play a role in inducing apoptosis. It was found, that gallic acid induces apoptosis in tumor cells with higher sensitivity than normal cells.[86, 87] It was also demonstrated by Inone et al[86] that intracellular ROS induced by gallic acid plays an important role in eliciting an early signal in apoptosis. H_2O_2 which is derived from $^{\cdot}O^{-}_2$ generated extracellularly may increase inracellularly Ca^{++} levels ROS and Ca^{++} elevation are induced independently, but both are required for the induction of apoptosis. Gallic acid has selective cytotoxicity against tumor cells with higher sensitivity than normal cells, because there is a great difference in the amount of the inhibitors generated by normal and tumor cells.[87]

Pharmacokinetic studies showed that polyphenols affect signal transduction pathway, inhibit cell proliferation, epidermal growth-factor and this inhibition was correlated with the inhibition of AP-1-dependent transcriptional activity.[88, 89] Other possible mechanism for cancer prevention activity of polyphenols has been proposed through inhibition of tumor necrosis factor.[90] It was demonstrated that trans-resveratrol decreases hepatocyte

growth-factor-induced HepG2 cell invasion by an as yet unidentified post-receptor mechanism.[91]

Billard et al[92] studied the antiproliferative and apoptotic effects of resveratrol, epsilon-viniferin (resveratrol dimer) and from vine-shots extract isolated polyphenol fraction on the normal and leukemic human lymphocytes in comparative investigation They established, that vine-shots extracts and resveratrol displayed comparable activity in inhibition of tritiated-thymidine uptake and reduction of cell recovery, but epsilon-viniferin exhibited only slight effects. On the other hand, it was already demonstrated earlier that polyphenols have a quenching effect on activated carcinogens e.g. benzo(a)pyrene derivatives[93] probably by eliminating of induced precursors. In vitro and in vivo animal and human studies suggest that green tea and red grape, wine polyphenols are photoprotective and can be used for the prevention of UVB light-induced skin damages such as melanoma, skin cancer photoaeging.[94-96] Castanas et al [97, 98] investigated the action of red wine polyphenols on human brest and prostata cancer cells. The results of their investigation showed that polyphenols decreased cell proliferation in a dose and time dependent manner at the picomol or the nanomolar range and could have a beneficial effect on brest cancer. These results supported the opinion that red wine-grape polyphenols have a higher antioxidant capacity then other polyphenols.

It was also demonstrated that resveratrol induces apoptosis through activation of p53 activity.[99] However, other experiments did not support the observation because the stimulation by polyphenols are relatively small and the anticarcinogenic property of polyphenols unlikely to be mediated by modulation of p53 gene expression.[100]
Resveratrol inhibits CYP 1A1 expression in vitro[101]by preventing the binding of the aryl hydrocarbon receptor to promoter sequences that regulate CYP 1A1 transcription. This is the possible chemopreventive activity of resveratrol.

As we have seen, there are multiple mechanism by which grape-wine green tea polyphenols produce inhibitory effect against carcinogenesis, Main question is which mechanisms are relevant to human cancer prevention. Polyphenols take part in different reaction in the biological system. They can react with peroxides, free radicals have metal chelating activity and have many beneficial biological effects through different biochemical reactions.

As polyphenols can take part in the Mannich and Lederer – Manasse reactions the question emerged recently weather they can influence they the one carbon pool, the methylation and demethylation processes and the endogenous formaldehyde metabolism. Few investigations were carried out on this field.

10. REACTION OF POLYPHENOLS WITH ENDOGENOUS FORMALDEHYDE, AMINES AND AMINO ACIDS IN BIOLOGICAL SYSTEM

Mannich reaction

In the original reaction, formaldehyde can react with the salt formation of ammonia and an oxo-compound containing an active hydrogen. The reaction is connected with the mobility of hydrogen atom of oxo-compound and the reaction can be carried out with salts of primary and secundary amines and also amino acids and the product is referred to

a Mannich base. Numerous active hydrogen compounds provide this Mannich-types reaction, which is an nonsymmetric condensation reaction (Fig. 4).

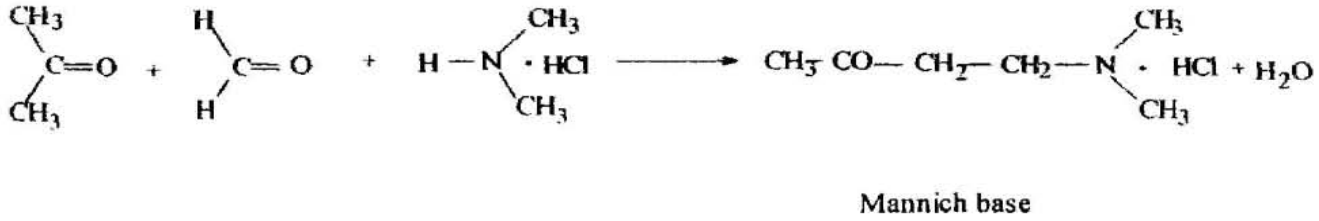
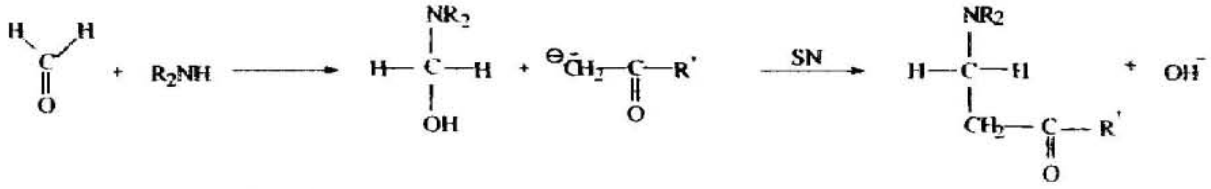
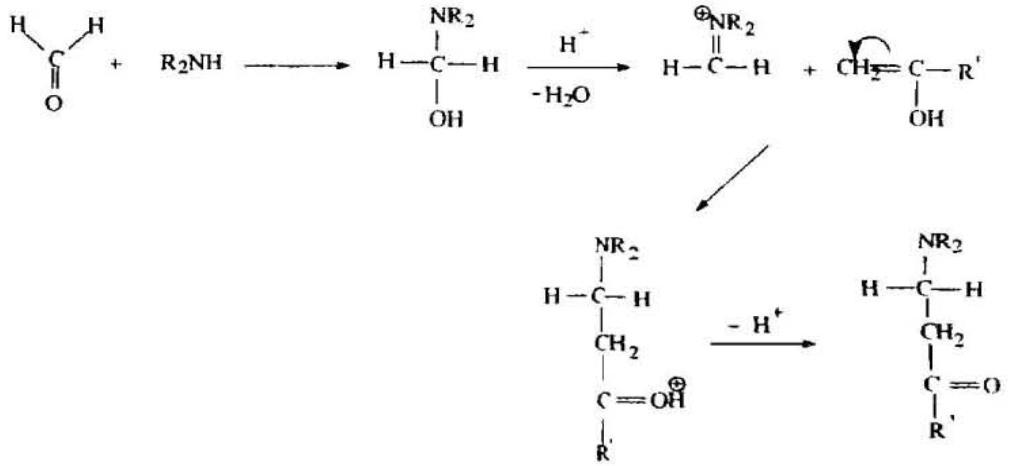


Figure 4. Mannich reaction

Investigation kinetic of the reaction have led to the following mechanism:



based - catalyzed reaction



acid - catalyzed reaction

The investigation of this mechanism shows that it is the free amine, not the salt which reacts even in acidic solution and that the active-hydrogen containing compound reacts as the enol when that is possible. These is kinetic evidence for the intermediacy of the imminium ion.[102]

It was recognized as early as 1917 by Mannich already that this condensation reaction of formaldehyde with ammonia in the form its salts and a compound containing an active

hydrogen proceed on biological system also.[103] Mannich could demonstrate his idea on the syntheses of arecolin under biological conditions,[103] which was confirmed by Schöpf and coworkers in their comprehensive work.[104]

Being the Mannich reaction a three component reaction all compounds are present in the biological system and it is today unambiguous, that the Mannich reaction is an important biosynthetic way of natural products, mainly of alkaloids and some of these pathways have been duplicated in the laboratory. Hydroxymethyl phenols react particularly easily with aliphatic amines or phenols with hydroxymethyl-amines originating amino methyl phenol derivatives.

Lederer – Manasse reaction

Phenols can react with formaldehyde in the presence of alkali to produce hydroxymethyl derivatives. The hydroxymthyl group enter into „o" or „p" position (Fig. 5), in addition other condensation products are formed.

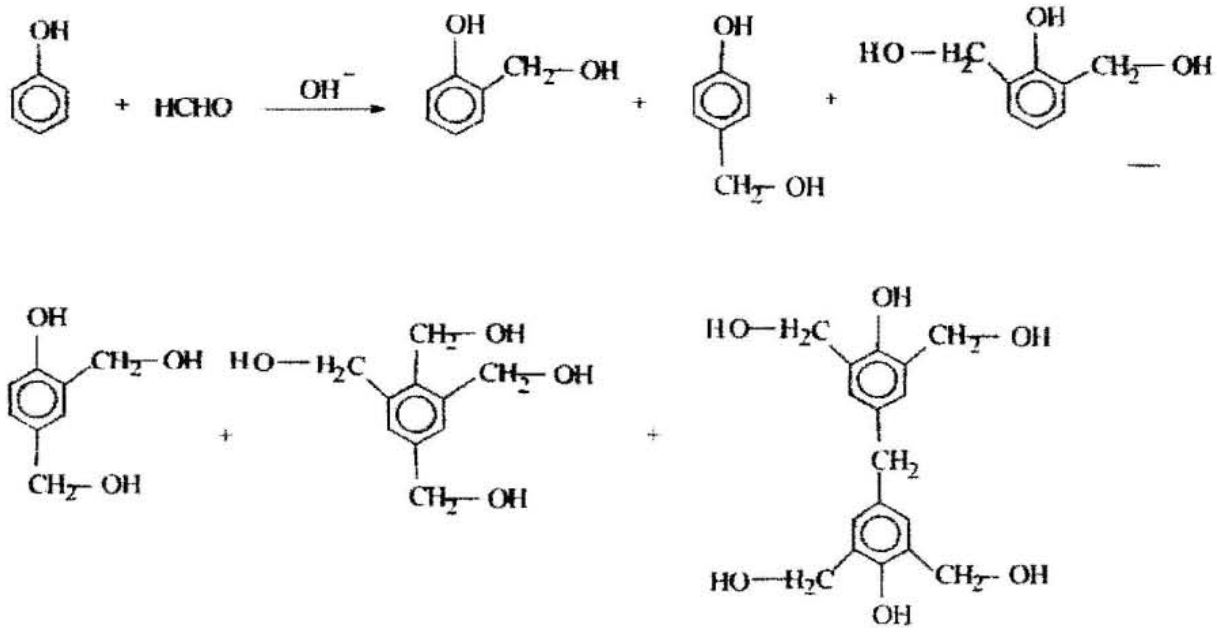


Figure 5. Lederer – Manasse reaction

Investigations were carried out in our laboratory with ^{14}C-labelled formaldehyde and gallic acid to study this reaction reaction in vitro. We could establish that the Lederer – Manasse reaction proceeds at pH 7.4 and on 37°C in physiological circumstances. (Fig.6.)

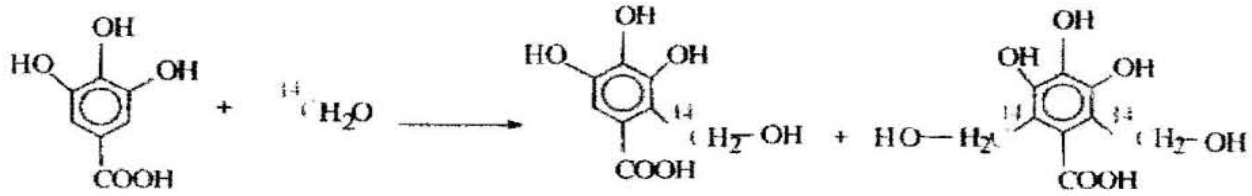


Figure 6. Reaction of gallic acid with $^{14}CH_2O$.

In the presence of H_2O_2 activated formaldehyde originates and the yield of the hydroxy-methylated product is much higher and formyl product is also produced. Our results suggest that the Lederer – Manasse and Mannich reactions proceed in physiological circumstances, because in the case of polyphenol application all compounds are present in the biological system including endogenous formaldehyde amines and amino acids.

The Fig. 7. and 8. show some of the reactions which can proceed in the biological circumstances in the presence of polyphenols, formaldehyde, amino acids.

Why is it important in the biological system ?

It is well known that the one-carbon units may be divided into three categories:

1. Hydroxymehtyl group — CH_2OH
2. Formyl group — CHO
3. Methyl group — CH_3

The chemical reactions of one-carbon units, their transfer from one substrate to another, their interconversion are the most important metabolic transformations. The metabolic reactions catalyzed by folic acid coenzymes and these reactions are concerned with the metabolism of the nucleic acids and with the metabolism of the proteins. In normal circumstances most of the C_1 groups carried by methionine are generated de novo in the folate dependent C_1 pathway, primarily from the β - carbon of serine by the enzyme serine hydroxymethyltransferase in the formaldehyde oxidation state forming 5,10 - methylen -tetrahydrofolate. 5,10-CH_2THF can also be produced nonenzymatically through a condensation reaction between formaldehyde and tetrahydrofolic acid.

5,10-CH_2THF acts as – CH_2-OH donor in hydroxymethylation of homocysteine to form methionine in a reduction process. Endogenous releasable formaldehyde is known to occur at low concentration in biological systems. Thus, in normal human blood 13 – 20 μmol/l and in urine 83 – 133 μmol/l.[105] In normal circumstances the source of the endogenous formaldehyde is the one- carbon pool. The capacity to form formaldehyde from 5,10-methylentetrahydrofolic acid is shared by formaldehyde forming enzyme and methylenreductase (5 - methylentetrahydrofolat – NAD - oxidoreductase). It has been demonstrated that endogenous formaldehyde is enzymatically formed from 5 - methyltetrahydrofolic acid and it is produced by 5 – methyltetrahydrofolate - N -methyl - transferase. Flavin - adenine dinucleotid is required as a cofactor as well as an electron acceptor.

Under defined condition S-adenosylmethionine can produce formaldehyde in human blood by enzymatic process. [106]

One of the three methyl groups of betaine an oxidation product of cholin can also serve as a methyl source of methionine. The other two groups are oxidized directly to formaldehyde by oxidative CYP demethylation.

Small amounts of formaldehyde may also be obtained in vivo from the oxidation of methanol, and from the reduction of format.

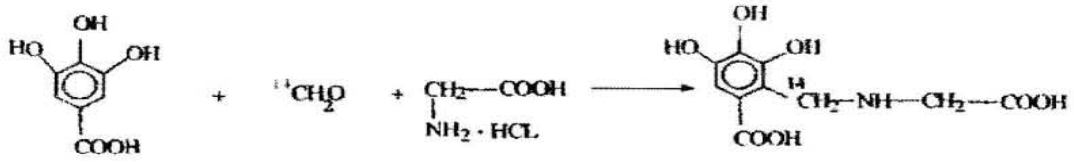
There are many other endogenous and exogenous formaldehyde precursors which can influence the normal endogenous formaldehyde level in biological system and this in some cases „activated" formaldehyde can take part in different uncontrolled reactions.
The most important exogenous formaldehyde precursors in the biological systems are the following:

- Different N – methyl compounds
- Aromatic amines
- Heterocyclic amines
- Azo dye amines
- Nitrosamines
- Hydroxylamines
- – methyl compounds
- S – methyl compounds
- $= N - CH_2 - N =$ compounds
- $= N - CH_2 - S -$ compounds
- $- O - CH_2 - C$ - compounds
- hydroxymethyl derivatives of amino acids
- hydrazines
- triazenes
- formats

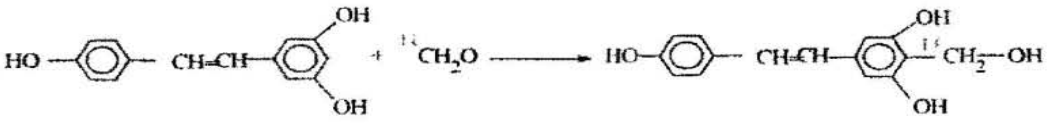
Some of these structures are substrates of different CYP enzymes. There are great differences in these metabolic reactions between the CYP enzymes. From the precursors produced formaldehyde by CYP enzymes will metabolized through format – carbon dioxide pathway. However, some of the precursors produced „activated" formaldehyde by CYP 2E1 or CYP 1 family particularly by CYP 2E1. The role of these enzymes in toxic activation of formaldehyde may be potential risk factor in the pathogenesis of different disorders particularly in cancer. Tyihák et al investigated the effect of resveratrol on the uncontrolled formaldehyde and presumed that resveratrol may exert a double effect in biological system. The elimination of formaldehyde with resveratrol may cause a cardioprotective effect and the reaction products between resveratrol and formaldehyde may act as a chemopreventive factor against cancer.[107] Earlier it was supposed, that elevated formaldehyde level would be the result of metabolic abnormalities of genetic origin,.[108] which may play a role in the clinical manifestation of schizophrenia. Later, other authors investigated the one carbon metabolism abnormalities in correlation with neurodegenerative disorders.[109-112]

Our experimental results suggest, that polyphenols cannot influence the normal endogenous formaldehyde level, but can raise the decreased total antioxidant status (TAS) to the normal 1.40 – 1.85 mmol/l level. We have diminished TAS in blood and urine samples of atherosclerotic, cancer and diabetic patients, and this lower level could be correct by adaptation of red grape, wine and green tae polyphenols.

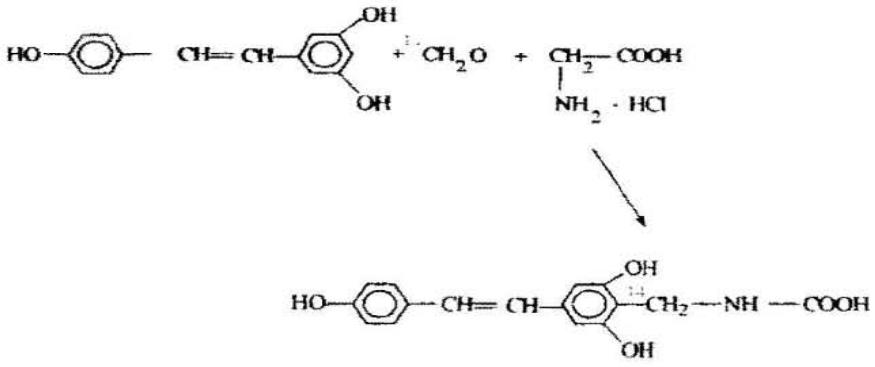
In hyperformaldehydism where uncontrolled „activated" formaldehyde is produced from different substrates by enzymatic process polyphenols can eliminate this toxic formaldehyde through Mannich and Lederer – Manasse reactions particularly. The investigation of biological properties of the Mannich and Lederer – Manasse products may be very important in the future.



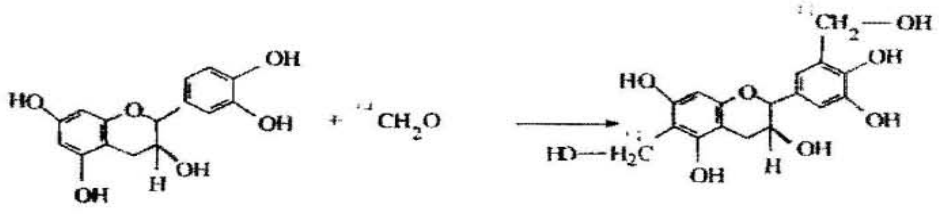
Gallic acid in Mannich reaction



Resveratrol in Lederer-Manasse reaction

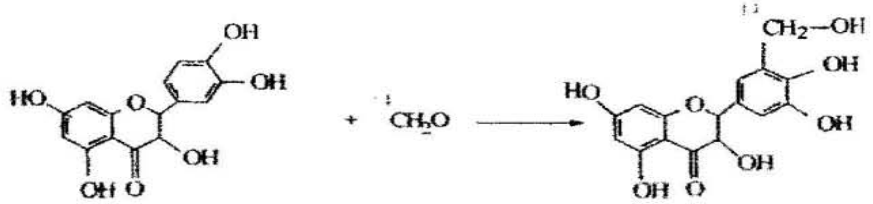


Resveratrol in Mannich reaction

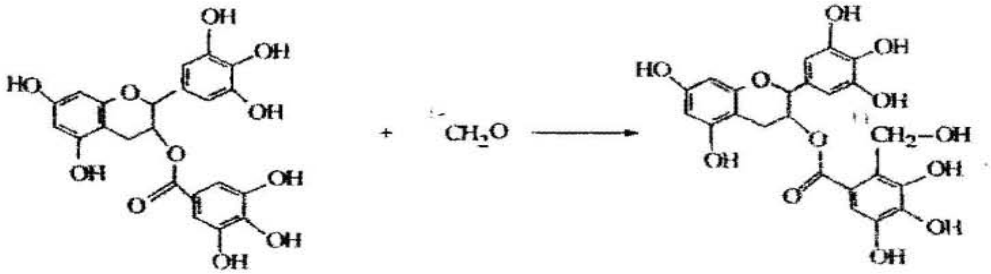


(-) -epicatechin in Lederer - Manasse reaction

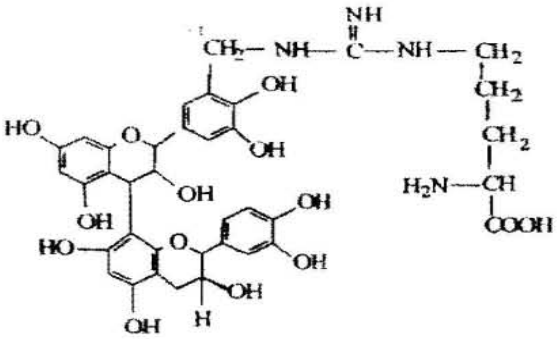
Figure 7. Reaction of different polyphenols with formaldehyde and amino acid.



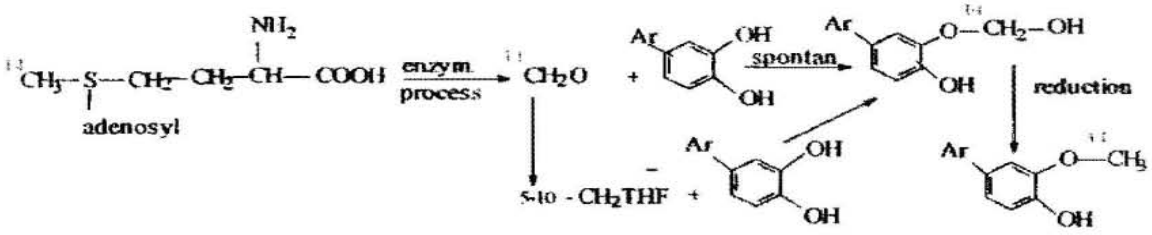
Quercetin in Lederer - Manasse reaction



Epicatechin gallate in Lederer - Manasse reaction



Mannich product of procyanidin



possible O - methylation of polyphenols

Figure 8. Reaction of different polyphenols with formaldehyde and amino acid.

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