# ЗБОРНИК

МАТИЦЕ СРПСКЕ ЗА ПРИРОДНЕ НАУКЕ

MATICA SRPSKA PROCEEDINGS FOR NATURAL SCIENCES

100

NOVI SAD 2001



#### МАТИЦА СРПСКА ОДЕЉЕЊЕ ЗА ПРИРОДНЕ НАУКЕ

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Зборник Майице срйске за йриродне науке издаје Матица српска Излази двапут годишње Уредништво и администрација: Нови Сад, Улица Матице српске 1 Телефон: 021/420-199 Proceedings for Natural Sciences published by Matica Srpska Published twice a year Editorial and publishing office: Novi Sad, Ul. Matice Srpske 1 21000 Novi Sad, Yugoslavia Telefon: 021/420-199

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## POVODOM IZLASKA 100. SVESKE ZBORNIKA MATICE SRPSKE ZA PRIRODNE NAUKE

Оснивачи Матице српске широко су схватали Матичине задатке. Поред белетристике и просвећивања народа, под појмом "књижества" подразумевали су и рад на науци. Ипак, покретању научних зборника претходио је дуг пут иако се од самог основања Матице кроз њен рад стално провлачила потреба за научним радом. Тако је, убрзо по оснивању Матице српске, Теодор Павловић приликом преузимања уредништва њеног главног, истовремено универзално културног органа Лешойиса, 1832. године, утврдио да се у Летопису "успјех и напредак Сербски у наукама и просвешченију јасно види". На основу схватања о повезаности науке и живота у Матици све јаче долази до изражаја тежња да се организује домаћа наука. Јован Ђорђевић 1858. године вели да Лешоџис треба начинити "научним органом за србске ствари". За време уредништва Јована Суботића Лешоџис испољава нарочито велико занимање за науку. Касније, почетком прошлог века, Тихомир Остојић пише: "Историја показује, а живот одлучно захтева, да Лешоџис буде народни орган који тражи чврсту подину у науци и познавању народног живота". И у наредном периоду често је истицана потреба за развојем научног рада у Матици. Тако је Књижевни одбор Матице српске 1912. године, на предлог ондашње младе генерације грађанске интелигенције, усвојио предлог да се Књижевном одељењу установе стручни зборови са циљем да дају полет књижевном и научном раду у Матици српској.

Упркос лутањима и неразумевањима у оријентацији Матичиног рада временом оправданост и потреба њене оријентације на научни рад у све већој мери долазе до изражаја. После Другог светског рата створени су услови за организовање Матице српске као научног друштва, иницијатора и организатора научноистраживачког рада у разним областима стваралаштва. Као резултат тих тежњи пре педесет година донета је одлука о покретању Зборника за *йриродне науке*. Оправданост те одлуке потврђује пола века успешног излажења овог часописа.

За протеклих педесет година *Зборник* је израстао у водећи национални научни часопис. У *Зборнику* је објављено 1.166 оригиналних научних радова из различитих области природних наука. Поред тога, објављено је 260 приказа и бележака, као и 21 некролог. Часопис је у протекло време редовно излазио. Са малим изузетком, објављиване су по две свеске годишње. За протеклих 50 година објављено је 100 свезака, што износи 17.929 страница штампаног текста.

Поред домаћих водећих научних стваралаца, у Зборнику су своје радове објавили и 73 инострана истраживача из 17 земаља света.

За протеклих 50 година излажења *Зборника* структура објављених радова по дисциплинама значајно се изменила. У првим годинама највише објављених радова било је из области биологије и географије, а касније се јавља све већи број наслова из области хемије и медицине. Анализом структуре објављених наслова за цео период излажења *Зборника* може се закључити да од укупног броја објављених радова на биологију отпада 57,9%. У оквиру биологије најзаступљенији су били радови из флористике и фитоценологије — 24%, затим физиологије биљака — 17,4% и зоологије кичмењака и бескичмењака по — 16,4%. Услед развоја нових области у оквиру биологије последњих година све су заступљенији ји радови из молекуларне биологије и биофизике.

Значајне разлике могу се уочити и у обиму радова: они су постали краћи, концизнији, без непотребних описа и детаља. Број аутора по раду се значајно повећао: док су у првих тридесет бројева скоро сви радови били самостални, у последњих двадесет бројева просечан број аутора по раду износи 3,2. За повећање броја аутора по раду има више разлога: један је мултидисциплинарни карактер објављених радова. Значајно се повећао и број цитираних аутора по раду. У првим бројевима Зборника по страници текста цитирано је у просеку 0,56 аутора, а у последњих петнаест бројева 1,7. Повећање броја цитираних аутора по раду указује на веома динамичан раст научних сазнања и комуникације у свету.

Зборник за *йриродне науке* је у току свог излажења три пута мењао наслов. Под насловом *Научни зборник Майице срйске — серија йриродних наука* појавио се 1951. године, па је као Зборник Майице срйске — серија *йриродних наука* излазио све до свог десетог броја, 1956. године, да би се затим, од 11. броја, појављивао под насловом Зборник за *йриродне науке*. Од 66. броја (1984. године) Часопис излази под насловом Зборник Майице срйске за *йриродне науке*.

Уредници *Зборника* били су: 1951. године проф. др Милош Јовановић, од 1952. до 1969. године проф. др Бранислав Букуров, академик, од 1970. до 1976. године проф. др h. c. Лазар Стојковић, а од 1977. до 1996. године проф. др Слободан Глумац.

Доминација енглеског језика у међународној комуникацији, посебно у природним наукама, као и Правилник Министарства за науку и технологију Републике Србије о рангирању и с тим у вези финансирању излажења часописа, наметнули су потребу за штампањем *Зборника* на енглеском језику. Први број на енглеском језику појавио се 1993. године. Истовремено, наметнула се потреба за променом у уређивању Часописа. Поред постојећег Уредништва, 1977. године је именовано и саветодавно тело Уредништва састављено од знаменитих иностраних истраживача.

Одувек се велика пажња посвећује размени *Зборника* са научним установама, библиотекама и рефералним центрима. *Зборник* се последњих година размењује са 63 институције из 23 земље света. Поред тога,

радови објављени у *Зборнику* реферисани су рефералним журналима у земљи и у иностранству, те достигнућа домаће науке у области природних наука постају део светске баштине сазнања. Размена *Зборника* истовремено доприноси афирмацији домаће науке и олакшава нашим истраживачима да прате страна научна достигнућа. Видан напредак на пољу информационе технологије последњих година омогућује много бржу и ефикаснију презентацију научних достигнућа него што је досад било уобичајено. С тим у вези намеће се потреба за презентацијом *Зборника* на интернету.

За протеклих педест година излажења *Зборник* је израстао у значајну трибину научних достигнућа у области природних наука у нас па и шире. Томе је свакако допринело новонастало окружење, оснивање научних института, факултета и универзитета и, уопште, развој научноистраживачког рада у нашој земљи. Радови објављени у *Зборнику* поред опште научне вредности и доприноса светској баштини знања у великој мери су допринели и бољем упознавању нас самих и природе која нас окружује: земљишта, клима, водотокова, ритова, пашњака, шума и целокупног биљног и животињског света. Значајно је истаћи да су ова сазнања допринела не само развоју наше земље, него такође и успостављању данас тако нужног хармоничног односа између човека и природе.

Динамичан развој природних наука у будућности ће пред Зборник постављати све замашније задатке. Пола века успешног појављивања Зборника уверава нас да ће овај часопис, захваљујући својим сарадницима, моћи успешно да одговори новим, све већим и тежим захтевима и да ће допринети даљој научној афирмацији и угледу не само Зборника него и Матице српске.

Матица српска ће и убудуће као афирмисана научна установа дати свој пун допринос развоју науке и других видова стваралаштва и на тај начин, следећи своје светле традиције, допринети нашем друштвеном и економском развоју. Даље успешно излажење *Зборника* биће део те велике и хумане мисије коју је Матица српска од свог оснивања следила.

> Главни и одговорни уредник *Р. Касшори*

## A WORD OF EDITOR IN CHIEF ON THE OCCASION OF THE 100<sup>TH</sup> ISSUE OF "MATICA SRPSKA JOURNAL OF NATURAL SCIENCES"

The founders of Matica srpska had tasked their brainchild with a broad scope of objectives. The term 'publishing' as they had used it implied not only belletristic and instructive publications but also scientific ones. Although the need for scientific work had been keenly felt from the very beginnings, it took a long time to publish the first scientific journal. In 1832, for example, Teodor Pavlović, on appointment for editor in chief of 'The Annals of Matica srpska', at that time Matica's principal publication of universal and cultural orientation, declared that the Annals , clearly demonstrate success and progress of Serbs in sciences and enlightenment". Aware that science and prosperity are closely linked, Matica vigorously advocated the necessity of organizing scientific work at the national level. In 1858, Jovan Đorđević declared that the Annals should be made ...a scientific instrument for the Serbian cause". During the term of Jovan Subotić as editor in chief, the Annals exhibited keen interest in scientific issues. Later on, at the beginning of the previous century, Tihomir Ostojić wrote: "History instructs us and life demands of us to make the Annals an instrument in the service of people, firmly based on science and the knowledge of the way our people live". In the subsequent period, Matica continued to advocate in favor of scientific work with unabated ardor. In 1912, in response to a motion coming from a group of young intellectuals, the Literary Board of Matica srpska passed a decision to establish expert panels within its Literary Department. The goal of the panels was to give impetus to literary and scientific work in Matica srpska.

In spite of meanderings and incomprehension of the orientation towards scientific work, Matica's efforts gradually grew to be understood and appreciated. After World War II, conditions were created to organize Matica srpska as a scientific society, a stimulant and organizer of research work in various scientific fields. The long expectations came to fruition when it was decided to start the Journal of Natural Sciences. Half a century of successful publishing activity fully justifies this decision.

Over last fifty years, the Journal grew into the national leading scientific publication. The Journal has published 1,166 original scientific papers from different natural sciences, 260 reviews and notices and 21 obituaries. The publishing of the Journal proceeded regularly. With small exceptions, two volu-

mes were issued annually. One hundred volumes were published over last 50 years, totaling 17,929 printed pages.

Contributors to the Journal have been leading researchers from the country as well as 73 foreign researchers from 17 countries.

During past 50 years, the profile of scientific disciplines covered by the Journal underwent significant changes. Biology and geography were the predominant disciplines in the early years, to be succeeded by chemistry and medicine. An analysis of the structure of papers published over the entire 50-year period has shown that papers from the field of biology take 57.9% of the total number. Within the field of biology, most frequent were papers dealing with floristics and plant communities, 24%, plant physiology, 17.4%, and the zoology of vertebrates and invertebrates, 16.4% each. In consequence to the developments in the biological studies, the number of papers from molecular biology and biophysics has increased proportionally in recent years.

Significant changes have also occurred in the volume of papers. They became more concise, avoiding unnecessary descriptions and details. The number of co-authors increased significantly. While in the first 30 issues almost all papers had a single author, the number of co-authors rose to 3.2 in the last 20 issues. There are several reasons for this increase, the multidisciplinary character of the papers being one of them. The number of citations per paper was also increased, from 0.56 authors quoted per page in the early issues to 1.7 in the last 15 issues. The increase in the number of cited authors is indicative of the dynamic growth of the scientific knowledge and communications in the world.

In its 50-year history, the Journal changed name three times. The first ten issues, released from 1951 to 1956, were published as 'Scientific Journal of Matica srpska — Natural Sciences Series'. The issues 11 to 65, released from 1956 to 1984, were published as 'Journal of Natural Sciences'. Since 1984 and the 66<sup>th</sup> issue the Journal is published under its present name, 'Matica srpska Journal of Natural Sciences'.

Chief editors of the Journal were Prof. Dr. Miloš Jovanović, from 1951 to 1952, Prof. Dr. Branislav Bukurov, Academician, from 1952 to 1969, Prof. Dr. h. c. Lazar Stojković, from 1970 to 1976, and Prof. Dr. Slobodan Glumac, from 1977 to 1996.

Domination of the English language in international communication, especially in natural sciences, and the rules of the Ministry of Science and Technology of the Republic of Serbia concerning the ranking and subsidizing of journals compelled the Journal to switch from Serbian to English. The first English issue appeared in 1993. The change in language called for a change in editorial policy. Consequently, in 1997, the Editorial Board was enlarged for a consulting commission made up of notable foreign scientists.

Attention has been given to the exchange of the Journal with scientific institutions, libraries and reference systems. In recent years, the Journal has been exchanged with 63 institutions from 23 countries. Also, papers published in the Journal are registered in several domestic and foreign reference journals. In this way the achievements of domestic natural scientists become part of the global scientific heritage. The exchange promotes the national science and helps domestic scientists follow scientific developments on the international

scale. Recent advances in information technology have made the presentation of scientific achievements faster and more efficient than ever before. Time has come to consider possibilities of Internet presentation of our Journal.

Over last 50 years, the Journal developed into an important scientific rostrum whose significance exceeds the national framework. This process was certainly facilitated by the new invigorating atmosphere, through the establishment of research institutes, colleges and universities and through the general development of research work in the country. In addition to their universal scientific significance, the papers published in the Journal helped us learn more about ourselves and the environment around us, soil, climate, watercourses, swamps, pastures, forests and the plant and animal life. The newly gained knowledge contributed not only to the development of the country but also to the reestablishment of harmony between the man and the nature.

The dynamic development of the natural sciences sets more and more complex tasks before the Journal. Half of century of successful work seems a convincing proof that the Journal's staff will be able to cope with these tasks, further confirming the good standing not only of the Journal itself but also of Matica srpska on the whole.

In harmony with its honorable tradition, Matica srpska is determined to continue making full contribution to the development of science and other forms of human creativeness, for the benefit of social and economic progress of the country. Future issues of the Journal will be part of that noble and humane mission.

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## STRESS-INDUCED INHIBITION OF REPRODUCTIVE FUNCTION EVIDENCE FOR INTERACTIONS BETWEEN THE FEMALE REPRODUCTIVE SYSTEM AND METABOLIC STRESS

ABSTRACT: This review will briefly discuss the stress-induced alterations of the GnRH/LH pulsatile secretion, and interactions between the female reproductive system and metabolic stress.

Experimental studies and clinical observations have shown that stress suppresses the reproductive function (RF) causing anovulation, inadequate luteal phase, infertility decreased libido and impotence. This is presumably an adaptive response devoted to conserving energy during hardship.

A variety of stressors (physical, physico-emotional, emotional, metabolic etc.) suppresses the RF acting on the hypothalamic-pituitary-gonadal (HPG) axis at all three levels. The most remarkable feature is the inhibition of pituitary preovulatory luteinizing (LH) surge and pulsatile LH secretion, as a consequance of markedly decreased episodic hypothalamic gonadotropin-releasing hormone (GnRH) secretion. Alterations in episodic release of GnRH appear to play a critical role in the inhibition of RF. Inhibition of GnRH/LH surge during stress conditions suggests that stress responsive hormones act primarily on the hypothalamic-pituitary (HP) component of the HPG axis causing anovulation i.e. hypothalamic/hypogonadotropic amenorrhea (HA) which is observed regardless of the types of stressors. For example, in response to metabolic stress: nutritional status, degree of fat stores, severe dietary restriction i.e. anorexia nervosa, energy availability, or heavy exercise, as ballet dancing and long distance running, failure in RF has been observed with prevalence of amenorrhea and infertility. Endocrine and neuroendocrine examinations have found that proximal cause of impaired RF in these women is HA, caracterized by disruption of pulsatile GnRH/LH secretion.

The HPG axis integrates information derived from the body's metabolic control centers and responds to maximize the organism's reproductive fitness. However, the physiological mechanisms linking metabolic center and reproduction remain poorly understood. It appears that leptin, a lipostatic hormone, may act as a metabolic signal to the HPG axis.

KEY WORDS: reproductive function, metabolic stress, hypothalamic amenorrhea

#### INTRODUCTION

Life exists by maintaining a complex dynamic equilibrium, or homeostasis, that is constantly challenged by intrinsic or extrinsic adverse forces of stressors. Stress is broadly defined as any stimulus that disturbs the homeostasis of the organism. Homeostasis is reestablished by a complex repertoire of physiologic and behavioral adaptive responses of the organism (S e l y e H ., 1939). The adaptive responses may be inadequate for the reestablishment of homeostasis or excessive and prolonged; in either case a healthy steady state is not attained and pathology may ensue.

Ample evidence has been accumulated showing that stress inhibits reproductive function (RF) acting at multiple levels, both centrally and peripherally. A countless of physical, physico-emotional, emotional etc. stressors suppress the RF acting on the hypothalamic-pituitary-gonadal (HPG) axis, particularly inhibiting pituitary pulsatile luteinizing hormone (LH) secretion as a consequence of markedly decreased episodic hypothalamic gonadotropin releasing hormone (GnRH) secretion, leading to absence of ovulation (amenorrhea) and disturbance of a normal estrus or menstrual cycle.

#### STRESS AND GnRH NEURONS

The function of HPG axis, essential for normal reproduction in humans, rodents and a variety of other species is primarily maintained by the activity of hypothalamic GnRH system secretion. The GnRH system is characterized by a peculiar anatomical location: it is composed of about a few hundred neurons distributed in the hypothalamus, extending from the preoptic area to the anterior and medial basal hypothalamic regions (Merchenthaler et al., 1984). GnRH producing neurons make synaptic connection with each other, and with numerous other neurons, and are believed to form a neuronal network that is responsible for the episodic GnRH secretion (Leranth et al., 1985). This episodic pattern of GnRH release is critical for normal secretion of anterior pituitary gonadotropins (LH and FSH). Tonic LH secretion, occurs at low levels in a pulsatile pattern in response to the episodic release of GnRH into the hypophyseal portal circulation. Increased pulsatile GnRH release (amplitude and frequency) is essential for the generation of the preovulatory LH surge (midcycle LH surge) that triggers ovulation. Alterations in episodic release of GnRH appear to play a critical role in the inhibition of RF, causing absence of ovulation i.e. amenorrhea, inadequate luteal phase, infertility etc. It has recently has been shown that even a modest slowing of GnRH pulsatility (from one pulse every hour to one every two hours) in women can significantly hinder the normal endocrine dynamics of the menstrual cycle and ovulation (Filicari et al., 1991). Also, pulsatile release of gonadotropins from the anterior pituitary cannot be maintained by continuous infusion of GnRH (Belchetz et al., 1978).

Pulsatile GnRH secretion and preovulatory LH surge, which are critical for ovulation, are controlled by increased level of ovarian estradiol; positive

feedback regulation, while pulsatile tonic GnRH and LH secretion are controlled by the negative feedback actions of gonadal steroids.

GnRH secretion is additionally modulated by various compounds: corticotropin releasing hormone (CRH), neurotransmiters, catecholamines, opiates, neuropeptide pituitary hormones, gonadal steroids, etc. Some of these compounds affect episodic GnRH, LH secretion and preovulatory LH surge in inhibiting (CRH, prolactin, cytokines, inhibitory amino acids) or stimulating (gonadal steroids, neuropeptide Y, excitatory amino acids) fashion (K r s m a n o v i c et al., 1996). The role of norepinephrine on GnRH/gonadotropin release has been a matter of debate, since both stimulatory and inhibitory effects have been reported.

Much work has been devoted to understanding the mechanisms, through wich stress inhibits HPG axis. In this respect one of the most important factor is CRH. CRH is the main regulator of pituitary adrenocorticotropin hormone (ACTH) secretion and coordinator of the stress response (Chrousos, 1998). Besides CRH various stress responsive hormones: EOP (Marić et al., 1991, 1995), prolactin (Simonović et al., 1994) and catecholamines (Calogero et al., 1998) affect HPG axis, acting at all three levels, but primarily on the hypothalamic-pituitary (HP) component of the HPG axis, causing decline of gonadotropins, LH surge, absence of ovulation i.e. hypothalamic amenorrhea (HA). Hypothalamic amenorrhea is observed regardless of the types of stressor. For example, complete blockade of the preovulatory LH profile and ovulation was observed after exposure of normal cycling rats to immobilization stress (Marić et al., 1992, Roozendaal et al., 1995).

#### HYPOTHALAMIC AMENORRHEA; INFLUENCE OF METABOLIC STRESS

In response to metabolic stress (nutritional status, degree of fat stores, severe dietary restriction i.e. anorexia nervosa, energy availability, or heavy exercise i.e. ballet dancing and long distance running, etc.) failure in reproductive function has been observed with prevalence of amenorrhea and infertility (Frisch, 1990) Endocrine and neuroendocrine examinations have found that the proximal cause of ovarian and menstrual cycle disfunction in these women is HA. Hypothalamic amenorrhea is caracterized by disruption of pulsatile secretion of LH by the pituitary and this is caused by the disruption of the pulsatile secretion of GnRH by the hypothalamus (L o u c k s, 1989).

Women of normal weight may also experience the so-called functional idiopathic hypothalamic amenorrhea (FHA).

Functional idiopathic hypothalamic amenorrhea is thought to be stress-induced if exercise or dieting with weight loss is not identified. Neuroendocrine disfunction has been found in women with FHA, suggesting that central regulation of multiple hypothalamic-pituitary hormones in response to various stressors is disturbed (B e r g a et al., 1989). A common denominator in FHA is suppression of GnRH secretion. Recent data suggest that energy availability or overall energy deficit may cause the suppression of GnRH secretion that occur in FHA (L a u g h l i n and Y e n, 1997). This so-called "energy availability hypothesis" holds that dietary energy intake is inadequate for the energy costs of both reproduction and locomation (L o u c k s, 1998).

The HPG axis integrates information derived from the body's metabolic control centers and responds to maximize the organism's reproductive fitness (I'Anson et al., 1991). However, the physiological mechanisms linking metabolic center and reproduction remain poorly understood. It appears that leptin, a lipostatic hormone discovered in 1994, may act as a metabolic signal to the HPG axis. Leptin which is secreted almost exclusively by adipocytes, is the product of an obese gene (Z h a n g et al., 1994) and is involved in the regulation of food intake, body fat and energy balance (P e 11 e y m o u n t e r et al., 1995). Several lines of evidence suggest that leptin might participate in the regulation of reproductive processes, by acting at multiple levels, both centrally and peripherally (Z a m e z a n o et al., 1997).

Leptin protein is found in follicular fluid with levels corresponding to those found in the serum (Cioffi et al., 1997) Leptin levels in peripheral blood samples vary throughout the menstrual cycle, with leptin levels peaking in the luteal phase, in a manner similar to estradiol and progesterone (Greis tho vel et al., 1998). The expression of the functional leptin receptors in ovarian follicular cells (Karlson et al., 1997) supports the possible direct involvement of leptin in the ovarian function (D u g g a l et al., 2000). Also, available evidence indicates that some actions of leptin on reproduction might derive from influences on specific brain areas (B a r a s h et al., 1996). High concentrations of leptin receptor have been found in the arcuate nucleus as well as other regions in the hypothalamus, (Hakansson et al., 1998) that have been implicated in the regulation of both feeding and reproduction (Kalra and Kalra, 1996). The precise site(s) where leptin might exert its central action(s) on the reproductive processes still remain(s) not completely elucidiated. Namely, it has been shown that leptin might modulate the activity of GnRH secreting neurons in a direct way, (M a g n i et al., 1999) also it has been shown that leptin may indirectly stimulate GnRH release by acting on interneurons impinging on GnRH secreting neurons (Y u W H. et al., 1997). Thus the net effect of leptin on this process would derive from the modulation of GnRH secreting nerve terminals present in the median eminence as well as of interneurons producing transmitters such as neuropeptide Y, proopiomelanocartin-derived peptides, nitric oxide and norepinephrine (Finn, 1998). It is well known that GnRH release is controlled by a complex hormonal system. In this context leptin also might be a modulator of the reproductive function at the GnRH levels as well as at other levels (pituitary, gonads) of HPG axis, probably interacting with other agents and facilitating their actions.

In different types of HA, amenorrheic athletes, in women with anorexia, in HA that are weight or exercise-induced, the syndromes associated with negative energy balance, low leptin levels and absence of diurnal rhythm of leptin have been reported. Since a common denominator in HA is suppression of GnRH secretion, depressed leptin level seen in HA raise the possibility that metabolic factors are present in this syndrome. Understanding the disregulation that occurs in HA may provide a clue to treating the amenorrhea and to understanding the central signal suppressing GnRH in hypothalamic amenorrhea (Warren et al., 1999).

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#### СТРЕСОМ ИЗАЗВАНА ИНХИБИЦИЈА РЕПРОДУКТИВНЕ ФУНКЦИЈЕ: МЕЂУЗАВИСНОСТ ЖЕНСКОГ РЕПРОДУКТИВНОГ СИСТЕМА И МЕТАБОЛИЧКОГ СТРЕСА

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#### Резиме

У приложеном прегледном чланку дат је кратак приказ стресом изазване инхибиције пулсаторне секреције GnRH/LH и интеракције између репродуктивне функције и метаболичког стреса.

Експериментални резултати и клиничке анализе су показали да стрес (сваки стимулус који ремети динамичку равнотежу — хомеостазис организма) инхибитарно делује на репродуктивну функцију (RF). Без обзира на врсту стресора (физички, физико-емотивни, емотивни, метаболички итд.) стресори инхибишу хипоталамо-хипофизну-гонадну осовину (HPG) делујући на сва три нивоа. Код жена инхибитарно деловање стреса је нарочито изражено на нивоу хипоталамо-хипофизног комплекса, а што се огледа у изостанку пулсативног ослобађања лутеинизујућег хормона (LH) хипофизе, као последица поремећене епизодне секреције, хипоталамо-гонодотропног ослобађајућег хормона (GnRH) у хипоталамусу. Последица оваквог поремећаја је изостанак овулације и појава хипоталамо-хипогонадотропне аменореје. Хипоталамо-хипогонадотропна аменореја се јавља без обзира на врсту стресора, а нарочито као одговор на метаболички стрес. Под метаболичким стресом се подразумева: начин исхране, строга дијета као што је то случај код анорексије нервозе, интензиван физички дуготрајан напор, нпр. код балерина, атлетичарки, итд.

Информације из метаболичког контролног центра интегришу се на нивоу хипоталамо-хипофизне-гонадне осовине. Међутим, физиолошки механизми који повезују ова два центра су слабо познати. Недавно откривање лептина (1994. год.) указује да би овај липостатички хормон могао деловати у интеракцији метаболичког центра и хипоталамо-хипофизне-гонадне осовине. Зборник Матице српске за природне науке / Proceedings for Natural Sciences, Matica Srpska Novi Sad, № 100, 21—30, 2001

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## PHYSIOLOGICAL AND MOLECULAR BASIS OF STRESS

"Stress is a result of civilization which man himself no longer can withstand" wrote Hans Selye, the author of modern concept of stress.

ABSTRACT: Stress causes changes in numerous physiological, molecular, biochemical and behavioral events, as a reaction of all living organisms to any homeostasis disturbance, by translating extracellular signals into a specific cellular response *via* the process of signal transduction. This paper briefly summarizes the data from a number of laboratories, including our own, providing results on important physiological and molecular activities of living organisms under different stress conditions.

The aim of this paper is to send the message that stress as a syndrome disturbs all vital functions of living organisms, leading to a broad spectrum of diseases and disturbances. Stress is the silent killer.

KEY WORDS: stress, HPA axis, glucocorticoid receptor, Hsps proteins, signal transduction

#### AND WHAT IS STRESS?

There is still no clear-cut definition for stress in the medical pathology textbooks, but current literature defines it as "the set of all organic reactions to physical, chemical, environmental, psychological (emotional), infectious, or other aggressions, which are capable of disturbing the homeostasis". Stress is a common phenomenon which is attracting increasing attention. The term "stress", although having a very broad meaning, generally describes a state of disturbed homeostasis, harmony and equilibrium. The disturbing forces, or inputs, are considered "stressors", while the counter-acting forces, or outcomes, are called the adaptation.

It is important to understand that stress always corresponds to a relation between the environment and the individual. It means that an aggression and a response have occurred and that an interaction took place, as it has been proposed by the Canadian physician Hans Selve, the creator of the modern concept of stress (S e l y e, 1946). In the face of danger, the organism changes its inner balance and priorities into a high physiological arousal, to enable these two functions. The fight-or-flight response (termed by Cannon and Selye in the 1930s) is a pattern of physiological responses that prepare the organism for emergency. When the external balance is disrupted, the body changes its internal balance accordingly. According to Cannon/Selye, the so-called physiological stress is a normal adaptation syndrome. When the response is pathological such as in an ill-adapted individual, or in a situation where the stress stimulus persists for a long time, an organic malfunction takes place, which may lead to transitory disturbances or to severe manifestations of disease. Activation of the stress system results in changes that allow the organism to adapt. See man et al. (1997) recently suggested to use *allostasis* to describe the process of adaptation to stress and developed a measure of allostatic load based on parameters reflecting levels of physiologic activity across a range of important regulatory systems. The allostatic load is the sum of a number of parameters, and this concept seems a more comprehensive assessment of major risks in the aging process.

It seems that stress will be the syndrome of the century, having in mind a broad spectrum of disturbances in all levels of organization of living organisms.

#### WHAT ARE THE FUNCTIONAL BASIS OF STRESS?

The scientist who has described for the first time the phenomenon of stress, Hans Selye, has described a generalized physiological response to stress. The hypothalamic-pituitary-adrenal (HPA) axis is the biological interface for neuronal and humoral communication between CNS and peripheral glands, organs responsible for mobilizing the stress response resulting in specific behavioral adaptation.

The perception of an imminent or traumatic event is made by the cortex, a part of the brain structure. This perception is mediated by an enormous network of neurons which comprise large parts of the brain, including the memory circuits.

Once the importance of the stimulus is determined, the cortex activates a subcortical brain circuit in one of its parts, the limbic system, by means of the neural structures which control emotion, as well the autonomous nervous system, responsible for the control of function of the so-called visceral systems (heart, blood vessels, eye pupils, stomach, intestines, glands, etc). These structures are mainly the amygdaloid bodies and the hypothalamus. Their activation will lead to many bodily changes as well as an increase in the secretion of the hormones adrenaline and noradrenaline by the medullar part of the adrenal glands.

We now know that vital chemicals carry messages between brain cells. In essence, they allow brain cells to "talk" to one another. On a typical day in

the brain, trillions of messages are sent and received. Most nerve centers receive input from different types of messengers, and the most important are serotonin, noradrenalin and dopamine. As long as this input is balanced, everything runs along on an even keel. Stress causes problems with brain messengers and the whole brain becomes distressed. Serotonin must work properly in order to maintain normal sleep and mood patterns. Inside of our brain there is a very precise "clock". The body clock is located deep in the center of the brain, in the pineal gland, which is a store of serotonin, the chemical "mainspring" of the clock. Each day, serotonin is converted right back to serotonin.

Noradrenalin has many important functions in the body's nervous system. The one that most concerns us here, however, is the role of noradrenalin in setting energy levels. And finally, dopamine seems to be concentrated in areas of the brain immediately adjacent to where the major endorphin-releasing mechanisms lie. Endorphins are responsible for regulating our moment-to-moment awareness of pain. When too much stress causes failure of dopamine function, it causes loss of body's natural "pain killer".

At the same time, the hypothalamus activates the pituitary. Under stressing stimuli, the main hormone produced by this gland is ACTH, also known as the "stress hormone". Carried by blood, ACTH increases the secretion of other hormones, called corticosteroids. These hormones have many actions over the body tissues, by altering its metabolism, the synthesis of proteins, the resistance to invaders of our organism by the immune system, inflammations provoked by infections, or damage of the tissues, etc. The degree of activation of this brain-pituitary-adrenocortical axis can be evaluated by measuring the level of cortisol, one of our inner corticosteroids. Cortisol is the main stressfighting hormone. When cortisol secretion is high, the body shifts to a "war footing".

The double discharge of hormones which are intensively bioactive, adrenaline and the corticoids has lead scientists to believe that the pituitary and the adrenals are the main mediators of stress.

Stress is associated with activation of the HPA axis. Stress first increases levels of corticotropin-releasing hormone (CRH) and other secretogogues in the hypothalamus. Release of CRH leads to subsequent stimulation of ACTH release from the anterior lobe of the pituitary, that, in turn, stimulates the release of glucocorticoids from the adrenal cortex. Similar effects are noted in aging, where there is a tendency for increased HPA activity and circulating glucocorticoids implicated in the occurrence of hippocampal pathology and memory deficits. The increased glucocorticoid levels have traditionally been ascribed to their physiological function of enhancing an organism's resistance to stress. Glucocorticoids exhibit immunosuppressive activity and are critically involved in adaptation to stress and in the control of metabolism. However, contrary to the traditional view, it has become increasingly clear that glucocorticoids at moderate to high levels also suppress defense mechanisms, such as the anti-inflammatory and anti-allergic activities.

Munck and colleagues (Munck and Naray-Fejes-Toth, 1992) clarified the permissive and suppressive effects of glucocorticoids as follows.

Permissive effects, which are usually stimulatory, are those by which glucocorticoids at basal levels "permit", or normalize responses to stress. In contrast, suppressive actions represent physiological function of glucocorticoids to protect the body against damage from its own activated defense mechanisms, preventing overshoot. Among many examples are the inhibition by glucocorticoids of the production of numerous mediators of defense reactions, including hormones such as antidiuretic hormone and insulin, as well as the immune cytokines and gamma-interferon, all of which are dangerous when in excess. Glucocorticoids suppress production of mediator and induce its receptors simultaneously in two opposing ways and mediator activity as a function of cortisol concentration can be described by a biphasic or bell-shaped curve. Based on these findings, it was suggested that the permissive and suppressive effects, which in juxtaposition appear paradoxical, can be viewed as complementary functions through which defense mechanisms are regulated by glucocorticoids over the full range of their basal and stress-induced concentrations, thereby preventing those reactions from overshooting to threaten homeostasis. Cortisol, as a main representative of glucocorticoid hormones, plays a major role in the intracellular communication and its importance for the survival upon a stress has been well documented long ago. The molecular basis of its action is well presented in Matić's review (Matić, 1995) and a better insight in the complexity of molecular events occurring in a cell during adaptation to unfavorable conditions are explained in many articles.

The events at the physiological level have the basis at the molecular level including activation of complete cellular machinery, including different signal transduction pathways within and between cells. Binding of signal molecule to receptor triggers subsequent intracellular signaling networks. Through a series of steps, the message from that signal gets transmitted and amplified within the receiving cell or molecule, often leading to activation or deactivation of specific transcription factors in the nucleus. Among the corticosteroids, the glucocorticoids are especially attractive because of a current list of their effects might include disparate items. The special roles of glucocorticoids in stress (M a t i  $\acute{c}$  et al., 1998), their relationship with shock proteins ( $\check{C}$  v o r o et al., 1998a; Butorović et al., 1994; Čvoro et al., 1998b), acute phase reactants (Poznanović et al., 1997), oncogenes, cancer, endocrine diseases, etc., make a spectrum of responses which can be elicited depending on the cell type, or environmental stimuli (Stanković et al., 1986; Trajković et al., 1981; Trajković et al., 1992; Dunđerski et al., 1996; Dunđerski, 1997).

A vast majority of diverse and widespread physiological effects of glucocorticoid hormones are mediated through the glucocorticoid receptor (GR) protein (T r a j k o v i ć, 1989), a transcription factor directly involved in the regulation of gene expression in glucocorticoid target tissues. *In vivo*, the hormone binding capacity of the receptor, the rate of its transformation to DNA-binding state, the affinity of the GR-GRE interaction and the efficiency of the receptor in transcriptional regulation are all subject to regulation enabling the final cellular response to glucocorticoid hormones to be turned in accordance with changing physiological and environmental conditions and instantaneous cellular demands (Munck et al., 1972; Vidović et al., 1996)

Steroid-free GR is assumed to be localized in the cytoplasm of target cells, where its two forms: active (i.e., capable of ligand binding) and inactive are maintained in equilibrium. The ligand binding to the receptor promotes its transformation from a weak to a tight DNA-binding form. The transformation process is accompanied by dissociation of the untransformed GR heterocomplexes, comprising some Hsps, an immunophiline and other proteins, and by conformational alteration of its steroid-binding domain and is followed by the nuclear import of the receptor occurring through nuclear pore complexes. Glucocorticoids and other steroid hormone receptors, when present in intact cells in non-activated, non-DNA-binding form, are associated with the dimmers of HSP90. These complexes contain also other heat shock proteins (Hsp70, Hsp56) and some others proteins. Although the Hsps were shown to chaperone the receptor, playing a role in its folding, stabilization and intracellular shuttling, the functional significance of the receptor-Hsps association is still under consideration. After the transformation, the receptor's ligand binding domain remains in a conformation incapable of steroid binding While the ligand binding provokes the GR transformation and nuclear import, the dissociation of the hormone from the receptor contrarily results in its efflux from the nucleus and reappearance within the cytoplasm.

The physical environment of organisms causing stress, damage or even death. Even minor environmental changes may hamper the physiological capacity of organisms to grow, reproduce, or interact socially. Not surprisingly, organisms have developed a variety of adjustments such as behavior, acclimation and heat stress responses, acute-phase responses, etc., that help buffering the physiological impact of environmental change. These adjustments, which can have a profound effect on evolutionary fitness, have been studied from diverse aspects, one of them being the molecular one. Molecular biological interest in the heat shock response was kindled by Ritossa's report (Ritossa, 1962) that brief heat treatment of *Drosophila* larvae induced dramatic alterations in gene activity as judged by the changes in puffing patterns observed in the salivary gland polytene chromosomes. Soon thereafter it become clear that all living organisms, from bacteria to man, respond at the cellular level to unfavorable conditions such as heat shock, or other stressful conditions of many different origins, by a rapid, vigorous and transient acceleration in the rate of expression of a small number of specific genes, termed heat shock genes. The products of these genes, commonly referred to as heat shock proteins (Hsps) or stress proteins, most of which are also present under normal circumstances but in lower concentrations, increase and accumulate in cells to reach, in some instances, fairly high levels. A great deal of circumstantial evidence supports the belief that the heat shock response is a rapid, but transient reprogramming of cellular activities aimed to induce or increase the synthesis of the Hsps which would ensure survival during the stress period, protection of essential cell components against heat damage and a rapid resumption of normal cellular activities during the recovery period. Some Hsps, including Hsp90 and Hsp70, serve fundamental cellular roles, chaperoning proteins during folding,

functioning and intracellular trafficking and are found *in vivo* in complexes with a number of transcription factors and protein kinases.

#### CAUSES AND CONSEQUENCES OF STRESS

In general, acute or chronic stress can be attributed to the biggest problems we encounter in the course of our life. Several researchers have noted that any kind of change in our daily lives, either good or bad, is among the most effective causes of stress. The intensity of stress will depend on the nature and the degree of change, which can go from major ones to relatively trivial life changes.

Certain events in our lives are so severe in terms of psychological stress that they are characterized as a trauma (lesion or damage). Recently, the mental health sciences have recognized the existence of a new syndrome called Post-Traumatic Stress Disorder (PTSD), a real disease, classified in the area of the major anxieties. The syndrome started to be studied in the USA, after return of veterans of the Vietnam war.

This does not apply to war veterans only. It is enough to see what happens to victims to the increasing levels of urban violence. Bombings, automobile accidents or aircraft crashes, earthquakes and floods, kidnapping, rape, child or spousal abuse, etc. are often the cause of post-traumatic stress disorder.

Physicians have recognized that chronic stress has three distinct phases, which occur sequentially one after the other in the case the stressing agents are not suspended:

*The acute phase*: The phase when the stressful stimuli start to act. Our brain and hormones respond quickly and we usually perceive the effects, but we are unable to see the silent working of repeated stress in this phase.

*The resistance phase*: The phase when the first mental, emotional and physical consequences of chronic stress start to appear. Loss of concentration, emotional instability or depression, heart palpitations, cold sweating, muscular pains or headaches are the usual telltale signs, but most of the persons do not relate them to stress, and the syndrome may progress to the last and most dangerous phase.

*The exhaustion phase*: This is the phase when the organism capitulates to stress, when organic and psychological diseases begin.

Since it is not possible in such a brief survey to list, let alone describe the entire array of the disturbances developing during stress situations, we will mention here only some of them:

*Stress and aging*: The researchers hypothesized that old age results from the accumulated stress experienced during an organism's life span (Platner, 1961).

Selye and colleagues have proposed and characterized aging as a systematic loss of adaptation (S e l y e, 1976). Aging organisms show an elevation of basal corticosterone level, a major glucocorticoid that plays a key role in the neuropathological effects of stress, and an impaired capacity to adapt to and recover from stress, suggesting that there is an age-related loss of sensitivity of the brain and pituitary to the negative feedback of high circulating levels of corticosterone. Glucocorticoids sensitize hippocampal structures, making hippocampal neurons less likely to survive coincident neurological insults, perhaps by inhibition of glucose transport leading to energy production decreases within cells (Lupien and Meaney, 1998).

In the 1960s, H a r m a n (1960), based on his findings that aging and ionizing radiation have similar effects on mutagenesis, cancer and cellular damage, proposed "free radical theory of aging". This theory proposes that free radicals are produced in aerobic metabolism and damage they inflict to biomolecules is a major contributor to aging. Oxidative damage to biomolecules produced by oxidants, including free radicals, has been postulated to be a type of endogenous damage, contributing not only to aging but also to a variety of diseases, especially the age-related degenerative diseases.

Stress, cancer and immune system: Stress may be related to the development and progression of cancer, in part due to suppression of immunity (Keller et al., 1981). Stress reduction has been considered as a factor in reducing the mortality rate of cancer patients, and it has been hypothesized that relief or prevention of chronic stress by cognitive therapy may be a promising method for cancer prevention (Cholst, 1996).

Psychological stress adversely affects the immune systems, or affects many features of cellular immune function, including cytokine production (Cooper, 1984). Cellular immunity has an important role in the regulation of wound repair. Pro-inflammatory cytokines, such as IL-1, IL-6, IL-8 and tumor necrosis factor (TNF), help to protect against infection, prepare injured tissue for repair and enhance phagocyte recruitment and activation. Psychological stress is important in the onset and course of major depressive disorders. Patients with major depression show immunosuppression as consequence of hypercorticolism. However, glucocorticoids are not always immunosuppressive, but may enhance certain components of the immune response.

Nutrition and stress: Nutritional status and stress are closely related. Nutrient deficiencies are stressful to the organism. Long-term protein undernourishment, especially if the individual is subject to stress or injury, can be detrimental. Previously outlined immune system malfunctioning in undernourished subjects, especially when protein intake is insufficient, is likely a secondary effect of the HPA overstimulation (Klebanov et al., 1995; Hjaijej et al., 1998; Flynn and Wu, 1997). Chronic food restriction leads predictably to hypercorticosteronemia, hypoglycemia and enhanced adrenal glucocorticoid content. Glucocorticoids follow a diurnal pattern that is more pronounced in animals under the chronic food restriction regimen. The peak elevation can be observed after one month of food restriction. The elevation in blood and adrenal corticosterone levels is a consequence of the fasting-induced adrenal hypertrophy in animals and a cause for reduced responsiveness to acute stress. The influence of chronic food restriction on basal neuroendocrine, immune and adipocyte functions, and the acute-phase response to endotoxic shock were also established.

Protein calorie malnutrition can result in a significant macrophage dysfunction (C u r t i s et al., 1995). It is believed that this malfunction is more likely to be a consequence of elevated glucocorticoids, rather than of the primary nutrient deficit. In a mouse study comparing standard 24% protein diet with a protein free diet, it has been observed that protein malnutrition after 7 days impaired the macrophage functioning (IL-6 levels were measured), and elevated serum glucocorticoid levels. Administration of the glucocorticoid receptor antagonist, RU486, prevented the impairment of the macrophage functioning. Under the same dietary conditions, exogenous glucocorticoid addition (subcutaneous pellets) reproduced the macrophage impairment.

In the end it can be concluded that stress and the consequent disorders, however extensively discussed as a general and unspecific syndrome, represent in fact the disturbances affecting all functions of an organism that could provoke development of most severe diseases.

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#### ФИЗИОЛОШКА И МОЛЕКУЛАРНА ОСНОВА СТРЕСА

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#### Резиме

Стрес изазива многобројне промене физиолошких, молекулских и биохемијских догађаја, укључујући и промене у понашању код виших организама, превођењем екстраћелијског сигнала у специфични одговор ћелије процесом трансдукције сигнала, као реакција сваког живог организма на поремећај хомеостазе. У овом раду учињен је покушај да се кратко сумирају резултати многобројних лабораторија, укључујући и нашу, који пружају доказе о важним физиолошким и молекулским активностима живих организама под различитим стресним условима.

Циљ овог рада је да се пошаље порука да је стрес синдром који ремети све виталне функције у живим организмима, доводећи до широког спектра обољења и поремећаја. Стрес је тихи убица. Зборник Матице српске за природне науке / Proceedings for Natural Sciences, Matica Srpska Novi Sad, № 100, 31—37, 2001

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### MOLECULAR ANALYSIS OF MICROSATELLITE-LIKE REPETITIVE DOMAIN AND N-TERMINUS OF *Glu-A*<sup>m</sup>3 GENE

ABSTRACT: Polymerase chain reaction (PCR) analysis of genomic regions corresponding to the *Glu-A*<sup>m3</sup> locus in six *Triticum monococcum* accessions revealed size variation among these DNA fragments. To confirm the presence of a compound microsatellite, DNA fragments have been cloned and subjected to sequence analysis. The size variation of DNA fragments differing in  $(CAG)_n(CAA)_n$  repeat composition was the only source of length variation and it corresponded to *Glu-A*<sup>m3</sup> allelic variation of B glutenin subunits.

Cloning and sequencing of 9-7 clone revealed a sequence with similarity of up to 96% to the previously published homeologous *Glu-3* sequence. This sequence also harbors two stop codons and therefore represents a pseudogene member of a multigene family encoding LMW GS in *T. monococcum*.

KEY WORDS: glutenins, PCR, pseudogene, T. monococcum

#### **INTRODUCTION**

Einkorn wheats-diploid species (2n = 2x = 14) of the genus *Triticum*, include A genome species *T. monococcum* and *T. urartu*. Like in other *Triticeae*, the einkorn wheat kernel is a rich source of protein due to large amounts of seed storage proteins deposited in the endosperm during seed development. A major fraction of seed storage proteins, prolamins, is composed of glutenins, capable of forming large proteinaceous aggregates held together by disulfide bonds and classified into high- and low-molecular weight glutenin subunits (HMW and LMW GS). The later are encoded by homeologous *Glu-3* loci, mapped to the short arms of group 1 homeologous chromosomes in the genus *Triticum* (G u p t a and S h e p h e r d, 1990). According to amino-acid sequences of LMW glutenin subunits, they have been classified into a group of

sulfur-rich prolamins, together with gamma  $\alpha/\beta$  and / gliadins (S h e w r y et al., 1995).

Both the genomic and cDNA Glu-3 clones from *T. aestivum* and *T. durum* (Colot et al., 1989; Cassidy and Dvorak, 1991) show extensive similarities in organization, with a more repetitive N-terminal domain and 3' located a modular C-terminal coding region containing most of the cysteine residues.

A number of cereal pseudogenes have been reported, including pseudogenes for two wheat HMW GS (F or d e et al., 1985; H a r b e r d et al., 1987), and gamma-gliadin (R a f a l s k i, 1986). A n d e r s o n and G r e e n e (1997) found that eight of twenty known  $\alpha$  gliadin genomic sequences were pseudogenes. It is well known that prolamin genes have regions of microsatellite-like repeats coding for glutamine residues. Point mutations such as transition C  $\rightarrow$  T could easily transform CAG and CAA codons in stop codons TAG and TAA, respectively. Predominance of C  $\rightarrow$  T transition could be explained by the fact that a large amount of DNA in higher plants is methylated. Up to 20% of cytidine could be found as 5-methyl-cytidine which could easily be incorrectly replicated as thymidine.

Here we report a detailed analysis of a microsatellite-like repeat within genomic DNA regions and a novel *Glu-3* sequence from *T. monococcum* that apparently encodes a LMW-GS but has undergone several mutations in recent evolutionary history thus becoming a psedogene.

#### MATERIAL AND METHODS

Plant material: The analyses were carried out on six *T. monococcum* accessions which were kindly provided by Vavilov Institute, Sankt Petersburg, Russia (accessions M1, M9, M13) and by Institute of Field and Vegetable Crops, Novi Sad, Yugoslavia (M2, M4, M5). Hexaploid wheat cultivar Chinese Spring was used as reference.

DNA extraction: Genomic DNA was isolated from young leaves of single plants essentially according to the mini-prep procedure of Lichtenstein and Draper (1988).

PCR amplification: PCR amplifications were carried out in a DNA Thermal Cycler (Techne) on 20 ng template DNA in a 50 l reaction containing 1X Taq buffer (Gibco BRL Life Technologies), 1.5 mM MgCl2, 200 nM primer, 200  $\mu$ M of each dNTP and 1 U of Taq polymerase (Gibco BRL Life Technologies). For amplification of the microsatellite region primers P1 and P2 (D e v o s et al., 1995) were used (forward primer 5'TCCCGCCATGAGTCA-ATC3' and reverse primer 5'TTGGGAGACACATTGGCC3'). The primer sequences used for amplification of N-terminus were according to V a n C a m p e n h o u t et al. (1996):

forward primer 5'CGCCGTTGTGGCGACAAGTA3' reverse primer 5'GTTCTTGTAGGATGATGGAGTAGG3'

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clone 9-7	1	GCC	GTT 	GTG	GCG	ACA	AGT	ACC	ATT 	GCG	саа   •	ATG	GAG	аст 
pTdUCD1	88	GCC	GTT	GTG	GCG	ACA	AGT	ACC	ATT	GCG	CAG	ATG	GAG	ACT
	40	AGT	TGC	ATC		GGT	TTG	GAG	AGA	CCA	TGG	CAG	GAG	CAA     CAA
	70	AGC	TGC	AIC	CCA	CAA	mac	ACA	mma	mmm	CCA	CAG	CAA	(TAA)
	166		11A     TTA	III CCA	III CCA		·   · CAC	ACA     ACA	111A 111 TTA	 TTT				·     CAA
	118	CCA	CCA	TTT	TCA	CAA	CAA	CAA	CCA	TCA				
	205	 CCA	····	 TTT	•   CCA	 CAA	 CAA	 CAA	•   CAA	•   CCA				
	145									TTT	TCG	CAG	CAA	
	229	CCA	••••	тса	CAA	CAA	CAA	CCA	тса		I ·   TTG	CAG	CAA	
	157	CAA	CCA	ATT	СТА	CCG	CAG	CCA	CCA	TTT	TCA	CAG	CAA	CAA
		111	111	111	111	111	111	1.1	111	111	111	111	111	111
	265	CAA	CCA	ATT	CTA	CCG	CAG	CTA	CCA	TTT	TCA	CAG	CAA	CAA
	196	CAA	CCA	GTC	TTA	CCG	CAA	CAA	TCA	CCA	TTT	TCG	CAG	CAA
	304	CAA	CCA	GTT	CTA	CCG	CAA	CAA	TCA	CCA	TTT	TCA	CAG	CAA
	235	CAA	CTA	GTT	TTA	CCT	CCA	CAA	CAA	CAA	TAC	CAA	CAG	CTT
	343	 CAA	 CTA	 GTT	 TTA	 CCT	CCA	CAA	CAA	CAA	 TAC	 CAA	 CAG	•     GTT
	274	CTG	CAA	CAA	CAA	ATC	CCT	ATT	GTT	CAG	CCA	TCC	ATT	TTG
	382	CTG	CAA	CAA	CAA	ATC	CCT	ATT	GTT	CAG	CCA	TCC	GTT	TTG
	313	CAG	CAG	СТА 	AAC	CCA	TGC	AAG	GTA	TTC	CTC	CAG	CAG	AAG •
	421	CAG	CAG	CTA	AAC	CCA	TGC	AAG	GTA	TTC	CTC	CAG	CAG	CAG
	352	TGC	AAC	ССТ 	GTA 	GCA	ATG 	CCA	GAA •	CGT 	СТТ 	GCT 	AGG	тса   ·
	460	TGC	AAC	CCT	GTG	GCA	ATG	CCA	CAA	CGT	CTT	GTC	AGG	TCG
	391	CAA	ATG	TGG  ·	CAG	CAG	AGC	AGT	TGC	CAT	GTG	ATG	CAG	
	499	CAA	ATG	TTG	CAG	CAG	AGC	AGT	TGC	CAT	GTG	ATG	CAG	CAA
	430 538		TGT	TGC		CAG								
	460	CAA	 	CAT	CAG	Ame	CCT	CCG	ATC	ACC	mac	mcc	ATC	300
	577	III CGC	III III TAT	GAT GAT	·  GTA	ATC ATC	III CGT	III GCC	ATC ATC	III ACC	III TAC	III TCC	ATC	 ATC
	508	CTA	CAA	GAA	С	5	7							
	606	•   TTA	 CAA	 gaa	l c	6	15							

Fig. 1. — PCR amplification of *T. monococcum* accessions. Bands larger than 180bp represent artifactual amplifications as revealed by hybridization to pLMWGT2 probe (Colot et al., 1989) under high stringency (data not shown). CS stands for Chinese Spring.
Amplification products were separated on 4% non-denaturing polyacrilamide gels in 1X TBE buffer and visualized by silver staining.

Southern blotting, hybridization, cloning and sequencing of amplification products were performed essentially according to T a n u r d z i c et al. (1998). Sequence pT-Adv clone 9-7 was compared with the GenBank database using BLAST ver. 2.0. 3 (Altschul et al., 1997).

# RESULTS

In order to investigate variability of Glu-3 locus, genomic DNA fragments corresponding to a microsatellite-containing region of Glu-3 genes were isolated by PCR using a set of locus-specific primers. PCR amplified pairs of DNA fragments in all accessions, except for M4 and M9 where only a single amplification product was observed within the fragments in the range from 132 bp to 170 bp (Figure 1).

To verify the assumption that the variability in Glu-3 locus is contributed at least in part by differences in the number of  $(CAG)_n(CAA)_n$  repeats outside of the repetitive region of Glu-3 genes, some DNA fragments amplified by PCR were cloned and subjected to sequence analysis. This confirmed the presence of a compound microsatellite with  $(CAG)_n(CAA)_n$  repeats (Figure 2). The number of repeats among those fragments varied from 9 to 17 and it was the only source of the observed sequence length variation (Figure 2). Sequences flanking the repeat were highly conserved among different Glu-3 alleles as well as between Glu-A3 alleles of bread wheat cultivar Chinese Spring (Figure 2).

band	F4																								R4
	⇔																								¢
1	cgt	gct	atc	atc	tac	tct	atc	atc	ctg	(cag)5	(caa)6	cag	ggt	cag	agt	atc	atc	caa	tat	cag	(caa)3	ccc	caa	cag	
2										(cag)1	(caa)8		-t-						gc-						
3				g						(cag)8	(caa),							g							
4										(cag)2	(caa)8														ttg
5				g						(cag)7	(caa)8							g							
6										(cag)5	(caa)6														
7	g									(cag) <sub>4</sub>	(caa)5														

Fig. 2. — Sequence alignment of *Glu-A*<sup>m3</sup> (CAG)<sub>n</sub>(CAA)<sub>n</sub> repeat-containing region. Deletions are marked by dots and identical bases by dashes. Position of the microsatellite-like repeat is shaded and arrows mark the primer positions. Band numbers 1—7 correspond to identically numbered PCR products in Figure 1.

Amplification of the 3' sequence of signal peptide, N terminus, proline-rich repetitive domain, and 5' sequence of C terminus using F6-R6 set of primers generated genomic fragments ranging in length from 450 to 550bp. A single amplification product was observed in accessions M1 and M2, while the other accessions had pairs of bands. Using the same primers, V a n C a m p e n h o u t et al. (1995) amplified a 554 bp-long PCR product from genomic DNA of *T. aestivum* cultivar Chinese Spring and showed by aneuploid analysis that the primers were specific for the A genome of this hexaploid cultivar. Chinese Spring was used as the control cultivar in the analysis reported here.

For further study PCR products were cloned in pT-Adv vector. Clone 9-7 (PCR product of M2 accession) was fully sequenced. The nucleotide sequence of clone 9-7 was compared with the nucleotide sequences present in a non-redundant set at GenBank. As predicted, the best matches were found with Glu-3 sequences from T. aestivum and T. durum (over 90% identical nucleotides with minimum number of deletions/insertions). Sequence of clone 9-7 showed repetitive structure of *Glu-3* genes of *T. monococcum*. The primary repetitive motif, P-O-O-P-P-F-S-O-O, showed high homology with the standard repetitive module of *Glu-3* genes from 4X and 6X wheats, and  $\alpha$  and gamma gliadins (S h e w r y et al., 1995). A detailed nucleotide sequence comparison with T. durum TdUCD1clone (GenBank accession No X51759) showed 96% of identical nucleotides with clone 9-7 (Figure 3) Clone 9-7 differs from TdUCD1 by one 24 bp deletion in the repetitive domain, and 22 point mutations. Twelve of them were transitions TC, CT, ten were found to be silent mutations. Clone 9-7 was classified as pseudogene because of two stop codons, which were found to be the result of two transitions TC at positions 95(TAG) and 115 (TAA).

#### DISCUSSION

Significant variability is present in *Glu-3* locus of einkorn wheats. L e e et al (1996) reported 60 different B-LMW GS patterns among einkorn wheat accessions. In spite of a significantly smaller number of accessions analyzed, our results confirm a large extent of variation present in *Glu-3* locus of *T. monococcum* with important implications for breeding programs oriented toward improved bread-making quality.

Majority of analyses aimed at understanding the variation in LMW GS size stressed the importance of the repetitive region within the *Glu-3* gene as a source of allelic variation (L e e et al., 1996; M a s c i et al., 1996). However, no compelling evidence has been presented so far that conclusively pointed out the repetitive region as the only source of variation among the expressed *Glu-3* sequences. Sequences of the microsatellite-like region reported herein suggest that *Glu-3* regions other then the repetitive region could be sources of allelic variation in *Glu-3* locus.

Significant variation in the observed sizes of PCR products, using the primers originally reported by Devos et al. (1995), should also provide new molecular markers for monitoring introgression of favorable *Glu-3* alleles form wild wheats at the early stages of modern wheat breeding programs.

The presence of pseudogenes in multigene prolamine family has been observed in maize for zeins (Heidecker and Messing, 1986), in wheat for HMW (Harberd et al., 1987), LMW (Lee et al., 1999),  $\alpha$  and gamma gliadin genes (Anderson and Greene, 1997; Rafalski, 1986). Lafiandra et al. (1984) have resolved at least 16 major  $\alpha$ -gliadin spots by 2-D PAGE of protein extracts from hexaploid wheat cv. Cheyenne and that number was considerably less than the estimated 150 genes (Anderson et. al., 1997). The possible explanations for the observed differences in protein products/gene number were that many of the family members were pseudogenes and/or that single protein bands could originate from multiple genes (A n - d e r s o n and G r e e n e, 1997).

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#### МОЛЕКУЛАРНА АНАЛИЗА МИКРОСАТЕЛИТСКОГ РЕГИОНА И N-КРАЈА *Glu-A*<sup>m</sup>3 ГЕНА

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#### Резиме

Применом ланчане реакције полимеразе (PCR) анализирани су геномски региони *Glu-A*<sup>m</sup>3 локуса шест популација врсте *Triticum monococcum*. Откривено је варирање у величини ових ДНК фрагмената и у циљу утврђивања присуства, форме и величине микросателита, ДНК фрагменти су клонирани, а одабрани клонови секвенцирани. Утврђено је да разлике у величини ДНК фрагмената потичу од различитог броја умножака (CAG)<sub>n</sub>(CAA)<sub>n</sub> микросателита и да разлика у величини одговара алелној варијабилности субјединица Б глутенина мале молекулске масе кодираних генима *Glu-A*<sup>m</sup>3 локуса.

Клониран и секвенциран 9-7 клон показао је сличност од 96% са већ објављеном секвенцом хомеологног *Glu-3* клона. Ова секвенца садржи два стоп кодона и представља псеудоген мултигене фамилије која кодира ЛМВ ГС код врсте *T. monococcum*.

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# CHARACTERISTICS OF BLOOD AND ADIPOSE TISSUE FATTY-ACID COMPOSITION IN OBESE PERSONS

ABSTRACT: Composition of fatty acids (FA) in obese persons was investigated in four fractions of blood lipids (FFA, cholesterol esters, triglycerides, and total lipid fraction) and subcutaneous adipose tissue samples taken from the anterior abdominal wall. The method of gas-liquid chromatography allowed separation of a number of saturated, mono- and polyunsaturated FA, five most important of them being analyzed in particular. In contrast to total FA, which are significantly increased only in the fraction of total serum lipids, the level of stearic acid (18:0) was significantly lowered in both the FFA fraction and serum total lipids, and especially in the adipose tissue of extremely obese persons. Of polyunsaturated FA, linoleic acid (18:2 n-6) showed a statistically significant decrease in practically all serum lipid fractions, the decrease of linolenic acid (18:3 n-3) level being characteristic of only adipose tissue. In contrast to this, contents of the polyunsaturated arachidonic acid (20:4 n-6) and nonessential eicosatrienoic acid (20:3 n-9) were significantly increased. The obtained results are practically identical to the findings for other pathological states related to the accelerated development of atherosclerosis (the sudden death of cardiovascular diseases, diabetes mellitus, and hyperlipoproteinemia IIb and IV).

KEY WORDS: obesity, fatty acids, adipose tissue, serum lipids

### **INTRODUCTION**

Composition of fatty acids (FA) in blood and other tissues depends on the pathways of FA synthesis and the processes of their elongation and desaturation on the one hand, and on the nutrition mode, i.e. amount and sort of alimentary fat intake (L e p s a n o v i c, 1997) on the other. Significant differences have been found for the adipose tissue composition in the Americans, Japanese, and some of European populations, which is certainly a consequence of the mode of nutrition and life in the geographically different environments (L e p s a n o v i c, 1982). It has been observed that the Japanese immigrants to the USA that had accepted American way of life and nutrition had fattyacid compositions similar to that of the native Americans (I n s u 11 et al. 1969). Quite similar results were also obtained for the Eskimo immigrants from Greenland to Denmark (D y e r b e r g, 1982). In our previous studies we have found that the persons taking mainly animal fats, if compared to those being mainly on vegetable diet, have a significantly increased content of total monounsaturated and a decrease of total polyunsaturated FA, among the later, especially marked decrease being characteristic of the essential linoleic acid (L e p s a n o v i c et al., 1986). In another study we have found that the persons being on a long-term strict ovolactovegetarian diet had significantly lower values of total saturated and monounsaturated FA, and significantly higher contents of polyunsaturated FA (being primarily a consequence of a significant increase in linoleic acid level) in comparison with the persons being on the diet characteristic of our geographic region (L e p s a n o v i c et al., 1992).

It has been unambiguously proved that the physical, chemical, and biological characteristics of various lipids are greatly influenced just by those FA that are in the form of their esters present in every lipoproteinic fraction. Numerous experiments and epidemiological studies on humans have shown that the altered content of certain FA plays a significant role in the genesis and development of coronary heart disieases, thromboembolic incidents, and atherosclerosis in general (W atts et al., 1995; K n a p p, 1997; K r i s - E th e r t o n and Y u, 1997; L i c h t e n s t e i n et al., 1998). Namely, a number of authors showed that the persons suffering from atherosclerosis of coronary and peripheral arteries have significantly lower concentrations of some polyunsaturated FA and significantly higher concentrations of some saturated long-chain FA (H u et al., 1997; C a g g i u l a and M u s t a d , 1997; P i e t i n e n et al., 1997).

Obesity, especially that of android type, is frequently associated with lipid disturbances that are characterized by the lowered clearance of hylomicron and increased contents of the lipoproteins rich in triglycerides, predominant population of small, dense, and markedly atherogenic LDL particles, and a decreased content of the HDL-cholesterol (mainly on account of the protective  $HDL_2$  subfraction) (V a n G a a 1, 1995). On the other hand, it is well known that obesity leads to numerous and heavy complications in various organs and organ systems, metabolic disturbances and heart diseases being most important among them. It is quite logical to assume that the mentioned changes in the metabolism of lipids and lipoproteins are connected with the changes in fatty-acid composition in blood and other media in the organism, which, understandably, arouses great research interest.

The aim of this work was to give a contribution to the study of fatty-acid composition in blood and adipose tissue in obese persons of our population and compare the findings with the results of the studies carried out in other environments and on other pathological states.

# MATERIAL AND METHODS

Studes of the serum fatty-acid composition in obese persons were carried out in the fraction of free fatty acids (FFA) (n = 25), fraction of cholesterol

esters (n = 10), triglyceride fraction (n = 10), and the fraction of total serum lipid (n =19). The results were ompared with those obtained for metabolically healthy persons of normal nutritional status, serving as control groups (n = 14, 30, 27 and 36, respectively). The groups of test subjects were composed of obese persons of both sexes and different age, having the body mass index (BMI) higher than 30.

Starting from the fact that fatty-acid composition in the adipose tissue may be essentially different in particular body parts, samples of adipose tissue were always taken from the anterior abdominal wall, just below the right rib arch (in the majority of cases, samples were taken in the course of cholecystectomy). Our investigations encompassed excision samples of adipose tissue of persons of both sexes and different age. The group of obese subjects consisted of 121 persons, which were divided into two subgroups according to the obesity degree: 83 moderately obese (BMI = 30.0-39.9) and 38 extremely obese (BMI  $\geq$  40). The obtained results were compared with those obtained for the metabolically healthy persons of normal alimentary status (control group, BMI = 20.24.9), as well as with the findings obtained for 16 undernourished subjects (BMI  $\leq$  20).

All the subjects involved in the adipose tissue investigations, as well as those of control groups, had been previously on a dietary regime characteristic of the Voivodinian population for a prolonged span of time.

Lipid extraction was carried out from the serum aliquot of 1.5 ml using organic solvents (Bligh and Dyer, 1959), and the particular fractions were separated by thin-layer chromatography, according to Gloster and Fletcher (1966). Fatty acids were transformed into their methyl esters using boron trifluoride.

Samples for adipose tissue analyses, taken by excision from the anterior abdominal wall, weighing 40 mg, were esterified with boron trifluoride.

Qualitative and quantitiative analyses of methyl esters of fatty acids from both media were carried out by gas-liquid chromatography on a HEWLETT PACKARD, model 7650 A chromatograph, connected on-line with a 3352 B HEWLETT PACKARD computer system.

The procedure applied allowed separation of over 20 fatty acids, but our study encompassed only 5 of them: stearic (18:0) of the long-chain saturated FA, linoleic (18:2 n-6), linolenic (18:3 n-3) and arachidonic (20:4 n-6) of the polyunsaturated essential FA, as well as the nonessential eicosatrienoic acid (20:3 n-9).

#### RESULTS

# Serum lipid fractions

In Table 1 are presented the results of the analysis of fatty-acid composition of total lipid fraction and FFA fraction of the obese subjects and compared with those for metabolically healthy and normally nourished persons, whereas Table 2 contains the corresponding results for the fractions of cholesterol esters and triglycerides. As can be seen, contents of total saturated FA are significantly increased (by 8%; p < 0.001) only in the total lipid fraction. As for the saturated stearic acid, its significant decrease was observed in the total lipid fraction, and especially in the FFA fraction (even by 28%; p < 0.001). On the other hand, no statistically significant changes in content of this FA were observed in the other two serum lipid fractions.

Tab. 1. — Mean values of percent content (percent of total fatty acids) fatty acids within total lipid fractions and FFA fractions of the blood in obese persons compared with normally nourished and metabolically healthy persons — control groups % — percent changes relative to control groups

	1	total lipid ser	um		free fatty acids (FFA) serum					
fatty acids	control groups	obese persons			control groups	obese persons				
	$\overline{x} \pm SD$	$\overline{x} \pm SD$	%	p <	$\overline{x} \pm SD$	$\overline{x} \pm SD$	%	p <		
C 18:0	7.47(0.93)	6.82(1.06)	-9	0.025	8.12(0.80)	5.84(0.62)	-28	0.001		
total saturated	28.51(1.40)	30.71(2.04)	8	0.001	35.17(2.66)	35.27(2.57)	_	n.s.		
total mono- unsaturated	25.60(2.68)	33.48(3.59)	31	0.001	25.38(1.47)	29.82(2.40)	17	0.001		
C 18:2 n-6	33.42(3.83)	25.09(4.50)	-21	0.001	19.14(1.88)	16.84(2.45)	-12	0.005		
C 18:3 n-3	0.53(0.24)	0.68(0.27)	28	0.05	0.71(0.23)	0.40(0.13)	-44	0.001		
C 20:3 n-9	1.23(0.33)	1.21(0.16)	-2	n.s.	0.14(0.17)	0.18(0.13)	29	n.s.		
C 20:4 n-6	7.34(1.21)	5.71(1.65)	-22	0.001	7.85(0.45)	7.62(1.10)	-3	n.s.		
total poly- unsaturated	44.57(3.42)	34.29(4.69)	-23	0.001	30.32(1.94)	26.87(2.59)	-11	0.001		

Tab. 2. Mean values of percent content fatty acids within cholesterol esters and triglycerides fractions of the blood in obese persons compared with normally nourished and metabolically healthy persons — control groups

	chol	lesterol esters	serum		triglycerides serum					
fatty acids	control groups	obese	persons		control groups	obese persons				
	x ±SD	$\overline{x} \pm SD$	%	p <	$\overline{x} \pm SD$	$\overline{x} \pm SD$	%	p <		
C 18:0	2.57(1,72)	4.05(1.94)	58	0.05	4.12(0.76)	4.76(0.98)	16	0.05		
total saturated	16.05(1.90)	17.00(1.47)	6	n.s.	32.11(2.75)	31.50(4.12)	-2	n.s.		
total mono- unsaturated	25.79(2.68)	27.51(3.27)	7	n.s.	49.54(2.73)	45.59(2.55)	-8	0.001		
C 18:2 n-6	53.30(3.71)	46.20(6.85)	-13	0.001	16.94(2.82)	18.90(5.75)	12	n.s.		
C 18:3 n-3	in trace									
C 20:3 n-9	in trace									
C 20:4 n-6	4.34(1.65)	6.16(2.56)	42	0.02	0.53(0.46)	0.87(0.34)	64	0.05		
total poly- unsatureted	57.64(4.00)	52.36(6.30)	-9	0.005	17.47(2.95)	19.77(5.70)	13	n.s.		

Total monounsaturated FA showed divergent changes: in the fractions of total lipid and FFA there was their significant increase, in the cholesterol ester fraction there were no statistically significant changes, whereas in the serum triglyceride fraction, their significant decrease (by 8%; p < 0.001) was observed. It should be pointed out that the major portion of the monounsaturated FA makes oleinic acid (18:1 n-9), and this is the acid whose content in the serum triglyceride fraction by far exceeded all other FA.

With the exception of the triglyceride fraction (in which they are normally present in lowest concentrations), in the other three serum lipid fractions of obese subjects, a statistically significant decrease of the content of total polyunsaturated FA was observed. This was primarily a consequence of a significant decrease in linoleic acid: by 25% (p < 0.001) in the total lipid fraction, by 12% (p < 0.005) in the FFA fraction, and by 13% (p < 0.001) in the cholesterol esters fraction. Content of this FA is by far the highest in the fraction of cholesterol esters, and the lowest in the triglyceride fraction, where no significant changes of its content were registered at all. Linolenic acid level was significantly decreased in the FFA fraction, whereby it should be remarked that in the fractions of cholesterol esters and triglycerides of healthy subjects too, it was registered only in traces. In contrast to this, the level of arachidonic acid was significantly increased in the fractions of cholesterol esters and triglycerides, and decreased in the fraction of serum total lipids.

# Adipose tissue

Results of the determination of fatty-acid composition in the adipose tissue are given in Table 3. If compared with the metabolically healthy and normally nourished persons, in the obese persons, irrespective of the obesity degree, no statistically significant changes were observed in the contents of total

Tab. 3.	Mean	values	of percent	t content f	atty	acids in	i adipo	se tissue	of	unde	rnou	rished	and	obese
persons	comp	ared to	normally	nourished	and	metab	olically	healthy	pei	sons	— 0	contro	l gro	ups

fatty acids	control group	undernourished			moderately obese			extremely obese		
	$\overline{x} \pm SD$	$\overline{x} \pm SD$	%	p <	$\overline{x} \pm SD$	%	p <	$\overline{x} \pm SD$	%	p <
C 18:0	5.40(1.21)	5.94(0.83)	10	n.s.	4.56(1.03)	-16	0.001	3.21(0.97)	-41	0.001
total saturated	28.11(2.26)	28.76(1.91)	2	n.s.	27.63(2.05)	-2	n.s.	25.34(1.95)	-10	0.001
total mono- unsaturated	54.07(3.23)	53.26(1.84)	-1	n.s.	54.42(3.72)	1	n.s.	56.02(3.33)	4	0.005
C 18:2 n-6	15.08(2.96)	15.04(2.32)	_	n.s.	15.05(3.84)	_	n.s.	15.67(3.14)	4	n.s.
C 18:3 n-3	1.61(0.19)	1.72(0.18)	7	0.05	1.48(0.24)	-8	0.001	1.31(0.27)	-19	0.001
C 20:3 n-9	0.17(0.09)	0.15(0.07)	-12	n.s.	0.32(0.38)	88	0.001	0.30(0.11)	76	0.001
C 20:4 n-6	0.44(0.12)	0.40(0.13)	-9	n.s.	0.52(0.14)	18	0.001	0.61(0.16)	39	0.001
total poly- unsaturated	17.57(3.02)	17.58(2.27)	1	n.s.	17.66(3.99)	1	n.s.	18.25(3.25)	4	n.s.

saturated and polyunsaturated FA, whereas monounsaturated FA showed only some increase (by 4%; p < 0.005) in the subgroup of extremely obese subjects. The same also holds for the comparison of the subjects of the two obese subgroups with those of the undernourished group. However, the analysis of contents of the particular FA showed significant changes if compared with those found in the normally nourished and undernourished persons.

Stearic acid content was significantly decreased already in the subgroup of the moderately obese (by 16%; p < 0.001), the decrease in extremely obese subject being even 41% (p < 0.001). It is interesting to notice that in the group of undernourished subjects content of this FA was increased, but with no statistical significance.

As for the group of polyunsaturated FA, it was found that the obese persons, both the moderately and extremely obese, had a significantly lower level of the essential linolenic acid (by 8% and 19%, respectively; in both cases p < 0.001) and significantly increased levels of arachidonic and the nonessential eicosatrienoic acid. In the group of undernourished subjects, just the opposite was observed. In contrast to the serum lipid fraction, the essential linoleic acid showed no significant changes in dependence of the alimentary status.

It can be seen from Table 4 that there is a statistically significant correlation between the levels of stearic acid and essential linolenic acid and the percentage of deviation from the normal body mass and thickness of the skin folds, both in the male and female subgroups. In contrast to this, there is no such correlation for the total FA, mono- and polyunsaturated FA and for arachidonic acid alone, whereas for eicosatrienoic acid the correlation exists only in the subgroup of female subjects with the skin folds thickness.

fatty agida	thickness of	the skin folds	deviation from the	normal body mass
Tatty actus	men	women	men	women
C 18:0	368**	401***	400***	402***
total saturated	155	258*	147	324**
total monounsaturated	.093	.018	.000	.154
C 18:2 n-6	021	.178	092	.058
C 18:3 n-3	270*	404***	240*	416***
C 20:3 n-9	049	.408***	.049	.249*
C 20:4 n-6	.036	.224	.012	.180
total polyunsaturated	.001	.141	138	.057

Tab. 4. The correlation coefficient between thickness of skin folds and percentage of deviation from the normal body mass and fatty acids content in adipose tissue

\*p < 9.05; \*\*p < 0.01; \*\*\*p < 0.001

# DISCUSSION

Numerous studies have shown that the changes in the percentage of particular FA, both in the adipose tissue and blood lipid fractions, are important for the genesis of coronary diseases and atherosclerosis in general (W atts et al., 1995; C aggiula and Mustad, 1997; Hu et al., 1997; Pietinen et al., 1997). These observations conform with our previous investigations involving patients with a high risk of the accelerated development of atherosclerosis: certain types of hyperlipoproteinemia, hypothyroidism, diabetes, as well as the patients who suddenly died of cardiovascular diseases (Djeric et al., 1989, 1993; Lepsanovic et al., 1979, 1980, 1996). A common basic characteristic of all these diseases was an increase in the level of atherogenic long-chain saturated FA, an exception being stearic acid which, contrary, exhibited a decrease. At the same time, the levels of polyunsaturated FA, primarily of the essential ones, are decreased. However, the literature data on the fatty-acid composition in obese persons are very scarce, whereby we can notice that such examinations in our country have never been carried out at all.

In the interpretation of the obtained findings one has to take first into account the differences in contents of the individual FA in particular blood lipid fractions (L e p s a n o v i c, 1982). Further, it is very important to notice that the changes in the fatty-acid composition in the adipose tissue are very slow, so that first significant alterations are observable not before one half of a year later. Quite opposite holds for blood lipid fractions, where the changes take place even a few days after the change in the diet. For this reason, it is quite understandable that the latter changes are of greater importance than the those observed in the adipose tissue.

Our investigations showed no significant changes in the percentage of total saturated FA in both of the examined media in comparison with the controls, and this practically holds also for the monounsaturated FA. The absence of the difference in contents of total saturated FA can be explained by the fact that one of them (stearic acid) showed a significant decrease in the total lipid and FFA serum fractions, and especially in the adipose tissue — this is being paralleled with the obesity degree. This decrease in some way masks the increase in content of the other saturated long-chain FA exhibiting an atherosclerotic effect, especially of palmitic (16:0), lauric (12:0) and myristic (14:0) acids. It is also interesting to notice that the percentage of stearic acid in the adipose tissue of the undernourished persons is increased in comparison with that of the normally nourished persons. Calculation of the coefficient of correlation showed that there is a significant correlation between the content of this FA in the adipose tissue and the alimentary status (r = 0.400 for males and 0.402 for females, in both cases p < 0.001). If we bear in mind the unambiguously established its antiatherogenic effect (Bonanome and Grundy, 1988; Yu et al., 1995), the fact that percentage of stearic acid in blood and adipose tissue of obese persons shows an increase is of particular importance. Namely, identical findings have also been obtained for other pathological states yielding the genesis and progress of atherosclerosis (Insull et al., 1968; Lang et al., 1977; Wood et al., 1987; Kris-Etherton and Yu, 1997).

If compared with the controls, contents of total polyunsaturated FA are significantly decreased in all blood lipid fractions (except for the triglyceride fraction), but not in the adipose tissue. Of special importance are, however, the changes in contents of the particular polyunsaturated FA, especially of the two essential ones — linoleic and linolenic. The level of the former is decreased in all the investigated blood lipid fractions, with the exception of the triglyceride fraction, where its content is normally the lowest. In contrast to this, the concentration of linolenic acid is significantly decreased in the adipose tissue and, similar to stearic acid, there is a negative correlation of its content with the thikness of the skin folds and alimentary status. Similar results were also obtained in a study of fatty-acid composition of blood lipid fractions and adipose tissue in the very obese persons of Sweedish population (R o s s n e r et al., 1989), as well as in the studies of the serum of obese children (D e c s i et al., 1996). Practically the same changes we registered in the persons who suddenly died of cardiovascular diseases, diabetic patients, and hyperlipoproteinemia of type IIb and IV.

In contrast to the above two FA, contents of arachidonic and eicosatrienoic acids showed an increase in some blood lipid fractions, and especially in the adipose tissue. The finding of a significant increase in the content of eicosatrienoic acid is of essential importance, because there are abundant pieces of evidence that this acid, in contrast to other polyunsaturated FA, exhibits an atherogenic effect (L e f k o w i t h, 1990).

The divergent changes observed with the highly-protective linolenic acid and atherogenic eicosatrienoic acid among the polyunsaturated FA is best illustrated by their significantly changed ratio in comparison with healthy persons, found in the adipose tissue (p < 0.001) and the FFA serum fraction (p < 0.001).

It has been shown experimentally that the increased alimentary intake of linolenic acid increases flexibility of the artery walls (M o r i et al., 1997) and decreases insulin resistance (S i m o p o u l o s, 1994; N e s t e l et al., 1997), the phenomenon which, in some other diseases, is regularly present in obese persons. Some works even suggest that the increased alimentary intake of this FA could have a protective effect in respect of the process of atherosclerosis (H u n et al., 1999). In view of this finding of special importance is the significant decrease of linolenic acid in the obese persons, observed in our study.

A question arises whether the investigation results obtained for the adipose tissue would be different if they were carried out on samples of the visceral and not subcutaneous adipose tissue. There are, however, data suggesting that the FA contents in the subcutaneous adipose tissue exhibit strong correlation with their values in the visceral adipose tissue (S c h o e n et al., 1996). Hence, we are also of the opinion that the results of our study of the fatty-acid composition in the adipose tissue samples taken from the anterior abdominal wall are sufficiently reliable.

## CONCLUSION

The presented results of the study of fatty-acid composition in blood and adipose tissue of obese persons, especially the observed significant decrease of content of stearic acid among the saturated FA and linoleic and linolenic among the polyunsaturated ones, as well as the increase in eicosatrienoic acid, indicate that the fatty-acid composition in obese persons is practically identical to the findings in other diseases that are also characterized by a progressive development of atherosclerosis.

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### КАРАКТЕРИСТИКЕ МАСНОКИСЕЛИНСКОГ САСТАВА КРВИ И МАСНОГ ТКИВА У ГОЈАЗНОСТИ

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#### Резиме

У гојазних особа испитиван је маснокиселински састав у 4 фракције крвних липида (FFA, естри холестерола, триглицериди и фракција укупних липида) и субкутаног масног ткива узетог с предњег трбушног зида. Анализе су вршене методом гасно-течне хроматографије помоћу које је издвајан велик број засићених, моно- и полинезасићених масних киселина (МК), од којих је посебној анализи подвргнуто 5 најзначајнијих. За разлику од укупних засићених МК, које су сигнификантно повишене само у фракцији укупних липида серума, стеаринска (18:0) МК сигнификантно је смањена у фракцији ФФА и укупних липида серума, а, нарочито, у масном ткиву, посебно у екстремно гојазних. Међу полинезасићеним МК, линолна (18:2 н-6) показује статистички сигнификантно смањење практично у свим липидским фракцијама серума, а линоленска (18:3 н-3) у масном ткиву. Супротно овоме, арахидонска (20:4 н-6) и неесенцијална еикосатриенска (20:3 н-9), од полинезасићених МК, сигнификантно су повишене. Добијени резултати практично су идентични налазима у другим патолошким стањима повезаним с убрзаним развитком атеросклерозе (изненада умрли од кардиоваскуларних обољења, шећерна болест, хиперлипопротеинемије IIб и IV).

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# INTERACTION BETWEEN CADMIUM AND NITROGEN AND ITS EFFECTS ON THE GROWTH AND PHOTOSYNTHETIC PIGMENTS OF YOUNG SUNFLOWER PLANTS

ABSTRACT: The interactions among different concentrations of nitrogen and cadmium and their effects on the growth of young sunflower plants in water culture were studied. Increases in the concentrations of nitrogen and cadmium in the nutritive solution brought proportional increases of these elements in plants. Maximum values of plant height, leaf area, fresh mass of the second pair of leaves and the content of chloroplast pigments, chlorophyll a+b and total carotenoids were obtained with the medium concentration of nitrogen (7.5 mM). The increases in cadmium concentration reduced the values of all parameters. The adverse action of the maximum concentration of cadmium (5.0  $\mu$ M) was lowest when the nitrogen provision to plants was optimal. The obtained results permit the conclusion that the optimal provision of nitrogen may mitigate the negative effects of cadmium on sunflower plants.

KEY WORDS: sunflower, cadmium, nitrogen, plant height, plant mass, leaf area, photosynthetic pigments

#### INTRODUCTION

Cadmium (Cd) is considered an important environmental pollutant. The anthropogenic sources of Cd are heating systems, urban traffic, cement factories, waste incinerators and byproducts of phosphate fertilizers. The annual volume of anthropogenic emission of Cd is 29.19 x  $10^3$  t (S a n i t a et al., 1999). Mineralization of rocks is another source of Cd in soil. Cd affects many physiological processes in plant. These effects are direct and indirect. Cd damages the photosynthetic apparatus, in particular the light harvesting complex II (K r u p a , 1988). Cd inhibits the oxidative mitochondrial phosphorylation

(Kessler and Brand, 1995). Furthermore, Cd interacts with the water balance (Kastori et al., 1992). Cd alters the synthesis of RNA and inhibits ribonuclease activity (Shah and Dubey, 1995) and produces oxidative stress (Hendry et al., 1992). Cd alters the activity of several enzymes such as glucose-6-phosphate dehydrogenase, malic enzyme, isocitrate dehydrogenase, Rubisco and carbonic anhydrase (Van Assche and Clijsters, 1990; Siedlecka et al., 1997).

Cd evidently affects the metabolism of nitrogen (K a s t o r i et al., 1997). Cd reduces the absorption of nitrate and its transport from roots to shoots and it inhibits the nitrate reductase activity (P e t r o v i ć et al., 1990; H e m a n - d e z et al., 1996). The plant cell resorts to a number of defense systems in response to Cd stress. One of them is the synthesis of proteins, metallothioneins, stress proteins (L e i t a et al., 1991) and phytochelatin (H o w d e n et al., 1995). The above literature sources state that there exists an interaction between Cd and nitrogen metabolism. This was further supported by P a n k o v i ć et al. (2000) who found that optimum nitrogen nutrition may mitigate and partially modify toxic effects of high Cd concentrations on photosynthesis. This paper deals with the effect of Cd on the growth of and the associated physiological parameters in young sunflower plants differing in the level of nitrogen provision.

# MATERIALS AND METHODS

#### Plant Material and Culture Conditions

Experiments were conducted with young sunflower plants (hybrid NS-H-26-RM) grown in greenhouse conditions. Seeds were germinated in the dark, at 25°C, on sterilized quartz sand, watered daily with deionized water. Seven days later, the seedlings were transferred to either 1) half-strength Hoagland solution (H o a g l a n d and A r n o n , 1950) (control, contains 7.5 mM N), 2) half-strength Hoagland solution containing 2mM N or 3) half-strength Hoagland solution containing 10 mM N. When the second pair of leaves started to emerge, each group of plants was divided into four subgroups which received the following Cd concentrations: 0, 0.5, 2.0 and 5.0  $\mu$ M CdCl<sub>2</sub>. Each treatment (subgroup) was established in 12 6-plant replications and grown until the second pair of leaves was fully expanded. The second pair of leaves was used for analyzing.

#### Plant Growth Analysis

For determinations of leaf area and the dry matter mass (DW), the sampled plants were dissected into roots, aboveground parts and the second pair of leaves. Leaf area was measured by an Automatic Area Meter (LI-3000, U-COR<sup>®</sup>, Lincoln, NE). Dry mass was determined after drying plant samples at 70°C to constant weight.

### Cadmium and Nitrogen Analysis

Plant samples were dried and digested in a mixture of concentrated nitric and perchloric acids (1:3). Cadmium content was measured by standard atomic absorption spectrophotometry. Nitrogen content was determined by the micro-Kjeldahl method.

# Pigments

Chlorophylls a and b and carotenoids were determined spectrophotometrically in the acetone extract of freshly harvested leaves, using molar extinction coefficients according to Holm (1954) and von Wettstein (1957).

The obtained results were statistically processed by calculating the least significant difference.

# **RESULTS AND DISCUSSION**

Increase in nitrogen concentration in the nutritive solution brought corresponding increases in the nitrogen contents in leaves, with all Cd concentrations (Tab. 1).

N (mM)		Cd (j	I	SD- 0.05	
(A)	0.0	0.5	2.0	5.0	0.01
2.0	2.61	2.98	3.11	3.68	0.79
7.5	3.88	3.86	4.09	4.52	
10.0	4.38	4.39	4.30	4.56	0.18
LSD	0.0	5	0.067	I SD	0.089
LSDA	0.0	1	0.123	LSD <sub>AxB</sub>	0.135

Tab. 1 — Effect of Cd on N content (% DW) in young sunflower plants differing in N provision

Increase in Cd concentration tended to increase the nitrogen content in plants, especially in those grown with the low and medium nitrogen concentrations. However, it cannot be concluded on the basis of the obtained results whether the increased nitrogen contents were the consequence of more intensive nitrogen uptake or merely the result of lower synthesis of organic matter and thus the less intensive dilution of the accumulated nitrogen. Heavy metals may inhibit or they may stimulate ion uptake. Their action is typically based on antagonism or synergism or it may depend on ionic metabolism. The results of P e t r o v i ć et al. (1990) and P o p o v i ć et al. (1996) indicated that high Cd concentrations inhibited the enzymes that govern nitrogen assimilation. In that way Cd might affect nitrogen uptake or the accumulation of inorganic nitrogen. According to a study of K e v r e š an (2000), Cd had a negligible effect on the nitrogen content in peas.

The Cd content in the leaves of young sunflower plants tended to increase in proportion with the increase of Cd concentration in the nutritive solution (Tab. 2).

N (mM)		$Cd~(\mu M)~(B)$	I SD-	0.05	
(A)	0.5	2.0	5.0	LSDB	0.01
2.0	4.68	18.48	78.23		10.11
7.5	5.28	22.73	81.03		
10.0	4.32	16.93	92.71		23.33
ISD	0.05	8.64	4	ISD	11.50
LSDA	0.01	15.85	5	LSD <sub>AxB</sub>	17.43

Tab. 2 - Cd content (mg/kg DW) in leaves of young sunflower plants differing in N provision

Changes in nitrogen concentration in the solution had no high effect on Cd accumulation. With the lower Cd concentrations, its content was highest at 7.5 mM N, but with the high Cd concentration its content was highest at 10.0 mM N.

Plant height was highly dependent on the concentrations of nitrogen and Cd in the nutrive solution (Tab. 3).

Tab. 3 — Effect of Cd on stem height (cm) of young sunflower plants differing in N provision

N (mM)		Cd (µ	M) (B)		ISD	0.05
(A)	0.0	0.5	2.0	5.0	LODB	0.01
2.0	17.83	16.04	15.67	14.13		2.35
7.5	26.67	21.71	19.92	18.08		
10.0	22.08	19.59	17.58	14.33		5.43
LSD	0.0	5	2.01	I SD		2.68
LSDA	0.01		3.69	LSD <sub>AxB</sub>		4.05

Plants were tallest with the medium nitrogen concentration. The high nitrogen concentration reduced plant height. Increases in Cd concentration reduced plant height with all levels of nitrogen provision. Growth rate reduction in the presence of Cd has been observed earlier (A i d i d and O k a m o t o, 1993). Growth rate reduction of plant elongation by Cd was particularly high in the case of the cells of the stem, because Cd irreversibly inhibited the proton pump responsible for the elongation process.

The contents of chloroplast pigments, chlorophylls a + b and carotenoids, were also found to depend on the concentrations of Cd and nitrogen in the nutritive solution (Tab. 4 and 5).

The content of chloroplast pigments was reduced significantly as the Cd concentration increased. Many researchers have observed this phenomenon earlier (L a r s s o n et al., 1998). The increase of nitrogen concentration from the low to the medium level increased the content of chloroplast pigments in all variants. The effect of further increase of nitrogen concentration on the content of chloroplast pigments depended on Cd concentration. It was noticea-

N (mM)		Cd (µ		LSD-	0.05	
(A)	0.0	0.5	2.0	5.0	LSDB	0.01
2.0	8.44	8.00	4.59	4.06		0.76
7.5	11.94	11.56	8.15	7.74		
10.0	12.91	9.52	8.18	4.28		1.75
LSD	0.0	5	0.65	LSD		0.86
LSDA	0.0	1	1.19	LSD <sub>AxB</sub>		1.30

Tab. 4 — Effect of Cd on the content of chlorophylls a + b (mg/g DW) in young sunflower plants differing in N provision

Tab. 5 — Effect of Cd on carotenoid content (mg/g DW) in young sunflower plants differing in N provision

N (mM)		Cd (µ	Ţ	SD 0.05	
(A)	0.0	0.5	2.0	5.0	0.01
2.0	5.75	5.52	4.10	3.67	0.45
7.5	8.24	7.48	5.81	5.71	
10.0	8.10	6.48	6.02	4.28	1.04
L SD.	0.0	5	0.38	ISD	0.51
LSDA	0.0	1	0.71	LSD <sub>AxB</sub>	0.78

ble that, under the conditions of optimum plant provision with nitrogen and the high Cd concentration, the pigment content was much higher than under the conditions of insufficient or excess nitrogen. These results confirm the finding of P a n k o v i ć et al. (2000) that optimum nitrogen nutrition could decrease the inhibitory effects of Cd in young sunflower plants. Further confirmation for this are the results obtained for the effect of nitrogen and Cd interaction on the area and mass of the second pair of leaves (Fig. 1 and 2).

Increase in Cd concentration caused lowest reductions in leaf area and mass when plant provision with nitrogen was optimal. The reductions were much higher when nitrogen supply was insufficient, and they were especially high when excess nitrogen was supplied. It is well known that excess nitrogen makes plants highly susceptible to stress conditions. This explains the results obtained with the high nitrogen concentration. Since Cd damages nucleoli (L i u et al., 1995), alters the synthesis of RNA (S h a h and D u b e y , 1995) and inhibits the nitrate reductase activity (M a t h y s , 1975), it manifestly affects the nitrogen metabolism. Negative effect of excess Cd concentration on nitrogen metabolism is more prominent in conditions of nitrogen shortage than in conditions of optimal nitrogen supply. Interaction between Cd and nitrogen seems to be primarily based on the effect of Cd on nitrogen metabolism, i.e., on the structural and soluble proteins which play an important role in all vital processes of plants.



Z2.0 mM N 7.5 mM N 10.0 mM N

Fig. 1 — Effect of Cd on the area of the second pair of leaves (cm²/plant) in young sunflower plants differing in N provision



Fig. 2 - Fresh matter mass of the second pair of leaves (g/plant) in sunflower

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#### ИНТЕРАКЦИЈА ИЗМЕЂУ КАДМИЈУМА И АЗОТА И ЊЕН УТИЦАЈ НА РАСТЕЊЕ И ФОТОСИНТЕТИЧКЕ ПИГМЕНТЕ МЛАДИХ БИЉАКА СУНЦОКРЕТА

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#### Резиме

У условима водених култура проучено је узајамно дејство кадмијума и азота на растење и садржај пигмената хлоропласта младих биљака сунцокрета. Са повећањем концентрације азота и кадмијума у хранљивом раствору повећао се њихов садржај у биљкама. Висина биљака, лисна површина и маса свеже материје другог пара листова и садржај пигмената хлоропласта, хлорофила а+б и каротеноида биле су највеће при средњој испитиваној концентрацији азота (7,5 mM). Повећање концентрације кадмијума смањило је све испитиване параметре. Неповољно дејство највеће испитиване концентрације кадмијума (5,0 µM) било је најмање при оптималној обезбеђености биљака азотом. На основу добијених резултата испитивања може се закључити да оптимална обезбеђеност азотом смањује неповољно дејство већих концентрација кадмијума код биљака сунцокрета.

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# MICROALGAL BIOMASS PRODUCTION DURING PURIFICATION OF THERMOMINERAL WATER

SUMMARY: The growth of microalgal species *Spirulina platensis, Scenedesmus quadricauda* and *Dunalliela* sp., cultivated in water samples from three Vojvodinian geothermal drill-holes, was examined for 15 days. Microalgae were cultivated in undiluted thermomineral water, in different dilutions (20%, 40%, 60%, 80%), as well as in the thermomineral water enriched with 5% liquid swine manure. The growth was characterized by specific biomass production for each microalgal species: in the case of *Spirulina platensis cultivation*, very significant biomass increase was measured in all treatments, especially in the one enriched with swine wastewater. Green microalga *Scenedesmus quadricauda* grew only in diluted thermomineral water samples. The growth of microalga *Dunalliela* sp. was strongly inhibited during the cultivation in thermomineral water and the dilutions. In the case of cyanobacteria *Spirulina platensis*, the effect of microalgal growth on the significant thermomineral water demineralization was noticed after 7 days of cultivation.

KEY WORDS: algal biomass, *Dunalliela* sp., purification, *Scenedesmus quadricauda*, *Spirulina platensis*, thermomineral water.

#### INTRODUCTION

According to the latest geothermal investigation in Vojvodina (M i l o s a v l j e v i ć et al., 1995), considering physical, chemical and geothermal characteristics of thermomineral waters, these waters can easily find an important role in agriculture (glass-houses warming), cattle-breeding (farms warming), fishery (fish-ponds), industry (technological warm water), bathing places (rehabilitation, sport, recreation and tourist centers), warming of the urban settlements, etc. Many local energy crises in the 70's sought solution in possible geothermal water employment as energy resource. It proved to be a successful replacement of other sources for producing electric energy, but its capacity was sometimes quite modest. Nevertheless, the advantage of thermomineral waters in energy production is significant in local utility, which can be treated

as more practical, economic and environmental-friendly solution. This is the main reason the world industry is interested in this problem investments (A k - sin & Milosavljević, 1984).

In spite of high potential application, thermomineral water may cause high levels of eutrofication in water recipients due to high concentration of mineral salts. During the thermomineral water treatment with microalgae, high quantity of algal biomass and mineral salts concentration decrease would be provided, since specific microalgae species can utilize most of the minerals for their growth (O b r e h t, 1988; O b r e h t et al., 1993). Special attention should be given to the cyanobacteria *Spirulina platensis*, because of its significantly easy cultivation and high biomass quality comparing with other microorganisms or higher plants (R i c h m o n d, 1986; V o n s h a k, 1997; H e n r i x o n, 1997). Green algae *Scenedesmus* and *Dunaliella* are actually used worldwide as producers of biomass in biotechnology (S o o n g, 1980).

In order to racionalize the thermomineral waters exploatation, the main aim of this research was to examine possible cyanobacteria (*Spirulina platensis*) and green algae (*Scenedesmus quadricauda* and *Dunaliella* sp.) application as biomass producers and thermomineral waters purifiters. Biomass could be considered as an alternative high calorie food or feed and source of different industriall important compounds. At the same time, high concentration of the minerals, as limiting factor in thermomineral waters usage, would be reduced during microalgal cultivation.

# MATERIAL AND METHODS

Microalgal strains *Spirulina platensis*, *Scenedesmus quadricauda* and *Dunaliella* sp. are part of the Microbiology laboratory collection (Institute of Biology, University of Novi Sad). The analyses of the total samples mineralization have been made after 3, 5, 7, 9 and 14 days during thermomineral water purification (demineralization) by microalga *Spirulina platensis*.

Cultivation of microalgae in undiluted and diluted thermomineral water (20%, 40%, 60%, 80%) and in undiluted water enriched with 5% liquid swine manure lasted for 15 days. Liquid manure was added in order to increase nitrogen and phosphorus concentrations. Cultivation was done in Erlenmeyer flasks, at the temperature of 30°C, with fluorescent light intensity of 50  $\mu$ molm<sup>-2</sup>s<sup>-1</sup>.

Spirulina platensis biomass production was detected indirectly by chlorophyll *a* concentration analysis (M a c k i n n e y, 1941). Biomass concentrations of *Scenedesmus quadricauda* and *Dunaliella* sp. were measured by direct absorbency analyses on spectrophotometer.

The presented results are the average values of five repeated measurements. Experimental data were subjected to analysis of variance (LSD — test).

Microalgal strains were cultivated in water samples taken from three geothermal drill-holes: Pb-1/H, Mk-1/H and Sr-1/H. Physico-chemical characteristics of the examined thermomineral waters are presented in Table 1.

PHYSICAL AND CHEMICAL WATER CHARACTERISTICS	Pb-1/H	Mk-1/H	Sr-1/H
Flow (l/min)	1200	740	700
Hardness (mol/m)	1.72	0.67	0.10
Temperature (°C)	49	51	55
pH value	7.4	7.8	7.7
Dry rest (mg/l)	5940	2390	2770
Total mineralization (mg/l)	5806	3048	3253
HPK (mg/l)	76	43	_
CO <sub>2</sub> (mg/l)	437.8	801.9	—
NH <sub>4</sub> (mg/l)	16	24.5	18.75
Inorganic nitrogen (mg/l)	10.86	23.5	—
Nitrates (mg/l)	0	0	49
Nitrites (mg/l)	0.02	0	0
Chlorides (mg/l)	2482	40.42	709
Carbonates (mg/l)	0	0	0
Bicarbonates (mg/l)	1214	2162	1470
Sulfates (mg/l)	0	20	0
Phosphates (mg/l)	0	0	0
SiO <sub>2</sub> (mg/l)	0.5	6.5	4.0
Fe <sup>3+</sup> (mg/l)	0.25	0.16	0.2
Fe <sup>2+</sup> (mg/l)	0	0.24	0.1
Na+ (mg/l)	2000	800	1000
K+ (mg/l)	40	5.5	6.2
Ca <sup>2+</sup> (mg/l)	17	9	4
Mg <sup>2+</sup> (mg/l)	10.6	10.5	10.3

Tab. 1 — Physical characteristics and chemical composition of thermomineral waters

# RESULTS AND DISCUSSION

The cultivation of microalgal strains was carried out in water samples taken from three geothermal drill-holes, in order to examine possible exploitation of thermomineral waters in Vojvodina. According to our results, microalgal biomass was very characteristic and specific for each microalgal species applied.

The increase of *Spirulina platensis* biomass (g chl a/ml) was directly correlated to the increase in thermomineral water concentration (Table 2).

Tab. 2 — The increase of Spirulina platensis biomass grown in different dilutions of thermomineral water compared with the control medium

THERMOMINERAL WATER (%)	Pb-1/H	Mk-1/H	Sr-1/H
20%	1.105	1.298	1.162
40%	1.421	2.055	1.374
60%	1.484	2.911	2.621
80%	2.210	3.476	2.637

100%	2.386	3.941	4.020
100% + 5% swine manure	3.473	4.357	2.116
CONTROL (SOT)	3.156	4.124	3.500

Data in bold: statistical significance of tested parameters compared with the control (SOT) at the confidence level of 0.05 (higher values)

Spirulina biomass was extremely high in the water samples taken from Pb-1/H and Mk-1/H (+ 5% liquid swine manure). It has been shown that cultivation of *Spirulina platensis* should be carried out in undiluted thermomineral water. Comparing the biomass quantity in control mineral nutritive medium (SOT), water samples from Sr-1/H demonstrated even better growing conditions for *Spirulina* cultivation (Table 1). Water samples from Mk-1/H and Pb-1/H showed to be less corresponding media, but swine manure compensated for the deficiency in important elements (S v i r č e v et al., 1996).

Green alga *Scenedesmus quadricauda* manifested very good biomass increase in water samples diluted up to 80% (Sr-1/H and Pb-1/H) and in all samples taken from Mk-1/H, with exception of the samples enriched with swine manure (Table 3).

Tab. 3 — Scenedesmus quadricauda growth in different dilutions of thermomineral water compared with the control medium

THERMOMINERAL WATER (%)	Pb-1/H	Mk-1/H	Sr-1/H
20%	0.511	0.345	0.418
40%	0.714	0.719	0.694
60%	1.149	1.362	1.217
80%	0.538	1.567	1.414
100%	0.406	1.722	1.109
100% + 5% swine manure	0.321	1.595	0.823
CONTROL ("148")	1.760	1.983	1.814

Comparing these results with the growth of the control culture in "148" mineral medium, the water sample Mk-1/H made this green alga cultivation the most efficacious.

In the case of *Dunaliella* sp., all thermomineral water samples inhibited green alga growth (Table 4).

Tab. 4 — Dunaliella sp. growth in different dilutions of thermomineral water compared with the control medium

Pb-1/H	Mk-1/H	Sr-1/H
0.314	0.294	0.318
0.389	0.349	0.308
0.286	0.364	0.339
0.311	0.401	0.618
0.305	0.411	0.406
0.319	0.447	0.433
2.680	2.735	2.842
	Pb-1/H 0.314 0.389 0.286 0.311 0.305 0.319 2.680	Pb-1/H         Mk-1/H           0.314         0.294           0.389         0.349           0.286         0.364           0.311         0.401           0.305         0.411           0.319         0.447           2.680         2.735

Generally speaking, *Dunaliella* sp. biomass quantity ( $\mu$ g chl a/ml) increases when undiluted water is enriched with swine manure. Much lower quantities of biomass have been produced in all water samples compared with the control ("148" medium).

The aims of microalgal cultivation in thermomineral waters were both biomass production of microalgae and water purification in order to decrease mineral concentration, which could cause high levels of eutrofication in water recipients. Accordingly, *Spirulina platensis* cultivation in thermomineral water samples has been followed by significant reduction of total minerals. Significant nutrient concentration decrease at the beginning of *Spirulina* cultivation followed by biomass increase after the seventh day have been observed during *Spirulina* cultivation in Pb-1/H and Sr-1/H undiluted water samples. Until the fourteenth day, the biomass production and water demineralization lasted unperceptible (Figure 1 and Figure 2).

*Spirulina platensis* biomass growth as well as the mineral concentration also indicated successful demineralization in Mk-1/H water sample until the seventh day. Sudden minerals increase and inhibition of biomass production observed after the seventh day (Figure 3) can be associated with eventual bacterial occurrence in algal culture.

In spite of encouraging results with Vojvodina thermomineral waters exploitation for the production of *Spirulina* biomass published twelve years ago (O b r e h t, 1988), there have been no attempts to commercialize the process. Vietnam is the only country with commercial production of *Spirulina platensis* in thermomineral waters (N g u e n h u n T h u o c et al., 1989). The chemical composition of Vietnamese waters is quite similar to those in Vojvodina. The cultivation of *Spirulina platensis* in thermomineral water could produce multi-



Fig. 1 — Comparison of *Spirulina platensis* biomass increase and total mineralization decrease in Pb-1/H water sample



Fig. 2 — Comparison of *Spirulina platensis* biomass increase and total mineralization decrease in Sr-1/H water sample



Fig. 3 — Comparison of *Spirulina platensis* biomass increase and total mineralization decrease in Mk-1/H water sample

ple benefits (C i f e r r i & T i b o n i, 1985): it can be used in the purification of thermomineral water, there are no unusable byproducts in the biomass production process (like with higher plants), the growth is extremely fast (in wet or dry climate), *Spirulina* is not competitive with other microorganisms and finally, *Spirulina* is a source of chemical compounds for the pharmaceutical industry and other industries (high percentages of proteins and vitamins). (D o - u m e n g e et al., 1993; V o n s h a k, 1997; H e n r i k s o n, 1997).

According to our results, green alga *Scenedesmus quadricauda* grew only in diluted water samples, the growth of *Dunalliela* sp. was inhibited during the cultivation in thermomineral water, while *Spirulina platensis* demonstrated significant reduction of total minerals and biomass increase after the seventh day of cultivation, which can be considered as strong points in favor of future *Spirulina* usage in thermomineral water purification.

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### ПРОДУКЦИЈА МИКРОАЛГАЛНЕ БИОМАСЕ ТОКОМ ПРЕЧИШЋАВАЊА ТЕРМОМИНЕРАЛНИХ ВОДА

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#### Резиме

Три врсте микроалги, Spirulina platensis, Scenedesmus quadricauda и Dunalliela sp., узгајане су у узорцима термоминералне воде узете из три војвођанска геотермалана извора: Pb-1/H, Sr-1/H и Mk-1/H. Након 15 дана култивације све три микроалгалне врсте показале су специфичан раст биомасе у неразблаженој термоминералној води, у узорцима различитог разблажења (20%, 40%, 60%, 80%), као и у узорку обогаћеном са 5% течног стајњака (ради повећања концентрација азота и фосфора). За микроалгу Spirulina platensis (Cyanobacteria) карактеристично је сигнификантно повећање биомасе у сва три узорка воде, погову у стајњаком обогаћеном узорку. Зелена алга Scenedesmus quadricauda адаптирала се само у разблаженим воденим узорцима (< 80%), док је раст микроалге Dunalliela sp. био инхибиран у сва три узорка термоминералне воде.

Испитиван је и ефекат култивације микроалги на деминерализацију (пречишћавање) термоминералних вода. У случају цијанобактерије *Spirulina platensis*, драстично смањење укупне минерализације уз значајно повећање биомасе примећено је након 7 дана култивације, што указује на практичну могућност примене цијанобактерије *Spirulina platensis* у пречишћавању термоминералних вода. Зборник Матице српске за природне науке / Proceedings for Natural Sciences, Matica Srpska Novi Sad, № 100, 67—70, 2001

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# ISOLATION OF *SALMONELLA ENTERITIDIS* FROM POULTRY FARMS IN VOJVODINA

ABSTRACT: Occurrence of salmonelosis increasingly endanger poultry production, since isolation the bacteria from poultry material was frequent during the last three years (1996—1998). During 1996, occurrence of *Salmonella* positive isolates was 4.9%, 3.9%, in 1996 and 1997, respectively, while Salmonellosis occurred in alarming 15.85% of total bacterial findings in 1998. During the three year period *Salmonella enteritidis* was increasingly prevailing in population of the bacteria, being isolated in 82%, 90.5% and 94.5% of total *Salmonella* isolates, during 1996, 1997. and 1998, respectively.

KEY WORDS: Salmonelosis, Salmonella enteritidis, poultry

# INTRODUCTION

Salmonelosis are very important infections that follow poultry production. Reports by WHO and FAO (1998) describe an alarmingly high occurrence of *Salmonella* mostly prevailing *Salmonella enteritidis*, *Salmonella typhimurium* and *Salmonella virhow* among findings.

It was established that 80% of all *Salmonella* isolated from animal material occurred in poultry samples (P i g u e t and P i r e s - G o m e s , 1989), coming from chicken in 41%, from ducks in 21% and from turkeys in 18% of Salmonella-positive samples. *Salmonella enteritids* was established in 82.7% of total Salmonella-positive findings in poultry. Other reports (M r d e n et al., 1997; O r l i ć et al., 1998; R a š i ć and O r l i ć , 1999) also indicate that frequent Salmonelosis does not only present an important economic problem in poultry production, but also a threat to human health (P a l i ć and O r l i ć , 1999). Types of *Salmonella* were the causative agents in 62.9% of the reported outbrakes of epidemic food poisoning in Yugoslavia from 1992. to 1996., and *S. enteritidis, S typhimurium* and *S. infantis* were most frequently isolated in 82.7%, 5.9% and 2.2 % of samples, respectively (E r s k i - B i l i ć and I l i ć , 1999). The findings of large number of *Salmonella* carriers can explane the transmision of the disease via meat and meat products obtained from infected chicken and give rise to the importance of constant monitoring of the *Salmonella* outbrakes and prevalence esspecially *S. enteritidis*.

The aim of the paper was to prove presence of *Salmonella* in poultry material and determine the type that is mostly represented.

# MATERIAL AND METHODS

Samples of livers and yolk material obtained from the transported chicken and juveniles chicken, and also livers of broilers were used for bacteriological testing. During a three year period a total of 5869 liver and yolk samples, 1510 suffocated chicken and 3196 eggs were evaluated.

The samples were examined by standard bacteriological procedures (M i - h a j l o v i ć 1983). Liver samples were cultivated on semi-liquid culture media (M r d e n et al., 1977), in selenite-buyon and after 24 hours of incubation, samples were transfered on Endo agar, Salmonella-Shigella culture and Brilliant green culture media. Samples obtained from suffocated chicken and eggs were cultivated on selenite-buyon, incubated for 24 hours and then transfered on Endo agar, Salmonella-Shigella culture and Brilliant green culture media. *Salmonella*-Shigella culture and Brilliant green culture media. *Salmonella*-suspective colonies (lactose-negative) were transfered on Kligler's duble agar. Biochemical properties and serotyping of *Salmonella* were also performed (M i h a j l o v i ć , 1983).

# **RESULTS AND DISCUSSION**

During 1996. Salmonella was established in 4.9% of total bacterial infections (Table 1). There were 3.9% of salmonella-positive findings in 1997, while an allarming increase of Salmonella occurence was established in 1998. when 15.8% of positives, mainly from liver samples, were reported (M r d e n et al., 1997; Stojanov and Orlić, 1998).

samples	No. of positives per total examined						
	1996	%	1997	%	1998	%	
livers, yolks	211/3039	6.9	142/2516	5.6	57/313	18.2	
suffocates	76/1085	7,0	23/314	7.3	15/111	13.5	
eggs	76/1085	0.1	1/1399	0.1	0/30	0	
total	189/5891	4.9	166/4229	3.9	72/454	15.8	

Tab. 1 — The occurrence of Salmonella in poultry material

The most frequently isolated type of *Salmonella* was *S. enteritidis* (Table 2.). While *S. enteridis* was isolated in 82% of all *Salmonella* findings during 1996. and 1997, in 1998. an increased percentage (94.5%) of the type was

Salmonella types	No. of positive per total examined					
	1996		1997		1998	
	n	%	n	%	n	%
S. enteritidis	238/289	82.0	150/166	90.5	68/72	94.5
S. typhimurium	29/289	10.5	5/166	3.0	1/72	1.5
S. virchow	9/289	3.0	1/166	0.5	2/72	2.5
S. infantis	13/289	4.5	10/166	6.0	72/72	100

Tab. 2 - Occurrence of Salmonella enteritidis in positive isolates from poultry material

established (M r d e n et al., 1997; P a l i ć and O r l i ć, 1999). Similar increase of *S. enteritidis* positive isolates was reported by other authors (P i g u e t and P i r e s - G o m e s, 1989, WHO, 1998). The allarming increase of *Salmonella* occurence in Yugoslavia can be a result of bad economic conditions in poultry production, non-systematic profilactic exposure, and non-controled rat population (R a š i ć and O r l i ć 1999), and by use of suspective fish meal in poultry food mixtures (R a d a n o v - P e l a g i ć et al., 1998). The favourable decrease of *Salmonella* occurence can be achived by systematic controle monitoring, epizootiologic screeninig, controle of food components and foodstufs (R a š i ć and O r l i ć, 1999) and by introduction of profilactic measures against *S. enteritidis* (P a l i ć and O r l i ć, 1999).

The allarming increase of occurence of Salmonelosis from 4.9% to 15.8% in 1998 gave rize not only to large economic losses in poultry production due to increased mortality and treatmant cost, but endangeres health of human population as well.

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## ИЗОЛАЦИЈА *SALMONELLA ENTERITIDIS* СА ФАРМИ ЖИВИНЕ У ВОЈВОДИНИ

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#### Резиме

Салмонелозе све чешће угрожавају живинарску производњу и узрочници су били често изоловани из материјала пореклом од живине, у трогодишњем периоду (1996—1998). Од укупно испитаних бактеријских инфекција у 1996. години, салмонеле су биле изоловане у 4,9% узорака, 1997. години код 3,9% узорака, док је у 1998. години забележен значајан пораст присуства салмонела у испитаним узорцима живине (15,8%). Од укупног броја изолата салмонела, у току трогодишњег испитивања, доминантна је била *Salmonella enteritidis* са 82% у 1996. години, затим следи тренд пораста од 90,5% у 1997. години, као и у 1998. години, када учешће *S. enteritidis* износи 94,5%. Зборник Матице српске за природне науке / Proceedings for Natural Sciences, Matica Srpska Novi Sad, № 100, 71—75, 2001

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# SYNTHESIS OF 2-ACETYL-3-METHOXY-17-TOSYLOXY-16,17-SECOESTRA-1,3,5(10)-TRIEN -16-NITRILE

ABSTRACT: The title compound has been obtained in two independent ways starting from 3-methoxy-17-tosyloxy-16,17-secoestra-1,3,5(10)-trien-16-nitrile (1). Treatment of 1 with HBr in  $Ac_2O$  gave the 3-acetoxy derivative 2. Action of NaOMe upon compound 2 afforded migration of the acetyl function, whereby the acetyl derivative 3 was obtained. Finally, methylation of 3 gave 2-acetyl-3-methoxy-17-tosyloxy-16,17-secoestra-1,3,5(10)-trien -16-nitrile (4). On the other hand, compound 4 was obtained by the condensation reaction of 1 with acetyl chloride in presence of AlCl<sub>3</sub>.

KEY WORDS: Steroids, D-secoestrone derivatives, 2-acetyl-D-secoestrone derivatives, methylation.

## INTRODUCTION

In an earlier paper [Petrovic et al., 1992] we have worked out a procedure for demethylation of 3-methoxy-17-tosyloxy-16,17-secoestra-1,3,5(10)-trien-16-nitrile (1, Scheme 1), by the action of concentrated hydrogen bromide in acetic acid. Under mentioned reaction conditions, intermediate acetoxy derivative 2 was formed, which was saponified to compound 3, using sodium metoxide in methanol. However, the saponification procedure resulted in formation of a minor product, to which, according to spectral data, structure 3 was prescribed.

The aim of this work was the chemical confirmation of the proposed structure of compound 3.

## EXPERIMENTAL

Melting points (mp) were determined in open capillary tubes on a Büchi SMP apparatus and are uncorrected. Infrared spectra were recorded in KBr pellets on a Perkin-Elmer M 457 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were determined in CDCl<sub>3</sub> on a Bruker WP 250 SY spectrometer and are reported in parts per million downfield from a tetramethylsilane internal standard. Mass spectra were recorded on a Finnigan-MAT 8230 (E.I. 70 eV) spectrometer.

## 3-Methoxy-17-tosyloxy-16,17-secoestra-1,3,5(10)-trien-16-nitrile (1)

3-Methoxy-17-hydroxy-16,17-secoestra-1,3,5(10)-trien-16-nitrile (1 g, 3.34 mmol) and tosyl chloride (1 g, 3.34 mmol) were dissolved in absolute pyridine (30 cm<sup>3</sup>). The reaction mixture was kept at room temperature for 50 hours and was then poured into 6N HCl (300 cm<sup>3</sup>). The precipitate was collected by filtration, washed with water, and air-dried, giving a crude p-toluenesulfonate ester **1** in a 93% yield. After crystallization from MeOH, 1.06 g (70%) of pure 3-methoxy-17-tosyloxy-16,17-secoestra-1,3,5(10)-triene-16-nitrile (1), mp 88–91°C, was obtained.

IR spectrum ( $v_{max}$ , cm<sup>-1</sup>): 3.050, 2.225, 1.605, 1.505, 1.375, 1.180; <sup>1</sup>H NMR spectrum ( $\delta$ , ppm): 0.95 (s, C-18 Me), 2.50 (s, Me from tosyloxy group), 3.50 (q, C-17 protons, J = 21.25 Hz), 6.60 to 7.80 (7 aromatic protons); mass spectrum (m/z): 453 (M<sup>+</sup>), 241 (M<sup>+</sup>-C<sub>2</sub>H<sub>4</sub>NO), 212 (M<sup>+</sup>-CH<sub>3</sub>CN-CH<sub>2</sub>-OTs-CH<sub>3</sub>). Anal. calculated for C<sub>26</sub>H<sub>31</sub>NO<sub>4</sub>S: C, 68.87; H, 6.84; N, 3.09. Found: C, 68.99; H, 6.80; N, 3.08.

#### 3-Acetoxy-17-tosyloxy-16,17-secoestra-1,3,5(10)-trien-16-nitrile (2)

Aqueous solution of HBr (38%, 60 cm<sup>3</sup>) was added dropwise to cooled acetic anhydride (300 cm<sup>3</sup>), maintaining the temperature bellow  $15^{\circ}$ C. To the solution compound **1** (1g, 2.21 mmol) was added, the reaction mixture kept for 24 hrs at room temperature and then refluxed for 4 hrs. The cooled reaction mixture was poured into ice-cold water (1000 cm<sup>3</sup>), the precipitate was filtered off, washed with water and air-dried. The crude product was chromatographed on a silica gel column (15g, benzene-ethyl acetate /9:1/),whereby 100 mg (10%) of starting compound was recovered and 345 mg (36%) of compound **2**, mp. 137—138°C, was obtained.

IR spectrum ( $v_{max}$ , cm<sup>-1</sup>): 2.245, 1.765, 1.360, 1.175, 670 and 560; <sup>1</sup>H NMR spectrum ( $\delta$ , ppm): 0.90 (s, C-18 Me), 2.30 (s, Me from acetate function), 2.50 (s, Me from tosyloxy group), 3.57—4.00 (q, C-17 protons), 6.80—7.90 (7 aromatic protons); mass spectrum (m/z): 481 (M<sup>+</sup>), 453 (M<sup>+</sup>-CH<sub>2</sub> = CH<sub>2</sub>), 43 (CH<sub>3</sub>C = O<sup>+</sup>). Anal. calculated for C<sub>27</sub>H<sub>31</sub>NO<sub>5</sub>S: C, 67.35; H, 6.49; N, 2.91. Found: C, 67.31; H, 6.60; N, 2.85.

## 2-Acetyl-3-hydroxy-17-tosyloxy-16,17-secoestra-1,3,5(10)-trien-16-nitrile (3)

To a solution of sodium (0.55 g, 23.9 mmol) in absolute methanol (120 cm<sup>3</sup>) compound 2 (3.1 g, 2.08 mmol) was added. The reaction mixture was

kept for 5 min. at room temperature, then poured into water (300 cm<sup>3</sup>) and acidified with acetic acid to pH 3. The precipitate was filtered off, washed with water and air-dried. The crude product (2.9 g, 71.5 %) was chromatographed on a silica gel column (300 g, benzene-ethyl acetate /4:1/), yielding 1.0 g (31.55 %) of compound **3** mp. 151—152°C.

IR spectrum ( $v_{max}$ , cm<sup>-1</sup>): 3.500, 2.940, 2.440, 1.350, 1.175, 970, 670 and 560; <sup>1</sup>H NMR spectrum ( $\delta$ , ppm): 0.95 (s, C-18 Me), 2.50 (s, Me from tosyloxy group), 3.55—4.00 (q, C-17 protons), 4.70 (s, C-3, OH), 6.60—7.90 (7 aromatic protons); mass spectra (m/z): 439 (M<sup>+</sup>), 156 (M<sup>+</sup>-CH<sub>2</sub> = CH<sub>2</sub>--Me-D-seco ring). Anal. calculated for C<sub>27</sub>H<sub>30</sub>NO<sub>5</sub>S x MeOH: C, 66.24; H, 7.00; N, 2.97. Found: C, 66.11; H, 6.61; N, 2.78.

#### 2-Acetyl-3-methoxy-17-tosyloxy-16,17-secoestra-1,3,5(10)-trien-16-nitrile (4)

a) Anhydrous aluminiumchloride (13 g, 100 mmol) was dissolved in a mixture of carbon disulfide (10 cm<sup>3</sup>) and acetyl chloride (2 cm<sup>3</sup>, 28 mmol) and a solution of 1 (1 g, 2.38 mmol) in methylene chloride (10 cm<sup>3</sup>) was added dropwise. The reaction mixture was stirred for one hour at room temperature, then poured into ice-cold diluted HCl (1:1, 200 cm<sup>3</sup>) and extracted with ether (3x50 cm<sup>3</sup>). The organic phase was washed with a solution (5 %) of sodium hydrogencarbonate, followed by water and dried over anhydrous sodium sulfate. After evaporation of solvent, the crude product (0.92 g, 83.6 %) was chromatographed on a silica gel column (100 g, benzene-ethyl acetate /9:1/), yielding 0.75 mg (68.2 %) of 2-acetyl-3-methoxy-17-tosyloxy-16,17-secoestra-1,3,5-(10)-trien-16-nitrile (4), mp. 106—107°C.

b) To a solution of 2-acetyl-3-hydroxy-17-tosyloxy-16,17-secoestra-1,3,5-(10)-trien-16-nitrile (**3**, 200 mg, 0.46 mmol) in an 1:1 mixture of freshly distilled methanol and acetone (10 cm<sup>3</sup>) potassium carbonate (200 mg, 1.45 mmol) and methyl iodide (0.30 cm<sup>3</sup>; 1.10 g, 7.33 mmol) were added. The reaction mixture was refluxed for 8 hrs and then diluted with water (50 cm<sup>3</sup>). The separated solid was filtered off, washed with water and air-dried. The crude compound **4** (195 mg, 94.7 %) was crystallized from methanol yielding 150 mg (72.8 %) of pure 2-acetyl-3-methoxy-17-tosyloxy-16,17-secoestra-1,3,5-(10)-trien-16-nitrile (**4**), mp. 105–107°C.

IR spectrum ( $v_{max}$ , cm<sup>-1</sup>): 3.000–2.870, 2.245, 1.730, 1.670, 1.190, 970, 690 and 570; <sup>1</sup>H NMR spectrum ( $\delta$ , ppm): 0.75 (s, 3H, C-18 Me), 2.25 (s, 3H, Me from acetyl group), 2.70 (s, 3H, Me from tosyloxy group), 3.55–3.80 (m, 2H, C-17), 3.65 (s, 3H, Me from methoxy function), 6.50–7.65 (m, 7H, aromatic protons).

## **RESULTS AND DISCUSSION**

As mentioned, the aim of this paper was the chemical approval of the structure of 2-acetyl-3-hydroxy-17-tosyloxy-16,17-secoestra-1,3,5(10)-trien-16--nitrile (3) (Scheme 1).



Scheme 1

For this purpose, compound **3**, obtained by known procedure [P e t r o v i c et al., 1992], was methylated using methyl iodide in presence of potassium carbonate, whereby 2-acetyl-3-methoxy-17-tosyloxy-16,17-secoestra-1,3,5-(10)-trien-16-nitrile (**4**) was obtained in a high yield. On the other hand, compound **4** was synthesized starting from **1** [P e t r o v i c et al., 1990], by treatment with acetyl chloride in presence of anhydrous aluminiumchloride as catalyst [N a m b a r a et al., 1970]. Samples obtained by methylation of **3**, as well as by the condensation reaction of **1** with acetyl chloride revealed same spectral data and manifested identical chromatographic behavior.

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#### СИНТЕЗА 2-АЦЕТИЛ-3-МЕТОКСИ-17-ТОЗИЛОКСИ-16,17-СЕКОЕСТРА-1,3,5(10)-ТРИЕН-16-НИТРИЛА

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#### Резиме

У раду су описана два независна пута за добијање 2-ацетил-3-метокси-17--тозилокси-16,17- секоестра-1,3,5(10)-триен-16-нитрила (4). Као полазно једињење послужио је 3-метокси-17-тозилокси-16,17-секоестра-1,3,5(10)-триен-16-нитрил (1) који је дејством бромоводоничне киселине у ацетанхидриду дао 3-ацетат 2, а овај је дејством натријум-метоксида преведен у 2-ацетил-дериват 3. Најзад, метиловањем једињења 3 добијен је производ 4. С друге стране, једињење 4 је добијено директно у реакцији кондензације једињења 1 са ацетил-хлоридом у присуству алуминијум-трихлорида.

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# DOUBLE BETA DECAY AND THE PROPERTIES OF THE NEUTRINO

ABSTRACT: Connection between the  $\beta$  decay processes and the properties of the neutrino is presented. It is shown that the present experimental data still do not prove the theoretical GUT predictions on the nature of the neutrino and that the Standard Model of Particles is not violated. The experimental results of the Novi Sad group on the double beta decay are also discussed.

KEY WORDS: neutrino, double decay

## INTRODUCTION

Since its prediction by Wolfgang Pauli 70 years ago, the neutrino remained until recent times one of the most ambiguous particles in physics. Although it is a companion of the very well known  $\beta$  radioactive decay, the neutrino is still the only fermion for which we do not know experimentally whether it is different from its antiparticle ( $\nu \neq \tilde{\nu}$ ), is it a Dirac particle (as predicted by the Standard Model of Particles) or is it identical to its antiparticle ( $\nu = \tilde{\nu}$ ) and therefore a Majorana particle. The exeptionally obtained low order of magnitude ( $10^{-47}$  m<sup>2</sup>) of the cross section for the weak interaction of the neutrino with matter (which is equivalent of a mean free path of about 300 light years in water) is of course the main reason why it is so difficult to measure the basic properties of this particle.

The neutrino was discovered in the experiments of R e i n e s and C o w a n , 1959, using high intensity  $(10^{13} \text{ cm}^{-2} \text{ s}^{-1})$  antineutrino beams from a nuclear reactor. They succeeded in detecting the reaction

$$\tilde{\nu} + p \rightarrow n + \beta^+$$
 (1)

as a definite proof of the neutrino existence. Having in mind that  $D\,a\,v\,i\,s$  , 1955, failed to detect the inverse  $\beta\text{-decay}$  of  $^{37}\text{Cl}$ 

$$v + {}^{37}\text{Cl} \rightarrow {}^{37}\text{Ar} + e^- \tag{2}$$

with a similar antineutrino beam, it was concluded that  $v \neq \tilde{v}$ .

The discovery of parity nonconservation in  $\beta$  decay (W u et al., 1957) led to the conclusion that the weak interaction mediating the  $\beta$  decay is purely left-handed. Therefore the neutrino (antineutrino) created in the  $\beta$  decay of a neutron has positive helicity (spin parallel to the momentum), while the neutrino needed for the inverse  $\beta$  decay must have negative helicity. So even if  $\nu = \tilde{\nu}$ , reaction (2) would be forbidden due to helicity mismatch.

These open questions about the properties of the neutrino are today closely related to the predictions of some grand unified theories (Fritzsch and M i n k o w s k i, 1981). These theories predict not only that the neutrino is a Majorana particle, but also that it has a non-zero rest mass and slight righthanded interactions.

Experimental investigations of the  $\beta$  decay processes may contribute significantly to the solution of the presented dilemmas of contemporary physics.

## SINGLE $\beta$ DECAY AND THE NEUTRINO MASS

The nuclear  $\beta$  decay is a spontaneous transformation of the atomic nucleus changing its charge by one unit. Today we know that the process is the consequence of the weak interaction. The weak interaction is mediated by the virtual vector boson W with the rest mass of about 90GeV/ $c^2$ . Thus the range of this interaction is less than 10<sup>-3</sup> fm, what means that the decay is a nucleonic process. We distinguish three types of  $\beta$  decay:

$$\begin{array}{l} n \rightarrow p + e^{-} + \tilde{v}, \\ \beta^{-} \ decay, \\ p \rightarrow n + e^{+} + v, \\ \beta^{+} \ decay, \\ e^{-} + p \rightarrow n + v, \\ electron \ capture \ (E. \ C.) \end{array}$$

$$(3)$$

According to the Standard Model, the lepton number ( $\alpha = +1$  for e<sup>-</sup>, v and  $\alpha = -1$  for e<sup>+</sup>,  $\tilde{v}$ ) is conserved in  $\beta$  decay, thus  $v \neq \tilde{v}$ . Although this prediction was not challenged by  $\beta$  decay investigations, the hypothesis on the zero rest mass of the neutrino was questioned in a series of experiments. In order to describe these measurements, we will start from the general formula for the  $\beta$  spectrum derived from the assumption that  $m_v = 0$ :

$$N(p)dp = C |M_{fi}|^2 (E_0 - E)^2 p^2 dp,$$
(4)

which states that the number of emitted  $\beta$  particles with the momentum p N(p), is dependent on the nuclear transition matrix element  $M_{\rm fi}$ , the total transi-

.

tion energy  $E_0$  and the energy *E* and momentum *p* of the  $\beta$  particles. For a zero-range weak force the transition matrix element can be rewritten as

$$\mathbf{M}_{\rm fi} = \mathbf{G}_{\rm B} \mathbf{M} \ (\boldsymbol{\psi}_{\rm n}, \, \boldsymbol{\psi}_{\rm l}), \tag{5}$$

where  $G_{\beta} \approx 10^{-4}$  keV fm<sup>3</sup> is the  $\beta$  decay coupling constant that gives the order of magnitude of the transition probability and *M* is a function of the nucleonic ( $\psi_n$ ) and lepton ( $\psi_l$ ) wave functions. The nuclear matrix element depends on the initial and final wave functions of the nucleus and cannot be calculated in a model independent way, but for some  $\beta$  transitions, which are called 'allowed', it is independent on the energy of the  $\beta$  particles. For such transitions the shape of the  $\beta$  spectrum can be described by the formula

$$X = \frac{N(p)}{Cp^2} = C \left(E_0 - E\right), \tag{6}$$

which gives a straight-line dependence of X on E (Figure 1, dashed line).

If we assume that  $m_v \neq 0$ , then instead of (6) we derive:

$$X \quad C(E_0 - E) \quad 1 - \frac{m_v c^2}{E_0 - E} \stackrel{2}{\longrightarrow} \stackrel{1}{\longrightarrow} .$$
 (7)

Thus the straight line will be distorted near the endpoint energy (Figure 1, solid line).

Such distortions were mostly sought for in the tritium decay  $({}^{3}\text{H} \rightarrow {}^{3}\text{He} + e^- + \tilde{v})$  and the most recent results (K a w a k a m i et al., 1989, Frits c h i et al., 1990) yield an upper limit on the neutrino mass  $m(\tilde{v}) < 11\text{eV}/c^2$ .



Fig. 1 — Shape of the  $\beta$  spectrum with  $m_v = 0$  (dashed line) and with  $m_v > 0$  (solid line)

We can mention that some anomalies in the  $\beta$  spectrum of <sup>14</sup>C and inner bremsstrahlung spectrum of <sup>55</sup>Fe observed in the experiments of N o r m a n et al., 1991, were interpreted as evidence for 17 keV/ $c^2$  mass neutrinos. This and some following ,,discoveries" were based on the assumption that most neutrinos emitted in decay have zero mass ( $m_v = 0$ ) and only a small fraction of them (about 1%) have big, 17 keV/ $c^2$ , mass. These results are not generally accepted, so we can conclude that from decay measurements we still do not positively know that the mass of neutrino is zero; all we can state is that it is most probably less than 10 keV/ $c^2$ .

## DOUBLE **B** DECAY

The double  $\beta$  decay is a simultaneous transition of two nucleons  $(2n \Leftrightarrow 2p)$ induced by weak interaction. In this decay at least two intermediate bosons are exchanged, so it is a second order-process. Due to a small value of the weak coupling constant  $G_{\beta}$  the probability  $W_{\beta\beta}$  for double  $\beta$  decay (which would be roughly  $W_{\beta\beta} \approx W_{\beta}^2 \approx G^4$ ) becomes extremely small leading to lifetimes with the order of magnitude of  $10^{20}$  years. Such a rare process can be detected only if the single decay is forbidden. Generally the even-even nuclei in the bottom of the isobaric decay chain have lower masses than their odd-odd  $Z \pm 1$  neighbors and thus their single  $\beta$  decay is energetically not possible. Such nuclei (generally considered as stable) are the best species for the investigation of  $\beta\beta$ decay.

One of the most extensively investigated (A v i g n o n e et al., 1991)  $\beta\beta$  decays, the decay of <sup>76</sup>Ge is presented in Figure 2.



Fig. 2 — The <sup>76</sup>Ge doble  $\beta$  decay.

There are four possible modes of  $\beta\beta$  decay:

a)  ${}^{A}_{Z}X {}^{\beta^{-}\beta^{-}} \rightarrow {}^{A}_{Z+2}Y + 2e^{-} + 2\tilde{v}$  double electron emission b)  ${}^{A}_{Z}X {}^{\beta^{+}\beta^{+}} \rightarrow {}^{A}_{Z-2}Y + 2e^{+} + 2v$  double positron emission c)  $e^{-} + {}^{A}_{Z}X {}^{EC,\beta^{+}} \rightarrow {}^{A}_{Z-2}Y + 8e^{+} + 2v$  electron — positron conversion d)  $e^{-} + e^{-} + {}^{A}_{Z}X {}^{EC,EC} \rightarrow {}^{A}_{Z-2}Y + 2v$  double electron capture In the decays (8) lepton number conservation is assumed. Such decays are usually denoted as  $2\nu\beta\beta$  decays because they assume the emission of two neutrinos or antineutrinos according to the Standard Model.

The double  $\beta$  decays can be detected by the geochemical method or by counting experiments. In the geochemical measurements, the accumulation of the decay products in a natural mineral of the parent element is measured. The accuracy of such results depends significantly on the long-term stability of the minerals in which the amounts of decay products are measured. So far only the measurements of noble gas isotopes in selenium and tellurium minerals yielded reliable results (M a n u e 1, 1991). Of course, such measurements cannot make difference between the  $2\nu$  and  $0\nu$  decay modes; their results give the  $2\nu + 0\nu$  half-lives.

In the counting experiments the spectrum and decay signatures of the emitted particles are measured by various types of detectors. In order to detect the very rare  $\beta\beta$  events, high efficiency and extra low background detection systems are needed.

As a result of several hundred decay measurements only the lower limits of the half-lives were mostly established. Only for 5 isotopes (<sup>76</sup>Ge, <sup>82</sup>Se, <sup>100</sup>Mo, <sup>116</sup>Cd, and <sup>150</sup>Nd) is the half-life for the  $2\nu\beta^{\Box}\beta^{\Box}$  determined (T r e t y a k and Z d e s k o, 1995, F i o r i n i, 2000) in the range  $10^{19} - 10^{21}$  years. The first recorded EC,  $\beta^{+}$  ( $0\nu + 2\nu$ ) half-life value ( $1.1 \pm 0.8$ ) x  $10^{19}$  years is a result of the experiment (B i k i t et al., 1995) on <sup>64</sup>Zn, which will be described later. The experimental results on the  $2\nu\beta\beta$  decay half-lives are necessary for the verification of the theoretical values of nuclear matrix elements. As will be shown later, reliable values of nuclear matrix elements are needed for the evaluation of the neutrino mass from the measured  $0\nu\beta\beta$  half-lives  $T_{1/2}$ .

## NEUTRINOLESS DOUBLE BETA DECAY

The neutrinoless double beta decay  $(0\nu\beta\beta)$  is a decay mode where the neutrino emitted in the first vertex is reabsorbed in the second one (Figure 3).



Fig. 3 — The  $0\nu\beta^{-}\beta^{-}$  decay

This process obviously violates the lepton number conservation law of the Standard Model. The first condition that enables this decay is that the neutrino is a Majorana particle ( $\nu \quad \tilde{\nu}$ ) so that the lepton conservation law becomes meaningless and all our descriptions of the  $\beta$  decay and weak interaction must be changed. But even if  $\nu \quad \tilde{\nu}$  the process will be still forbidden due to helicity mismatch in the first and second vertex. In order to allow the change of the neutrino helicity it is necessary to assume that the neutrino has a finite mass (and thus no definite helicity) and/or that the weak interaction has a right-handed component. In left-right symmetric models, the weak interaction is mediated by  $W_1^{\pm}$  and  $W_2^{\pm}$  bosons that are mixtures of left ( $W_L^{\pm}$ ) and right-handed ( $W_R^{\pm}$ ) bosons:

$$W_1^{\pm} \cos \Phi W_L^{\pm} + \sin \Phi W_R^{\pm}$$

$$W_2^{\pm} - \sin \Phi W_L^{\pm} + \cos \Phi W_R^{\pm}$$
(9)

where  $\Phi$  is the mixing angle.

According to this theory we see mainly left-handed bosons with the mass  $M_{\rm L} = 90$  GeV because the mass of the right-handed boson  $M_{\rm H}$  is much bigger. The transition probability for the 0y88 decay is (Faessler, 1991) is

$$W(0\nu\beta\beta) \quad \frac{\ln 2}{T_{1/2}} \quad \left| M_m \langle m_\nu \rangle + M_\Phi \langle tg\Phi \rangle + M_M \langle M_L^2 / M_H^2 \rangle \right|^2, \tag{10}$$

where  $M_{\rm m}$ ,  $M_{\Phi}$ , and  $M_{\rm M}$  are nuclear matrix elements and  $(m_{\rm v})$  is the effective neutrino mass. In the 0v $\beta\beta$  process we have only two emitted particles (instead of four in the 2v $\beta\beta$  decay) and the phase space will increase by a factor of 10<sup>6</sup> relative to the 2v $\beta\beta$  decay. Thus the nuclear matrix elements M (which include the phase space factor) for the 0v $\beta\beta$  decay will have at least the same order of magnitude as for the 2v $\beta\beta$  process. If we further suppose that the mass of the heavy boson  $M_{\rm H} > 1$  TeV, the last term in (12) will be negligible, which for the low values of the effective mixing angle (tg $\Phi$ ) < 10<sup>-8</sup> allows the direct evaluation of the neutrino mass from the measured half life.

For the  $0\nu\beta\beta$  decay mode the neutrino and antineutrino terms in (8) should be omitted. Thus the sum of the energies of the two e<sup>-</sup> and two e<sup>+</sup> in (8a) and (8b) will be monoenergetic as well as the energy of the e<sup>+</sup> in (8c). This fact enables the experimental recognition of the  $0\nu\beta\beta$  decay mode. As can be seen from (8d), no particle is emitted in the (0 $\nu$ , EC, EC) decay. After the capture of two electrons (mainly from 1s orbits) the parent nucleus  ${}^{A}_{Z}X$  transforms to the daughter  ${}^{A}_{Z-2}Y$  in an excited state (V e r g a d o s, 1983). The energy of this state is determined by the parent-daughter mass difference and the binding energy of the electrons and it is not equal to the energy of any eigenstate of  ${}^{A}_{Z-2}Y$ . Thus the energy of the  $\gamma$  ray deexciting this state is different from the known  $\gamma$  ray energies, which enables the unique detection of this process.

Today the most sensitive method (Klapdor-Kleingrothaus and Staudt, 1995, Baudis, 1999) for the investigation of the  $0\nu$  mode is the measurement of the  $0\nu\beta^{\Box}\beta^{\Box}$  decay of <sup>76</sup>Ge by large mass ( $\approx 10$  kg) HPGe spec-

trometers enriched with more than 80% <sup>76</sup>Ge. The 100% efficiency for the electrons emitted in the sensitive volume of the detector, deep underground positioning, and heavy shielding enabled to reach a lower limit for the half life  $T_{1/2} > 5.6 \times 10^{24}$  years, which is equivalent to the mass limit ( $m_v$ ) < 0.65eV. Although the 0v $\beta\beta$  process is still not detected, the performed measurements established the most stringent limit of the neutrino mass.

## DOUBLE BETA DECAY MEASUREMENTS IN NOVI SAD

The Novi Sad low-level gamma spectroscopy laboratory is an on-ground located facility equipped with a massive 25 cm wall thickness, 1 m<sup>3</sup> useful volume, pre WWII cast iron shield. Thus in the laboratory only  $\gamma$  ray events from  $\beta^+\beta^+$  can be measured and the background can be reduced by coincidence methods.

### Coincidence detection of positrons

In the cases of  $(\beta^+\beta^+)$  and  $(EC^+, \beta^+)$  two or one positron are emitted, respectively. These positrons annihilate in the voluminous sample material emitting simultaneously two  $\gamma$  rays with the energy of 511 keV in opposite directions. The coincidence counting of these  $\gamma$  rays enables the determination of the double  $\beta$  decay half-life. In order to extend the detection limits towards the  $10^{20}$  y half-life region, large quantities of sample material and efficient and good resolution detectors must be used. Although the coincidence method significantly reduces the background, passive and active shielding is necessary. In order to reduce the number of chance coincidences a fast-slow coincidence system is required.

The setup built in the low-level laboratory in Novi Sad is schematically presented in Figure 4.

The cylindrical sample is sandwiched between a 3" x 3" NaI(Tl) and a 25% nominal efficiency HPGe detector. The signals from the detectors are split. Through the constant fraction discriminator (CFD) and the amplitude and rise time compensated discriminator (ARC) fast timing signals are obtained and led to the time to amplitude converter (TAC). The fast time spectrum is through the analog to digital converter (ADC3) collected in the third section of the multichannel analyzer memory. The true coincidence signals from the single channel (SCA) section of the TAC are coincidenced with the 511 keV part of the HPGe spectrum derived from the timing single channel analyzer (TSCA). The signal from the slow coincidence unit (COINC) gates the ADC1 which processes the spectrum from the NaI(Tl) detector. Thus the intensity of 511 part of the coincidence spectrum from ADC1 measures the number of positrons from the sample. In order to suppress the contribution of annihilation events from environmental rays and cosmic rays the two detectors are placed in the hole of a 9" x 9" annular NaI(Tl) detector (ANNULUS). The signals from this detector are used for anticoincident gating of the TAC and all ADCs. In this way most of the events generated by rays not coming from the sample are electronically rejected. The whole detector assembly was operated in the iron passive shield.



Fig. 4. — The experimental setup for coincidence detection of positrons

As shown in B i k i t et al., 1999, one of the significant contributions to the background of the system is the NaI(Tl) detector itself. Natural radionuclides especially from the  $^{232}$ Th series and from  $^{40}$ K elevate the background of the HPGe detector.

Despite the passive and active shielding, 511-511 keV background coincidence events are still generated at the sample position and in the detectors. The measurement of this background is not simple. Three types of background measurements were performed: one with no material between the detectors and two with blank samples (Fe and Cu). No statistically significant difference between these results was obtained. The average value of all these measurements, derived for 6700 ks counting, was  $71(6) \times 10^{-3}$  c/ks.

The coincidence efficiency of the system  $\varepsilon = R_c/A$  ( $R_c$  — true coincidence count rate, A — calibrated <sup>22</sup>Na source activity) was  $\varepsilon = 1.9\%$ . This value was corrected for different samples by Monte Carlo methods.

The <sup>50</sup>Cr 
$$\rightarrow$$
 <sup>50</sup>Ti decay

The double  $\beta$  decay of <sup>50</sup>Cr is presented in Figure 5. The mass difference between the nuclei <sup>50</sup>Cr and <sup>50</sup>Ti is  $\Delta mc^2 = 1183.3$  (1.2) keV. This energy is besides the (EC, EC) decay sufficient for an (EC,  $\beta^+$ ) process with the transition energy of 144 keV. This energy is far from the 1554 keV energy of the

first excited state of <sup>50</sup>Ti, so the (EC,  $\beta^+$ ) decay can lead only to the <sup>50</sup>Ti ground state. The decay might be enhanced due to the fact that the daughter is a *Z* magic one. The 4.34% abundance of <sup>50</sup>Cr in natural Cr offers modest detection limit for the measurement of the (0v + 2v) half-life with natural Cr.



Fig. 5. — The (0v + 2v) decay scheme of <sup>50</sup>Cr

The  $\phi = 70$  mm and h = 25 mm thick p. a. grade Cr<sub>2</sub>O<sub>3</sub> sample contained 4.7 g of <sup>50</sup>Cr. The spectrum of this sample measured for 2562 ks is presented in Figure 6. The integration of the 511 keV area confirms what can be visually concluded: there is no statistically significant difference between the sample and the background count rate. So from the parameters of the experiment only a lower limit of the half-life could be determined (B i k i t et al., 2000):

 $T (0v + 2v) \ge 1.03 \text{ x } 10^{18} \text{ y } (68\% \text{ confidence level})$ 

This is currently the best limit for the <sup>50</sup>Cr (EC,  $\beta^+$ ) decay.



Fig. 6. - The Cr (solid squares) and background (open squares) spectrum

The mass difference between the parent and the daughter in this decay is  $\Delta mc^2 = 1.0965$  MeV. This energy besides the (EC, EC) mode allows for the (EC,  $\beta^+$ ) electron-positron conversion process with the lepton energy of only 66 keV. The tiny energy reduces the phase space, thus increasing the half-life. The small lepton energy suppresses also the contribution of right-handed amplitude to the probability of the (0v) decay mode (V e r g a d o s 1983).

On the other hand, the high abundance (49%) of  ${}^{64}Zn$  in natural Zn and the favorable spin sequence of the ground states involved ( $0^+ \rightarrow 1^+ \rightarrow 0^+$ ) motivated us to set up the experiment.

The  $\phi = 70$  mm and h = 25 mm cylindrical sample of p. a. grade Zn had the mass of 350 g. The coincident spectrum of this sample measured for 1720 ks is presented in Figure 8. As can be seen in the 511 keV region, the sample spectrum has a bigger area than the background one. By integration of the net Zn spectrum an area of 0.06(4) counts/ks was found. For the conditions of our experiment this area is equivalent to the half-life (Bikit et al., 1997).

 $T_{1/2} (0v + 2v) = (1.1 \pm 0.9) \text{ x } 10^{19} \text{ y } (99.7\% \text{ confidence level}).$ 

This value is the first reported positive result for the half-life of the (EC,  $\beta^+$ ) double decay process. The unexpectedly low value of the half-life is still not explained theoretically. The enhancement of the transition probability might be sought from the fact that the daughter nucleus is a *Z* magic one.



Fig. 7. — The results of the  $^{64}\text{Zn}$  (EC,  $\beta^+)$  mesurements

The mass difference for this decay (Figure 9) of  $\Delta mc^2 = 680.1. \pm 0.6$  keV allows only for the (EC, EC) process. The neutrinoless mode of this decay leaves the daughter nucleus with excitation energies  $668.1 \pm 0.1$  keV (K, K — decay),  $673.4 \pm 0.1$  keV (K, L — decay) and  $678.1 \pm 0.1$  keV (L, L — decay). These energies are not the energies of an eigenstate of <sup>54</sup>Mn; they are less than the energy of the first excited 2<sup>+</sup> state of <sup>54</sup>Mn. The detection of such "wrong" energies from iron sample would confirm the 0v decay process.

Making use of our passive cubic shaped iron shield as iron sample/source with the HPGe detector in the center, we could set up a very sensitive experiment for this decay. The 22 t iron shield contains 5.4% <sup>54</sup>Fe. Taking into account the self-absorption in iron and the geometry of the measurement, the detection efficiency was estimated. According to this calculation we see with unit efficiency 32 g of <sup>54</sup>Fe.



Fig. 8. — The  ${}^{54}\text{Fe} \rightarrow {}^{54}\text{Mn}$  decay scheme

The 670 keV region of the Fe spectrum measured for 281 days is presented in Figure 10. As can be seen the KK line lies on the tail of the <sup>214</sup>Bi background line, while the spectrum is flat in the KL and LL line regions. In none of these regions a total absorption peak is registered, so only upper limits on the line intensities could be set. The limiting intensity led to the result (B i - k i t et al., 1998)

 $T_{1/2}$  (0v, K, K) > 4.4 x 10<sup>20</sup> y (68% confidence level)

Our value is two orders of magnitude more stringent than the reported values for the (0v) <sup>196</sup>Hg decay, but still it is far from the theoretically predicted half-life of 1.5 x 10<sup>27</sup>.



Fig. 9. — The 670 keV region of the Fe spectrum

## CONCLUSION

Despite the significant efforts of the nuclear physics community worldwide the fundamental properties of the neutrino are still not known. So many fundamental problems of contemporary physics as the solar neutrino problem, the neutrino oscillation problem and the problem of missing mass in the universe are still unsolved. Significant progress is expected from future experiments (Fiorini, 2000).

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## ДВОСТРУКИ БЕТА РАСПАД И ОСОБИНЕ НЕУТРИНА

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#### Резиме

У овом раду представљена је веза између процеса бета распада и особина неутрина. Показано је да садашњи експериментални подаци још увек не потврђују предвиђања теорија великог уједињења о природи неутрина, као ни да није нарушен стандардни модел честица. Продискутовани су и експериментални резултати новосадске групе у вези са двоструким бета распадом.

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# THE MECHANISM OF OSCILLATORY ION TRANSPORT AND BIOPOTENTIAL ACROSS THE EXCITED CELL MEMBRANE

ABSTRACT: This paper presents results obtained in studies on oscillations of the membrane potential in several model-objects: *Nitelle* cells and maize (*Zea mays* L.) root cells. The possibility to induce different classes of oscillations, single and local impulses of the membrane potential in dependence on effects of stimuli that cause membrane excitation is also presented. The author explains the phenomenon of membrane potential oscillating by inducing the excited state of cell membrane, and then he presents some parameters that characterise such a membrane state. It is stated that oscillating of the membrane potential is caused by oscillatory ion transport processes across the excited cell membrane. Possible mechanisms of oscillatory transport processes and membrane potential oscillations across the excited cell membrane are also presented.

KEY WORDS: plant cell, root tissue, cell membrane, excited state, transport processes, ions, oscillations, impulses, membrane potential.

## INTRODUCTION

It is a generally known fact that phenomena and movements of oscillatory and rhythmic nature occur under within almost all fields of physics and physical chemistry. Oscillations are such movements and processes at which, magnitudes of physical quantities determining them, occur at exactly or approximately equal periods of time. Hence, any system disturbed from equilibrium starts to oscillate under certain conditions. Systems with one or two degrees of freedom are the basis for the analysis of oscillations. Regardless of different types of oscillatory processes, each oscillatory system can be described by a physical quantity whose displacement from its equilibratory value depends on coordinates and time.

The unique mathematical model is used in description of all oscillatory processes, but, homogenous differential equations of the second order with

constant coefficients are mainly applied for discrete systems, while partial differential equations with variable coordinates and time are applied for continuous systems [Andronov et al., 1966; Crawford, 1984; Tihonov et al., 1972].

On the other hand, oscillations and rhythms are one of principal characteristics of living organisms. Rhythm, as a type of regularity, with a patterned increase and decrease of certain parameters in the curse of time, has been detected at all levels of organisation: molecules, cells, tissues, organs, organisms and the population [Bjunning, 1964; Bioteux et al., 1977]. Overall bioelectric studies on plant models contributed to a discovery of the phenomenon of membrane potential oscillating. Today, the occurrence of membrane potential oscillating ( $\psi$ , mV) in plants is more systematically studied and analysed and therefore it is a very actual and contemporary scientific topic. This statement is supported by facts presented in numerous monographic papers [Kols et al., 1993: Kranvsky and Zhabotinsky, 1981: Sweenev, 1969; Žabotinskij, 1974], as well as in several scientific conferences on both, general and scientifically orientated issues on oscillatory, rhythmic, wave and auto-wave phenomena. Scientific conferences elaborated a mathematical and physical basis of oscillatory processes [B h a t n a g r, 1979; Frank et al., 1996; Seljkov et al., 1971; Vorobiev et al., 1968; Žabotinskij, 1967] initiating, in such a way, many issues on the theoretical base of oscillatory, wave and rhythmic processes and thereby indicating the nature of their occurrence, propagation and programmed inducements.

The phenomenon of membrane potential oscillating on the plasmalemma and tonoplast of intact plant cells was discovered in 1965, but was somewhat later announced in a scientific journal [R a d e n o v i ć et al., 1968] and since then we have been working on the following: (a) improvement of existing and development of new bioelectric and chemical measurement methods of the membrane potential, (b) observing effects of different stimuli on occurrence of membrane potential oscillating, (c) registering new and different classes of the membrane potential oscillations, (d) determination of dependence between occurrence of membrane potential oscillating and oscillatory transport processes across the excited cell membrane. Furthermore, it has been worked on the ascertainment of elements, parameters and functions that could be unique in the description and analysis of oscillatory mechanisms in physics and biology, i.e. biophysics. Such an approach, if successful, could provide solutions that can enable our better understanding of the diverse nature of all oscillatory and rhythmic phenomena, and creation of a unique image, which might provide rational modes of studying these phenomena through parameters of their analogy.

Results, presented in this paper, obtained in our both, previous and present, studies impose some actual issues of interests for further experiments and theoretical considerations of the mechanism of oscillatory ion transport across the excited cell membrane.

## MATERIAL AND METHODS

*Nitelle* cells and maize (*Zea mays* L.) root tissue were used as plant objects in the experiments. Growing conditions, preparation of the objects, their treatment and maintenance prior to and in the course of measuring had already been described in our studies [R a d e n o v i ć et al., 1968; R a d e n o v i ć and P e n č i ć, 1970; R a d e n o v i ć, 1974; R a d e n o v i ć et al., 1977].

Bioelectric signals: single impulses and oscillations of the membrane biopotential ( $\psi$ , mV) were registered after the method, which was also described, in principle and details, in our previously published papers [R a d e n o v i ć et al., 1968; R a d e n o v i ć and P e n č i ć, 1970; R a d e n o v i ć, 1974; R a d e n o v i ć and V u č i n i ć, 1976; R a d e n o v i ć et al., 1977; R a d e n o v i ć et al., 1980].

## RESULTS

## Single impulses and oscillations of the membrane potential induced by mechanical excitement of the cell membrane

All initial measurements of bioelectric occurrences, and thereby, single impulses, i.e. their sequences in the form of the membrane potential oscillations, were performed on the objects (plant cell, tissue segments or plant organs) kept in a standard solution consisting of 1.0 mM KCl + 10.0 mM NaCl. A rhythmic bioelectric response (single impulses or the membrane potential oscillations) was obtained under stated conditions and in the beginning of the experiment in over 15% of all performed bioelectric experiments.

Fig. 1A, B and C, presents bioelectric signals in the form of oscillations of the membrane potential caused by a mechanical stimulus (excitement of the cell membrane) by cell impalement by microelectrode. The base level of oscillations of the membrane potential ( $\psi$ , mV) induced in such a way tend towards the establishment of the stationary level of -150 mV (Fig. 1A), i.e. a somewhat lower level of -135 mV (Fig. 1B) and sometimes even a lower level of about -115 mV (Fig. 1C), which correspond to the limits of a plant physiological state [Vorobjev et al., 1968]. The establishment of the stationary level of the membrane potential oscillations is performed by a gradual increase in the course of time between the two successive impulses (Fig. 1A, B and C). This incidence is very often accompanied by an occurrence of local impulses (Fig. 1A and C). Local impulses, within membrane potential oscillations, often occur at the initial part of the oscillation (Fig. 1A, B and C), but they can also be detected in other parts of the membrane potential oscillations, sometimes even in a form of bursts, whereby they establish a certain relation towards normal impulses (Fig. 1A). Rhythmic bioelectric signals: impulses, normal and local, sequences of these impulses (Fig. 1A, B and C), are classified into typical examples of the membrane potential oscillations induced by membrane excitation by means of the mechanical impalement.



Fig. 1A, B, C — Oscillating pattern of the membrane potential induced by a mechanic excitation of the cell membrane and external solution: 1.0 mM KCl + 10.0 mM NaCl

# Single impulses and oscillations of the membrane potential occurring "spontaneously"

Single impulses, their small sequences and sometimes even a longer oscillation of the membrane potential could occur all at once, almost "spontaneously" — induced by nothing in the moment of electro-physiological measurements under conditions deferring from the physiological state. As an illustra-



Fig. 2A, B, C — Occurrence of sequences of single impulses in a form of a special membrane potential oscillations generated "sponteously" and with an external solution: 1.0 mM KCl + 10.0 mM NaCl

tion of the stated three patterns of the membrane potential oscillations are presented in the Fig. 2A, B and C. These patterns show some similarities, but also differences and as such, could be classified into a group of the membrane potential oscillations that occur "spontaneously". The membrane potential oscillations (Fig. 2A), were formed to the smallest extent, occurred only by "spontaneous" changes. Its occurrence was most probably influenced by membrane injuries, concentration "shock" and a phenomenon of spontaneity; its base level increases firstly, then sharply decreases and ends in a form of a damped oscillation. Moreover, the membrane potential oscillation can occur after a shorter stationary state (Fig. 2B). Although its occurrence ensues almost "spontaneously", it ends by establishing normal stationary state with a base level of about -120 mV. The most typical pattern of the membrane potential oscillations with a status of spontaneity is presented in the Fig. 2C. A modified sequence of single impulses, that passes into a sequence of local impulses is also observable in the Fig. 2C. This oscillating of the membrane potential occurred at a somewhat lower level of the membrane potential.

## Damped oscillations of the membrane potential induced by concentrations of chosen cations

The stationary level of the membrane potential can be established even without oscillation occurrences. As above stated, the establishment of the stationary level of the membrane potential is often accompanied with single impulses (Fig. 3A) that occur in smaller sequences and then cease. If the stationary level of the membrane potential is affected by a higher concentration of KCl



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Fig. 3A, B, C — Occurrence of damped oscillations of the membrane potential induced by a substitution of concentrations in the object external medium, with potassium:
Photo A: standard solution (♥: 1.0 mM KCl + 10.0 mM NaCl) solution KCl (b: 10.0 mM KCl)
Photo B: standard solution (♥: 1.0 mM KCl + 10.0 mM NaCl) solution KCl (b: 10.0 mM KCl)
Photo C: standard solution (♥: 1.0 mM KCl + 10.0 mM NaCl) solution KCl (b: 10.0 mM KCl)

(b: 10.0 mM KCl) under such conditions (Fig. 3A), the damped oscillation of the membrane potential will appear. Naturally, there are other cases (Fig. 3B), whereby oscillations of the membrane potential occur on the beginning of the experiment, when only the standard solution is applied. Its impulses become more infrequent and at the end they cease and the membrane potential reaches the stationary level that complies with physiological standards (Fig. 3B). Under conditions when the stationary level of the membrane potential is exposed to a higher KCl concentration (b: 10.0 mM KCl) (Fig. 3B) a smaller sequence of impulses of a damping nature occurs in the region of the membrane potential can occur at the regular substitution of solutions differing in their concentrations (Fig. 3C). The above mentioned examples with damped oscillations of the membrane potential were induced by potassium ions.

Naturally, the occurrence of oscillations of the membrane potential can be induced by other biogenous cations, first of all, nitrogen ion (Fig. 4A and B). Namely, when the stationary level of the membrane potential is established at the standard physiological level, then a standard solution is substituted in the external medium with a solution of 10.0 mM NaCl, and subsequently, single impulses occur, become denser and pass in the membrane potential oscillation with the damping trend (Fig. 4A). A somewhat different character of the membrane potential oscillation, also induced by sodium (10.0 mM NaCl), is presented in Fig. 4B. In the beginning, the oscillation amplitude gradually rises with increasingly greater time between oscillation impulses. Such a condition is changed in the middle of the membrane potential oscillation (Fig. 4B). Namely, symmetrical changes pertaining to the time between impulses and the magnitude of amplitudes (Fig. 4B).



Fig. 4A, B — Occurrence of damped oscillations of the membrane potential induced by sodium: ▲ 10.0 mM NaCl

## Single impulses and oscillations of the membrane potential in maize root tissue

Bioelectric-chemical approaches in experiments with maize root tissue could lead to results on the membrane potential. Such results have been often encountered for the last ten years. However, oscillations of the membrane potential on plant tissue, in our case, on maize root tissue is much more difficult to induce [R a d e n o v i ć et al., 1980]. Figure 5a, b, c, c and e presents five mutually different oscillations of the membrane potential registered on maize root tissue. These oscillations were recorded under the equal conditions regarding external solution, tissue and other technical properties. The position of a measuring microelectrode was unlike, i.e. less certain. Namely, since root tissue is still a matter of dispute, a microscope is used and it is difficult to be sure about the position of the working part of the microelectrode in relation to the membrane. As it can be assumed, several positions of the microelectrode are possible, hence, no wonder, the following impulses occur: single impulses and associated local impulses (Fig. 5a), associated single impulses and se-



Fig. 5a, b, c, d, e — Formation of different single and local impulses, as well as, membrane potential oscillations on the maize root tissue subjected to a nutritive solution: 0.1 mN KH<sub>2</sub>PO<sub>4</sub> + 1.0 mN NaHCO<sub>3</sub> + 0.4 mN CaCl<sub>2</sub> + 0.2 mN Mg(NO<sub>3</sub>)<sub>2</sub>

quences of local impulses (Fig. 5b), irregular oscillations of the membrane potential (Fig. 5c), then completely regular oscillations of the membrane potential (Fig. 5d), then regular oscillations of the membrane potential very similar, first of all, in respect to the frequency, to oscillations in chemical reactions (Fig. 5e).

## DISCUSSION

Results obtained on rhythmic bioelectric signals (single impulses, local impulses, sequences of single and local impulses, membrane potential oscillations), presented in this study are only a smaller part of our long-term programme on membrane potential oscillations in plants. Although presented compressedly, these results offer an opportunity to analyse many issues not sufficiently known yet.

Some parameters of membrane potential oscillations had already been presented in our previous papers [R a d e n o v i ć et al., 1977; R a d e n o v i ć, 1983; R a d e n o v i ć and V u č i n i ć, 1985]. However, this occasion should be used to point to these parameters with regard to determination of elements of analogy between oscillations in biology and physics [V u k s a n o v i ć et al., 1998]. These parameters encompass: base level of membrane potential oscillations, impulse spike potential (a level up to which the membrane depolarises when single or successive impulses are generated), amplitudes of single or successive impulses are generated), amplitudes of single or successive impulse to a subsequent or a preceding impulse in the chosen the membrane potential oscillation, impulse interval (duration between two succeeding impulses), the number of impulses in the membrane potential oscillation, the frequency of the membrane potential oscillation and duration of the membrane potential oscillation formation. Stated parameters can be easily observed in the presented results (Fig. 1-5).

In addition, attention should be given to issues such as kinetics of single impulses and kinetics of the overall membrane potential oscillation, as well as, to the nature of appearance and behaviour of rhythms of bioelectrical signals [Damjanović and Radenović, 1971; Radenović and Pen-čić, 1970; Radenović and Vučinić, 1976]. Furthermore, attention should be paid to effects of ion concentrations on generating of membrane potential oscillations [Radenović, 1985b; Volkov and Misjuk, 1968; Vorobiev et al., 1967, 1968; Vučinić et al., 1998; Vuletić et al., 1987]. The above stated issues and parameters, characterising membrane potential oscillations, are directly related to transport processes occurring in the excited cell membrane [Radenović, 1998].

As already mentioned, systems with one or two degrees of freedom are the basis for the analysis of the oscillatory mechanism. Besides, different characters of movements of lipids, proteins, pigments and other complex-bound structures contribute to mechanisms of the total transport processes across the excited cell membrane [R a d e n o v i ć, 1998]. These characters of movements could be: lateral movement (typical for lipids and proteins), rotary movement (typical for proteins specialised for ion transport) and flip-flop movement (typical for lipids and proteins regulating transport processes from one to other side of the excited membrane). When the degree of cell membrane excitation is higher, the mentioned characters of movements (of lipids, proteins and other molecules) are significant in respect of intensity, dynamics and diversity, reflecting on total transport processes [K o l s et al., 1993; R a d e n o v i ć, 1998].

As it is already known, ion transport across the excited cell membrane is characterised by passive and active processes. Diffusion is considered to be a dominant bearer of passive processes and is often expressed as simple, limited and facilitated diffusion. Simple diffusion consists of ion transport processes through the lipid two-layer, pores in both proteins and the lipid two-layer. Limited diffusion occurs in a form of ion transport processes through pores with charged groups on proteins. At last, facilitated diffusion occurs in a form of ion transport processes with a mobile carrier, a fixed carrier and exchange diffusion. It is obvious that there are at least two initiators of occurrence of the stated ion transport processes with passive characteristics. A concentration gradient and a membrane potential gradient are these initiators. Naturally, when these two gradients are coupled, then ion transport processes can take place from spots of a lower to spots of a higher concentration [Radenović, 1998], what is one of properties characteristic for active ion transport processes across the excited cell membrane. Besides, these processes, also, occur against chemical and electrochemical gradient. These processes can take place only with a certain energy consumption. These processes do not occur independently, but always alongside with the hydrolysis processes of ATP, i.e. on the account of consumption of energy accumulated in macroenergetic ATP bonds, i.e. by ATPase.

Gained results, presented in this study, indicate that membrane potential oscillations occur under particular conditions (Fig. 1–5). Moreover, the well--known dependence of membrane potential and ion transport processes across the excited cell membrane is already presented in this study. Different possibilities of ion transport processes across the excited cell membrane are set out as well. Considering all stated, there are still some unavoidable questions, such as: When and why do membrane potential oscillations occur? What is a direct cause for their occurrence? What is their physiological role?

Based on the presented results and discussion, as well as, on our overall information, we advance the following hypothesis:

— Membrane potential oscillations occur when the cell membrane is excited. The state of excited membrane is usually accompanied by the activities of ions ( $K^+$ ,  $Na^+$ ,  $Cl^-$ ) that are not constant in the cell phases: vacuole, cytoplasm and cell wall.

— The usual ion transport processes are disturbed: first of all, diffusion (concentration gradient is altered), then electrodiffusion (electrochemical potential gradient is changed), biocurrents (electric potential gradient is altered), fluid flow (hydrostatic pressure gradient is modified). These states determine the cell membrane excitation. Hence, when the cell membrane is excited, then membrane potential oscillations and single impulses occur. These oscillations occur in a form of particular classes [R a d e n o v i ć, 1985a]. At the same time under such conditions, oscillating of proteins starts in the cell membrane, and they rhythmically perform oscillatory ion transport processes across the excited cell membrane. Ion transport processes adopt a co-operative character, which induce conformational changes of active ion channels that stretch and contract and in such a way rhythmically modify a transport capability of the excited cell membrane for ions.

- Oscillatory changes occur in supplying energies (electric, osmotic and chemical) to plant cells, tissues and organs.

— The bonds between membrane transport processes and metabolism are disturbed, i.e. weaken. This is particularly related to self-regulation of a material within the cell, organ and organism.

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## МЕХАНИЗАМ ОСЦИЛАТОРНОГ ТРАНСПОРТА ЈОНА И БИОПОТЕНЦИЈАЛА КРОЗ ПОБУЂЕНУ ЋЕЛИЈСКУ МЕМБРАНУ

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#### Резиме

У овом раду дају се резултати вишегодишњих проучавања биоелектричних осцилација мембранског потенцијала на неколико модел-објеката: побуђене мембране *Nitelle* и ткива корена кукуруза (*Zea maays* L.).

Показани су могућност и услови изазивања различитих класа осцилација, појединачних и локалних импулса мембранског потенцијала у зависности од деловања различитих стимулуса: механичко побуђивање ћелијских мембрана, побуђивање мембране концентрационим шоком (веће концентрације: K<sup>+</sup>, Na<sup>+</sup> и Cl<sup>-</sup> и њихове смеше).

Аутор објашњава појаву осциловања мембранског потенцијала уз претходно довођење ћелијских мембрана у побуђено стање, а затим наводи неке од параметара који такво стање ћелијске мембране карактеришу.

Посебно се тврди да је осциловање мембранског потенцијала условљено осцилаторним транспортним процесима јона кроз побуђену ћелијску мембрану.

Објашњени су могући механизми осцилаторног начина транспортних процеса кроз побуђену ћелијску мембрану.

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# GEOCHEMISTRY AND MINERALOGY OF SOME THERAPEUTICAL MUDS AND CLAYS IN SERBIA

ABSTRACT: There are many resorts with therapeutical muds in Serbia. They are located in various parts of Serbia and are characterized by different geological settings. Mineral composition of the therapeutical mud from Gamzigradska Banja is: quartz, feldspar, calcite and clay minerals (illite, smectite, kaolinite). The therapeutical mud from Soko Banja contains calcite, quartz, feldspar and clay minerals (mostly smectite, illite and kaolinite are less abundant). Mud from Bujanovačka Banja consists of feldspars, quartz, calcite and clay minerals (mostly smectite). All muds are used as coatings in treatment of rheumatism.

There are some therapeutical clays on the Serbian market. Mineral composition of clay for internal use is the following: kaolinite, illite and illite-smectite. Clay for external use contains illite, kaolinite, and smectite. Another clay from the Belgrade area (clay of Miocene age) is used in external and internal manner. It consists of almost pure smectite. Medical paste made from clay is composed of illite, kaolinite, quartz and feldspars.

Chemical composition of therapeutical muds and clays from Serbia is in accordance with their mineral composition. All studied samples are characterized by prevailing of trivalent iron and calcium in relation to divalent iron and magnesium. The highest concentrations of Cr and Ni were found in clay from Mirijevo, and of Zn, Pb and Cu in therapeutical mud from Gamzigradska Banja.

KEY WORDS: therapeutical mud, clay, clay minerals, trace elements, Serbia.

## INTRODUCTION

There is a long tradition in using of peloids (common name for therapeutical muds and clays) in Serbia. Such folk tradition has noticed in some songs, beliefs and rituals. In some ethnological records can be found a brief description of the clay type: dry, not swelling, white colored (*bolus alba* = white clay), brick or ceramic clay, etc. Ethnologists often explain using of muds and clays by their absorbing properties. In many cases peloids improve digestion (internal use) or relieve rheumatic pain (external use). Therefore, using of muds and clays can be assigned as a type of empirical ethnomedicine. On the other hand, there are many beliefs on therapeutical influence of soils and clays
in treatment of snake sting and bee sting (Dapović, 1995). There is still no rational explanation for such application. In older tradition can be found another belief that soil of graves of some saints is also therapeutical soil.

Geophagy or geophagia (eating of earth or soil or clay) is much more studied from ethnological, chemical, medical, and pharmaceutical point of view (H a l s t e d, 1968; R ö m e r, 1975). Beside rational or empirical causes of this phenomenon, scientists have also found some superstition elements, i.e. magical aspects in the eating of soil or clay. Historical account of geophagia with a review of literature was given by C o o p e r (1957), and therapeutical clays by V e n i a l e (1996). A symposium devoted to therapeutical clays from different point of view was held in 1996 in Italy (papers are published mostly in Italian).

Muds are in therapeutical use in some resorts in Serbia. They are usually mixed with mineral water and applied by coating of certain body parts.

## MATERIALS AND METHODS

Samples of therapeutical muds from Soko Banja, Gamzigradska Banja and Bujanovačka Banja were taken. Therapeutical clays from the Timok river (bottom sediments), Mirijevo (a part of Belgrade) and two clays (for external and internal use) for sale in pharmacy (original source probably in the vicinity of Valjevo, central Serbia) were also studied. Medical paste made from clay, known under the name *Tedonal*, was investigated. Non-treated samples were investigated after overnight drying at 50°C. Size fractions were obtained by wet sieving (sandy fraction 50–2000  $\mu$ m) and decantation (silt 2–50  $\mu$ m and clay fractions < 2  $\mu$ m).

Mineral composition of muds and clays was studied by XRD (Philips PW1710, Cu irradiation) of bulk samples, oriented samples, treated with glycerol and heated at 450°C for 2 hours. Chemical composition is obtained by wet analysis (major elements) and by atomic absorption spectrophotometry (trace elements). Values of pH and Eh were measured after mixing of 15 g sample with 45 ml deionized water for 2 hours. Content of carbon and humus is determined by using 0.1N KMnO<sub>4</sub>, and cation exchange capacity by 1N ammonium acetate.

### **RESULTS AND DISCUSSION**

### Mineral composition

### Gamzigradska Banja

Gamzigradska Banja is located in eastern Serbia and as a resort is known probably from Roman period. There are several mineral springs with temperature  $32-42^{\circ}$ C. Water is rich in Ca, Na, Mg, K, Fe, HCO<sub>3</sub>, Cl and SO<sub>4</sub> and uses for drinking and bathing (M a r k o v i ć, 1980). Therapeutical mud is of

greyish color with temperature of 35.5°C. After mixing with mineral water it uses for coating of body parts in treatment of rheumatism, sciatica and some skin diseases.

According to granulometric composition this mud can be defined as clayey sand (Table 1). Values of pH and Eh are shown in Table 2. The content of carbon is 1.49% (humus 2.56%), and cation exchange capacity is 25.78 me/100g.

Tab. 1 — Granulometric composition (wt.%) of the rapeutical muds and clays from Serbia

Location	Sand	Silt	Clay
Gamzigradska Banja	80.74	2.46	16.80
Soko Banja	7.82	35.99	56.19
Bujanovačka Banja	56.71	37.13	6.16
Timok river	2.42	44.18	53.40

Mineral composition of the sandy fraction (50–2000  $\mu$ m) of therapeutical mud is the following: quartz, calcite, feldspars and small amount of clay minerals. Silt fraction (2–50  $\mu$ m) shows similar composition: quartz is dominant, and calcite and feldspars are less abundant. X-ray diffraction (XRD) study of oriented samples of clay fraction (< 2  $\mu$ m) gave the following composition: kaolinite, illite, smectite, and small amount of interstratified illite-smectite (Fig. 1).



Fig. 1. - XRD patterns of the clay fraction of therapeutical mud from Gamzigradska Banja

### Soko Banja

Soko Banja is located in a very nice landscape of eastern Serbia. There are many mineral springs with different temperature  $(40-46.5^{\circ}C)$ . Mineral water is characterized by higher content of Ca, Na, Mg, K, HCO<sub>3</sub> and SO<sub>4</sub>, as well as by high radioactivity. Therapeutical mud is prepared by mixing of yellowish clay from the vicinity with mineral water. It uses for treatment of rheumatism.

According to granulometric composition this mud can be defined as aleuritic clay (Table 1). Values of pH and Eh are shown in Table 2. The content of carbon is 0.39% (humus 0.67%), and cation exchange capacity is 19.88 me/100g.

Location	pH	Eh
Gamzigradska Banja	8.05	154
Soko Banja	8.11	167
Bujanovačka Banja	7.96	166
Timok (outer zone)	8.05	161
Timok (inner zone)	7.65	123
Mirijevo	8.02	188
"Tedonal"	7.63	175

Tab. 2 - Values pH and Eh (mV) of therapeutical muds and clays from Serbia

Mineral composition of sandy and silty fractions of therapeutical mud is similar: calcite, quartz, feldspars and clay minerals. Calcite is dominant mine-



Fig. 2. - XRD patterns of the clay fraction of therapeutical mud from Soko Banja

ral, while quartz and especially feldspars are little abundant. Clay minerals were studied by XRD using standard procedure. Smectite is the most abundant mineral (very broad reflection between 4 and 7  $^{\circ}2\Theta$ ). Illite and kaolinite are present in small amounts (Fig. 2).

#### Bujanovačka Banja

Bujanovačka Banja is situated in southern Serbia and probably used in Roman and the Middle Ages. Temperature of mineral water is 42°C, and it contains a high amount of Na, K, Ca, Mg,  $HCO_3$ ,  $SO_4$  and Cl. Therapeutical mud is of black color, and in Turkish period the place is known as Black Mud. This resort is very popular for treatment of chronic rheumatism and certain skin diseases.

According to granulometric composition this mud can be defined as aleuritic clay (Table 1). Values of pH and Eh are shown in Table 2. The content of carbon is 0.35% (humus 0.60%), and cation exchange capacity is 39.88 me/100g.

Mineral composition of bulk sample of mud is the following: feldspars, quartz, calcite, clay minerals and pyrite. Smectite is almost only clay mineral in the mud, and small amount of kaolinite is probably present (Fig. 3).



Fig. 3. - XRD patterns of the clay fraction of therapeutical mud from Bujanovačka Banja

#### Timok River (near Zvezdan)

Small occurrence of therapeutical clay is known in the Timok riverbed near village of Zvezdan (eastern Serbia). It is greyish and plastic clay which local people use for coating of rheumatic body parts. Sample is divided into two subsamples: the first one is analyzed immediately after taking, but the second one is studied after one year (sample was stayed in clean air atmosphere).

According to granulometric composition this mud can be defined as aleuritic clay (Table 1). Values of pH and Eh are shown in Table 2. The content of carbon is 0.84% (humus 1.44%), and cation exchange capacity is 55.18 me/100 g.

Mineral composition of both subsamples is identical: quartz, feldspars, calcite, smectite, illite and kaolinite. After standard procedure for oriented samples it was established that smectite is dominant clay mineral (very broad reflection), while kaolinite and illite are very less abundant (Fig. 4).



Fig. 4. - XRD patterns of the clay fraction of therapeutical clay from the Timok River

### Mirijevo (Belgrade)

Therapeutical clay in Mirijevo (a part of Belgrade) represents a poorly litified Sarmatian (Miocene) sediment. It occurs in bed of 30—40 cm thickness and characterized by greyish-yellowish color (Grubin et al., 1995). This clay is locally used for coating of rheumatic body parts and also for internal diseases (by drinking of suspension in water).

Values of pH and Eh are shown in Table 2. The content of carbon is 0.13% (humus 0.22%), and cation exchange capacity is 27.73 me/100g.

Mineral composition of bulk sample is: smectite, illite, quartz, calcite and feldspars. In clay fraction smectite is dominant mineral, but illite is present in very small amount.

There are three clays for sale on Serbian market. The first two clays (for external and internal use) come to the market from private producer. They are similar in color, mineral and chemical composition. "External" clay consists of smectite, illite, kaolinite, and hydrobiotite (Fig. 5). Chemical composition is the following: SiO<sub>2</sub> 68.05%, Al<sub>2</sub>O<sub>3</sub> 25.15%, CaO 0.62%, Fe<sub>2</sub>O<sub>3</sub> 1.70%, K<sub>2</sub>O 3.12%, MgO 0.68% and TiO<sub>2</sub> 1.09% (after certificate in package). "Internal" clay contains illite, kaolinite and interstratified illite-smectite. Its chemical composition is: SiO<sub>2</sub> 60.90%, Al<sub>2</sub>O<sub>3</sub> 22.52%, CaO 0.30%, Fe<sub>2</sub>O<sub>3</sub> 1.79%, K<sub>2</sub>O 3.00%, MgO 0.86% and TiO<sub>2</sub> 0.92% (after certificate in package). According to all data it is not clear why there is a difference in their use.



Fig. 5. - XRD patterns of the clay fraction of therapeutical clay for external use

The third clay is actually medical paste ("Tedonal") produced from clay and additional pharmaceutical compounds (methyl salycilate and other). Values of pH and Eh are shown in Table 2. The content of carbon is 0.86% (humus 1.48%), and cation exchange capacity is 12.38 me/100g. Its mineral composition is: illite, kaolinite, quartz, feldspars, and probably admixtures of talc and illite-smectite (?).

## Chemical composition of therapeutical muds and clays

Chemical composition of the studied therapeutical muds and clays is presented in Table 3. Content of silica varies in a broad range (21.81–62.78%) depending on the content of calcite. All samples are characterized by prevailing of  $Fe^{3+}$  and Ca in relation to  $Fe^{2+}$  and Mg. Mud from Soko Banja is rich in calcite and the content of CaO is very high. Analysis of two subsamples of therapeutical clay from the Timok river show some differences. After one year clay is changed in color (from greyish to reddish) indicating oxidation of  $Fe^{2+}$  to  $Fe^{3+}$ .

	1	2	3	4	5	6
SiO <sub>2</sub>	48.58	21.81	43.34	62.78	40.50	40.54
TiO <sub>2</sub>	0.44	0.32	0.94	0.94	0.84	0.84
$Al_2O_3$	14.43	7.59	17.42	12.01	17.40	17.32
Fe <sub>2</sub> O <sub>3</sub>	5.75	4.40	6.51	5.09	6.20	5.87
FeO	0.75	0.49	0.73	0.61	1.96	1.47
MnO	_	_	_	_	_	_
MgO	3.24	5.06	3.66	1.75	3.13	4.07
CaO	6.55	28.02	7.30	5.02	9.25	9.90
Na <sub>2</sub> O	1.10	0.48	1.93	2.10	0.50	0.48
K <sub>2</sub> O	1.58	1.70	2.35	1.40	1.90	1.10
$P_2O_5$	0.24	_	0.15	0.60	0.15	0.25
$H_2O^+$	14.70	28.40	10.11	5.46	10.76	12.22
H <sub>2</sub> O-	2.22	2.13	4.82	1.50	7.92	6.88
	99.56%	100.40%	99.26%	99.26%	100.51%	100.94%

Tab. 3. - Chemical composition of some therapeutical peloids and clays from Serbia

1 — mud, Gamzigradska Banja; 2 — mud, Soko Banja; 3 — mud, Bujanovačka Banja; 4 — clay, Mirijevo; 5 — clay, Timok River; 6 — clay, Timok River (after 1 year)

Trace element content is presented in Table 4. Nickel shows small variations — from 52 to 75  $\mu$ g/g, and only sample from Mirijevo is rich in this element (145  $\mu$ g/g). The average nickel content in clays is 55  $\mu$ g/g (T u r e k i a n and W e d e p o h 1, 1961). The content of chromium is similar (60—90 g/g) and only clay from Mirijevo is characterized by high content (240  $\mu$ g/g). The average chromium content in clays is 90  $\mu$ g/g (T u r e k i a n and W e d e p o h 1, 1961). The high content of Ni and Cr in clay from Mirijevo can be explained by the presence of ultramafic and mafic rocks in the vicinity. These rocks are enriched in Ni, Cr, Co, Cu and other elements.

Tab. 4. — Trace element content ( $\mu g/g)$  in some therapeutical muds and clays from Serbia

Location	Ni	Cr	Pb	Li	Zn	Cu
Gamzigradska Banja	75	90	66	80	179	234
Soko Banja	65	75	23	35	79	36
Bujanovačka Banja	70	80	23	24	86	71
Timok	70	60	23	36	115	125
Mirijevo	145	240	33	60	143	123
"Tedonal"	52	85	28	49		

The average content of lead in clays is 23  $\mu$ g/g (W e d e p o h 1, 1978). The studied samples show the range 23–33  $\mu$ g/g, and only peloid from Gamzigradska Banja is enriched in this element (66  $\mu$ g/g). It may be originated from lead tubes for water transport or from the presence of illite where K can be easily replaced by Pb. Lithium occurs in a broad range — from 24 to 80  $\mu$ g/g, as well as zinc (79–179  $\mu$ g/g). Higher content of Zn in the Gamzigradska Banja sample can be explained by the volcanic activity in that area in the geologic past (Upper Cretaceous volcanic rocks). The average content of copper in clays is 45  $\mu$ g/g (T u r e k i a n and W e d e p o h 1, 1961). The studied samples contain 36–234  $\mu$ g/g Cu. High content of Cu in Gamzigradska Banja is expected, because that area belongs to the Carpathian-Balkan copper geochemical and metallogenic province enriched in this element. Geological setting of the area of Soko Banja (limestone and other sedimentary rocks) influenced the low Cu content (36  $\mu$ g/g).

### CONCLUSIONS

Therapeutical muds from Serbia are characterized by different mineral composition. Smectite is dominant clay mineral, while other clay minerals are less abundant. Other minerals (quartz, feldspars, calcite) are usually present in sand and silt fractions. Their abundance depends on geological setting: calcite is dominant mineral when there is limestone in the vicinity of resort (e.g. Soko Banja). Quartz and feldspars occur in those parts of Serbia where magmatic and metamorphic rocks are prevailing. Therapeutical muds are mainly used as coatings in treatment of rheumatism.

Therapeutical clays vary in mineral composition. There is no clear difference in composition of clay for external and internal use. Two types of clays can be derived: the first one is composed from almost pure smectite, and the second one is mixture of many clay minerals: kaolinite, illite, smectite and interstratified minerals. It seems that clays for internal use rich in smectites can cause certain health problems after the long term use due to high sorbing and exchange capacity of smectites.

Chemical composition of therapeutical muds and clays from Serbia varies depending on their mineral composition. All studied samples are characterized by prevailing of trivalent iron and calcium in relation to divalent iron and magnesium. The highest concentrations of Cr and Ni were found in clay from Mirijevo, and of Zn, Pb and Cu in therapeutical mud from Gamzigradska Banja. Trace elements content is very important for the internal use of clays.

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### ГЕОХЕМИЈА И МИНЕРАЛОГИЈА НЕКИХ ЛЕКОВИТИХ БЛАТА И ГЛИНА СРБИЈЕ

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#### Резиме

У Србији има више бања са лековитим блатом. Оне се налазе у различитим деловима Србије и одликују се различитом геолошком грађом. Минерални састав лековитог блата из Гамзиградске бање је: кварц, фелдспат, калцит и глиновити минерали (илит, смектит, каолинит). Лековито блато из Сокобање садржи калцит, кварц, фелдспат и глиновите минерале (углавном смектит, илит и каолинит су мање заступљени). Блато из Бујановачке бање састоји се од фелдспата, кварца, калцита и глиновитих минерала (углавном смектита). Та блата се користе за лечење реуматизма. Неколико лековитих глина се налази на тржишту у Србији. Минерални састав глине за унутрашњу употребу је следећи: каолинит, илит и илит-смектит. Глина за спољашњу употребу садржи илит, каолинит и смектит. Глина из Миријева (Београд) за спољашњу и унутрашњу употребу састоји се скоро од чистог смектита. Медицинска паста од глине садржи илит, каолинит, кварц и фелдспат. Хемијски састав лековитих блата и глина у складу је са њиховим минералним саставом. Одликују се превлађивањем тровалентног гвожћа и калцијума у односу на двовалентно гвожће и магнезијум. Највеће концентрације никла и хрома има глина из Миријева, а цинка, олова и бакра — лековито блато из Гамзиградске Бање.

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# MINERALOGY AND AGROCHEMISTRY OF MAGNESIUM IN SOILS OF VOJVODINA, ŠUMADIJA AND NORTHERN POMORAVLJE

ABSTRACT: Magnesium is an important major constituent element of biosphere, hydrosphere and lithosphere. In biosphere it is an essential macronutrient in plants, for animals and for men. Magnesium deficiency in humans could be linked to pathogenesis of cancer or might be related to sudden cardiac deaths in areas with soft water.

The total content of Mg and Ca was determined in  $HClO_4 + HF$  digests by AAS method in the selected soil samples, whilst the 1M  $NH_4OAc$  extraction was carried out to assess the Mg and Ca bioavailability to plants from different soils. The mineralogy of bulk soil samples was obtained by XRD analysis by using SIMENS 2 kW diffractometer in Co K $\alpha$  radiation. The DRX Win 1.4c (1996) computer package was used to identify and quantify soil mineralogy. All data obtained were statistically analyzed by computer package STATISTICA for Windows 4.3b (1993). The raster maps showing the distribution of Mg were drawn by computer using UNIRAS subroutine.

This investigation has shown that the soils investigated have available Mg content between 10 and 93 mg/100g, with an average of 40.8 mg/100g and could be considered as Mg sufficient for plant production. Available Mg is primarily associated with carbonate fraction (dolomite) and to a lesser extent with exchangeable (clay minerals) and silicate forms (chlorite, mica, amphibole). Total content of Mg varies between 0.20 and 1.26%, with an average of 0.603%. The Ca/Mg ratio has shown a large scale variation between 2.5:1 and 78.8:1. Only 10% of soils have Ca/Mg ratio lower then 5:1 or optimal ratio for plant nutrition, whilst 90% of soils were found to have the ratio above 5:1 which, in case of extremely high values, might be a limiting factor in plant growth and influence an adequate supply of Mg to animals and men. This investigation have pointed out a necessity of wider research in soil mineralogy and chemistry of our soils, which are considered as one sufficient in available magnesium.

KEY WORDS: soil mineralogy, magnesium, calcium, Ca/Mg ratio, distribution maps.

## 1. INTRODUCTION

Magnesium is one of the most prevalent element in the earth's crust. It is the eighth most abundant of the elements and estimates of its content have ranged from 2.0% to 2.5%. It is an important major constituent of biosphere, hydrosphere and nearly all rock types of the lithosphere. According to R a nk a m a and S a h a m a (1950), in the upper lithosphere it is a lithophile element with a biophile tendencies. Average concentrations of magnesium in the earth's crust, selected rock types, water and soils are listed (L o w t o n, 1955; V i n o g r a d o v, 1959; M e n g e 1, 1979. and U r e, 1982) in Table 1.

Tab. 1. –	- Abundance	of M	Magnesium	in	Various	Natural	Environments	$(10^{4})$	mg/kg)
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Lithosphere	2.5	Sandstone	0.9
Ultrabasic rocks	20.4	Marl and clay	2.1
Basic rocks	4.6	Carbonates	4.7
Granites	0.6	Soils	0.6
Shale	1.9	Sea water	0.13

The highest magnesium contents are recorded in the earliest rocks to crystallise, particularly ultrabasic and basic rocks, peridotites, gabbros and basalts, whilst more acid rocks such as granites, granodiorites, trachytes and rhyolites contain only very small amounts of magnesium. Shales and marls contain similar amounts of magnesium to those found in igneous rocks, whilst sandstones and residual sediments in general contain less magnesium. According to R a n k a m a and S a h a m a (1950) and G o l d s c h m i d t (1950), the content of magnesium in sedimentary rocks varies from a traces in some sandstones to 3800 mg/kg in limestone, 11000 mg/kg in clays and up to 13% in dolomites.

In the igneous silicate rocks, magnesium is present in the divalent state associated largely with the ferromagnesian and accessory minerals. In the process of weathering progressive leaching occurs. It is removed from the zone of weathering as bicarbonate. The solubility and mobility of Mg is relatively high, irrelevant of pH and Eh conditions. The precipitation of dissolved magnesium in the form of carbonates is controlled by pH and Eh of solution and follows the precipitation of calcium. In geochemistry of surface processes of leaching, gleying and surface organic matter accumulation, together with soil properties such as reaction — pH and redox potential — Eh, affect the distribution, the form and the mobility of magnesium and calcium in soil.

According to M e n g e 1 and K i r k b y (1979), in areas with humid climate, average magnesium content in sandy soils is usually 0.05%, while clayey soils contain up to 0.5%. Magnesium is a constituent of more than 180 minerals although only sixty can be classed as magnesium-bearing ores. The most common naturally occurring sources of magnesium are the minerals: olivine, pyroxene, serpentine, magnesite, dolomite, brucite, as well as biotite and hornblende. It is also constituent of clay minerals such as: vermiculite, illite, montmorillonite and chlorite. In some soils it is present as magnesite —  $MgCO_3$  or dolomite CaCO<sub>3</sub>.  $MgCO_3$  while some soils from arid and semiarid regions could contain significant amounts of epsomite  $MgSO_4$  7H<sub>2</sub>O.

Magnesium in soils could occur in water soluble, exchangeable and non-exchangeable forms. There is a constant balance between these three forms. The greatest part of magnesium is found in residual, non-exchangeable form, bound to primary or clay minerals. The other two water soluble and exchangeable forms, are the major source of available magnesium to plants. The availability of soil magnesium to plants is controlled by the following factors: amount of available Mg, soil reaction (pH), CEC, soil texture, type of soil collides and ratio of magnesium to the other elements e.g. Ca and K in soil solution, H a b y (1990).

In biosphere it is naturally a major essential macronutrient and is vital to both plant and for animal life (Mengel and Kirkby, 1979). Chlorophyll pigment in plants is a Mg-porphyrin complex, and all enzymatic reactions in animals and man that are catalyzed by ATP require Mg as a co-factor. Oxidative phosphorylation, DNA transcription, RNA function, protein synthesis and critical cell membrane functions are all dependent upon optimal Mg concentrations, Thorton (Ed.) (1983).

Magnesium deficiency in humans has been receiving increasing attention. Several reports indicate that Ca and Mg concentrations in human tissues vary with the hardness of municipal water supplies (M c M illan, 1978). These suggest an important geochemical influence. Since cardiac arhytmias are serious manifestation of Mg deficiency, a hypothesis of sudden cardiac deaths in geographic areas with soft water (low in Mg content) might be related. Magnesium deficiency has been linked to pathogenesis of cancer both experimentally in animals and epidemiologically in man. Also, there is evidence that Mg may relate to formation of kidney stones. According to Blondell (1980), there are some evidence that geochemical availability may relate to human tissue Mg levels, which is essential to the hypothesis that the geochemistry of Mg might relate to human disease.

### MATERIAL AND METHODS

During large scale sample collection, for the project financed by the Ministry of Science and Technology, an orthogonal regular 10 x 10 km grid was used to avoid bias in site location. From the set of about 5000 samples a collection of samples from Vojvodina, Šumadija and Northern Pomoravlje was taken to represent the most important soils. Selection was restricted to the samples from the arable layer with 0-25 cm depth.

After collection, samples were air-dried and sieved to pass through a 2 mm sieve. A 50 g subsample of soil was obtained by conning-and-quartering and then ground, to less then 150 mesh, in an all-agate planetary ball-mill. To-tal concentration of Mg and Ca in  $\text{HCIO}_4$  + HF digests were determined by AAS analysis. The 1M ammonium acetate extraction was carried out to assess the magnesium and calcium bioavailability to plants from different soils.

The mineralogy of bulk soil samples was obtained by X-ray diffraction analysis (XRD) of random-packed powder mounts, after they were ground and freeze-dried. XRD patterns were obtained by using SIMENS 2 kW diffractometer, with iron filtered Co K radiation. DRX Win 1.4c (1996) computer package was used to identify and quantify soil mineralogy. Characterization of minerals in soils were carried out according to the method described in Brindly and Brown (Ed.) (1980) and Klute (Ed.) (1986). All data obtained were statistically analyzed by computer package STATISTICA for Windows 4.3b (1993).

The classes used to represent the data on the map were chosen from the box and wiskers analysis. Thus, the map has the 5 classes and a raster map showing the distribution of magnesium was drawn by computer using UNI-RAS subroutine.

### **RESULTS AND DISCUSSION**

### Mineralogy of the soils

The mineralogical composition of the bulk samples of the soils investigated is complex and it is presented in the table 2. The predominance of quartz, mica, associated with altered feldspars (plagioclase and orthoclase), carbonate (calcite and dolomite), and minor to trace amounts of chlorite, clay minerals, hornblende and rare goethite and talc may indicate the mineralogy and variety of parent rocks e.g.: loess, schists, flisch sediments and sandstones. Magnetite, ilmenite and other accessory minerals (garnets, epidote, apatite, pyroxene) are found in heavy mineral fractions. Variation in quartz content might reflect weathering processes that have developed during pedogenesis. The soils with stronger weathering processes (distric cambisols and luvisols) contain higher quartz content in comparison to less weathered soils such as fluvisols, rankes and regosols, on the same and similar parent rocks.

It is evident that soils from Vojvodina (chernozem, halomorphic soils, semigleys, humogleys and eugleys) have more abundant micas and chlorite compared to the southern soils of Šumadija and Pomoravlje, where eutric and distric cambisols and luvisols occur, which contain less mica and chlorite. Further comparison of the northern to southern areas has shown that northern areas or Vojvodina soils (chernozems and semigleys) comprise up to twice higher carbonate contents then southern areas or Šumadija soils (distric cambisols, luvisols and pseudogleys). In northern areas dolomite dominates over calcite in semigleys and cernozems, whilst calcite is more abundant then dolomite in fluvisols, humo- and eugleys. In the southern areas in some eutric cambisols and some luvisols calcite is only mineral present in the bulk soil, whilst in other soil carbonates are absent due to complete leaching as in more acidic distric cambisols. This trend could be only explained by preferencial leaching of carbonates, particularly dolomite, in the areas with higher annual precipitation.

The major weathering process in the soil in a wet, slightly acidic environment involved the disappearance of micas and chlorite from the surface horizons, and their conversion to expandible minerals such as vermiculite and smectite. The degree of micas and chlorite alteration has correlated with the development of brownised and humous varieties of the soils.

	Quartz	Plagioclase	Orthoclase	Chlorite	Mica + Illite	Smectite + Verm.	Mixed Layers	Kaolinite	Calcite	Dolomite	Goetite
Chernozem	53.8	8.7	0.7	6.0	19.0	0.9	0.3	1.0	4.5	4.9	0.2
Halomorphic soils	57.3	10.4	0.7	4.7	20.6	0.5	0.4	1.1	1.2	2.3	0.2
Semigley	52.5	8.5	0.6	5.4	19.8	1.8	0.2	1.5	4.3	5.1	0.2
Humo- + Eugley	52.9	11.2	0.7	5.3	21.7	2.6	0.3	1.7	1.7	1.5	0.3
Fluvisol	51.2	10.4	0.6	5.7	22.4	3.3	0.2	2.2	2.3	1.7	0.2
Pseudogley	67.6	6.4	0.5	4.6	16.5	1.3	1.3	1.1	0.1	0.1	0.1
Eutric Cambisol	63.5	8.9	0.8	3.7	16.5	2.3	0.2	2.3	0.8	0.2	0.3
Vertisol	70.3	8.8	1.4	2.5	10.8	4.0	0.3	1.1	0.2	0.0	0.3
Distric Cambisol + Luvisol	69.1	7.4	0.8	4.7	13.5	2.3	0.3	1.3	0.3	0.0	0.4
Regosol + Ranker	58.9	6.9	0.8	4.7	18.9	2.5	0.4	1.9	2.5	2.1	0.4
All soils	57.6	8.8	0.7	5.0	18.7	2.0	0.3	1.6	2.5	2.6	0.2

Tab. 2 — Average mineral composition (%) of the bulk samples of the soils investigated

The results of the X-ray diffraction analyses of the clay fraction are presented in the Table 2. It has revealed abundant illite associated with smectites and mixed layer silicates (MSS) of the illite/smectite (10-14) and chlorite/vermiculite (14-14) types. They are accompanied by minor amounts of vermiculite, kaolinite, chlorite, quartz, feldspars and rare calcite, dolomite and goethite. A small amount of talc was detected only in several specimens from luvisols and rankers developed on Paleozoic schists in Šumadija. The persistence of talc in fine clay throughout some luvisol profiles could also suggest a high stability of this mineral within soils in strongly leaching and weathering conditions.

Illite as a weathering product of micas is following their spatial distribution. It is more abundant in less weathered soils such as chernozem and semigleys, whilst it is up to twice less abundant in the distric cambisols and luvisols, where strong weathering have occurred during pedogenesis. On the other side smectites could be inherited from parent rocks as in vertisols formed on lacustrine sediments in Šumadija or geochemically developed in highly moist, temporarily flooded or water logged soils such as: semi-, humo- and eugleys and fluvisols in Vojvodina. Vermiculite occurs as an illite and/or chlorite transformation product in soils with slightly acid weathering conditions such as distric cambisols, luvisols and pseudogleys. Interstratified minerals of the (10-14) and (14-14) types are abundant in the soils (halomorphic soils and pseudogleys), where particular weathering occurs, indicating initial stages of illite and chlorite transformations to either smectite or vermiculite. Kaolinite occurs either as inherited as an alteration product of feldspar in rankers and regosols or it occurs as direct weathering product of feldspars in the soils with strong leaching conditions such as distric cambisols and luvisols.

# Agrochemical results

The results of 1M ammonium acetate soluble magnesium extracted from the soils (A horizon) and within soils are presented on Table 3 and Graph 1. Spatial distribution of available magnesium content over area investigated is presented on agrochemical map in Figure 1.



Fig. 1 - Agrochemical map of available magnesium

This investigation has shown that the soils investigated have available magnesium content between 10 and 93 mg/100 g, with an average of 40.8 mg/100 g. All investigated soils are well-supplied with available magnesium and could be considered as magnesium sufficient for plant production. It appears that available magnesium is primarily associated with carbonate fraction (dolomite) and to a lesser extent with exchangeable (clay minerals) and silicate forms (mica, chlorite, amphibole).

On the agrochemical map generally two areas could be distinguished, the first is where soils are formed on recent loess and alluvial sediments, as in Vojvodina and Pomoravlje, and the second where soils are developed on Mesozoic and Neogene sediments consisting of mudstones, sandstones, flisch and limestones that run throughout the most of Šumadija.

Soil tunos	Number	Inte	rvals	Average	St.	
Son types	of samples	min	max	Mg	Dev.	
Ranker, regosol and rendzina	6	3	38	22,8	8.83	
Distric Cambisol and Luvisol	5	23	40	32.4	8.46	
Vertisol	5	15	38	26.8	9.36	
Cambisol	14	23	70	37.3	12.96	
Halomorphic soils	5	25	53	36.8	11.19	
Pseudogley	5	10	45	32,8	13.74	
Chernozem	20	28	65	41.7	10.00	
Semigley	20	20	75	43.4	14.00	
Fluvisol	8	22	80	48.0	20.90	
Eugley and Humogley	12	23	93	57.8	20.62	
All analysed soils	100	10	93	40.81	16.12	

Tab. 3 - Statistical summary for available magnesium (mg/100g) classified according to soil type

Initial soils (rankers and regosols), soils developed on Neogene sediments as vertisols, some eutric and distric cambisols and pseudogleys contain less than the average amount of available magnesium (10-40 mg/100 g). On the other side soils formed on loess and alluvial sediments (chernozem, semigley, fluvisol and humo- and eugleys) are comparatively rich (40-93 mg/100 g) in available magnesium.

The results of AAS analysis of total magnesium in the soils (A horizon) are presented on table 4. Spatial distribution of total magnesium content over area and within soils investigated is presented on geochemical map in Figure 2.

Total content of magnesium varies between 0.20 and 1.26%, with an average of 0.603%. Ratio of available to total magnesium content in soils has indicated that the greater part (80 to 98%) of magnesium was present in residual forms bound to lithogenic fraction. It appears that total magnesium content is primarily associated with carbonate fraction (dolomite) as in soils developed on recent alluvial sediments and loess in Vojvodina and to a lesser extent with and silicate forms (mica, chlorite, clay minerals, amphibole) as in soils formed



Total Magnesium (%)	<0.2	0.20 - 0.30	0.30 - 0.40	0.40 - 0.65	0.65 - 0.80	>0.8
Raster label						

Fig. 2 — Agrochemical map of total magnesium

on Neogene and Mesozoic sediments that run throughout the most of Šumadija, with an exception of regosol and rendzina which are formed on parent rocks rich in carbonates.

The Ca/Mg ratio of available forms has shown a large scale variation between 2.5:1 and 78.8:1. Only 10% of soils have Ca/Mg ratio lower then 5:1 or optimal ratio for plant nutrition, whilst 90% of soils were found to have the ratio above 5:1 which might be a limiting factor in growth of some plants and cause some problems in agricultural production and influence an adequate supply of magnesium to animals and men.

From statistical correlation analyses it is evident that total magnesium content is closely correlated to mica+illite (r = 0.87) and chlorite (r = 0.74) contents as well as to the carbonate — dolomite (r = 0.72) content of the soils investigated. Available magnesium is also positively correlated to micas + illi-

	Number	Inte	rvals	Average	St.
Son types	of samples	min	max	- (%) Mg	Dev.
Vertisol	5	0.20	0.52	0.352	0.133
Distric Cambisol and Luvisol	5	0.32	0.43	0.346	0.052
Pseudogley	5	0.25	0.57	0.422	0.116
Cambisol	14	0.36	0.68	0.476	0.115
Halomorphic soils	5	0.35	0.85	0.568	0.193
Eugley and Humogley	12	0.20	1.00	0.614	0.206
Ranker, regosol and rendzina	6	0.39	1.25	0.655	0.333
Chernozem	20	0.25	1.00	0.666	0.176
Fluvisol	8	0.24	1.00	0.740	0.268
Semigley	20	0.26	1.26	0.744	0.264
All analysed soils	100	0.20	1.26	0.603	0.235

Tab. 4 — Statistical summary for total magnesium content (%) classified according to soil type

te (r = 0.70) and chlorite (r = 0.60) but to a lesser extent to smectite + vermiculite (r = 0.33) and dolomite (r = 0.33).

The correlation of total and available magnesium contents to minerals in the clay fraction has shown slight difference to that for bulk soil mineralogy. Total magnesium content has revealed a high positive correlation to fine dolomite particles in the clay fraction (r = 0.72), whilst available magnesium has shown a slight positive correlation with smectite (r = 0.39) and dolomite (r = 0.38) in all soils investigated.

Analysis of correlation data for specific soils has pointed out semigley as the only soil with a positive correlation of both total and available magnesium with dolomite in the bulk soil, whilst in the clay fraction they are correlated to clay chlorite. The weathering processes may effect soil mineralogy in the soils as well as soil mineral correlation to total and available magnesium contents. Fluvisols, chernozem and eutric cambisols have shown positive correlation between magnesium and micas + illite and chlorite in the bulk soils, whilst in the clay fractions magnesium correlates with interstratified minerals, smectite and vermiculite, respectively. Magnesium is correlated to smectite in both bulk and clay fraction in luvisols, distric cambisols and pseudogleys. Vertisols, humo- and eugleys have shown in bulk samples magnesium correlation to chlorite, amphiboles and interstratified minerals of (14—14) types, whilst in clay fraction magnesium has positive correlation to illite and interstratified illite/ smectite.

### CONCLUSIONS

Magnesium is an important major constituent element of biosphere, hydrosphere and lithosphere. It makes up to 2.5% by weight of the Earth's crust and is invariably present in all soils. The greatest part of magnesium occurs in the crystal lattices of numerous minerals, which are the main source of magnesium in nature.

In biosphere it is an essential macronutrient in plants, for animals and for men. Magnesium deficiency in humans could be linked to pathogenesis of cancer, or might be related to sudden cardiac deaths in areas with soft water, M c M illan (1978).

This paper is concerned with the magnesium content in the most important soil types of Vojvodina, Šumadija and Northern Pomoravlje.

The available magnesium in soils investigated is primarily associated with carbonate fraction e.g. mineral dolomite (CaCO<sub>3</sub>. MgCO<sub>3</sub>), and to a lesser extent with exchangeable (clay minerals) and silicate forms (mica, chlorite, amphibole). The greatest part (80 to 98%) of magnesium was present in residual forms bound to lithogenic fraction.

This investigation has shown that soils in Serbia contain between 0.20 up to 1.26% Mg, with an average content of 0.603%. The available magnesium content varies between 10 and 93 mg/100 g, with an average content of 40.8 mg/100 g Mg.

Taking in account both total and available magnesium contents it could be concluded that the soil investigated contain enough magnesium for proper plant nutrition. However, great importance on magnesium availability have ratios between available cations Ca and K with Mg.

The Ca/Mg ratio of available forms has shown a large scale variation between 2.5:1 and 78.8:1. Only 10% of soils have Ca/Mg ratio lower then 5:1 or optimal ratio for plant nutrition. The K/Mg ratio of available forms is on average 1.1:1, and would not affect magnesium uptake, whilst the ratio higher than 4:1 could be considered as a limiting factor in available magnesium uptake and utilisation by plants.

About 90% of soils were found to have the ratio above 5:1 which might be a limiting factor in growth of some plants. This could become a problem in the case of a wide Ca/Mg ratios and when available magnesium content is near the limit of deficit (< 10 mg/100 g), as well as to the plants sensitive to available magnesium content in soils.

This paper has shown a need for the wider investigation of chemistry and mineralogy of soils in Serbia, which are considered in general as well supplied with magnesium. However, in the case of deficit of available magnesium in soils with high ratios of Ca/Mg and K/Mg, it could a limiting factor in plant physiology, particularly to the plants with higher demand for magnesium as well as favourable ratios of available cations such as: Ca, K, Na, H and Mg.

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### МИНЕРАЛОГИЈА И АГРОХЕМИЈА МАГНЕЗИЈУМА У ЗЕМЉИШТИМА ВОЈВОДИНЕ, ШУМАДИЈЕ И СЕВЕРНОГ ПОМОРАВЉА

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#### Резиме

У раду су анализирани садржаји и дистрибуција укупног и приступачног магнезијума у земљиштима Војводине, Шумадије и северног Поморавља.

Магнезијум је врло важан елемент у грађи биосфере, хидросфере и скоро свих стена литосфере, где изграђује многобројне минерале, који су основни извор магнезијума у природи. У биосфери магнезијум је неопходан макрохранљиви елеменат за биљке, животиње и људе. Недостатку магнезијума у исхрани људи посвећује се све већа пажња, јер може да се повеже са патологијом рака и болести срца у подручјима са меком водом — M с M illan (1978).

Основни извор приступачног магнезијума у нашим земљиштима је првенствено минерал доломит, а у мањој мери минерали глина и други алумосиликати: хлорити, лискуни и амфиболи. Највећи део (80—90%) магнезијума у испитиваним земљиштима је везан за литогену — силикатну фракцију.

Ово истраживање је показало да земљишта у Србији садрже укупни магнезијум од 0.20 до 1.26% са просеком од 0.603%, док садржај приступачног магнезијума варира између 10 и 93 mg/100 g са средњим садржајем од 40.8 mg/100 g. Ако се узму у обзир садржаји укупног и приступачног магнезијума, може се сматрати да су наша земљишта овим елементом обезбеђена сасвим довољно за правилну исхрану биљака. Међутим, велику важност за приступачност магнезијума имају и односи приступачних катјона калцијума, калијума и магнезијума.

Однос приступачног Ca/Mg варира у широким границама између 2.5:1 и 78.8:1. Међутим, само 10% испитиваних земљишта има Ca/Mg однос нижи од 5:1, т.j. оптималан однос за исхрану биљака. Однос К/Mg је просечно износио 1,1:1, што указује да овај однос неће негативно утицати на усвајање Mg. Само односи већи од 4:1 уз дефицит приступачног магнезијума сматрају се негативни за усвајење Mg.

Око 90% земљишта има повишен однос, изнад 5:1, што може бити ограничавајући чинилац за раст биљака и проузроковати неке проблеме у пољопривредној производњи и да тако утиче на снабдевање људи и животиња неопходним магнезијумом. Ови проблеми настају при сувише широким односима (Ca/Mg) и када је садржај приступачног Mg близу границе дефицитарности (< 10 mg/100 g Mg).

Ово истраживање указало је на потребу ширег проучавања хемије и минералогије наших земљишта, за која је досад сматрано да су добро снабдевана магнезијумом, јер у мањем броју случајева може да дође до поремећаја у усвајању Мg (при мањој приступачности Mg, уз повећање односа Ca/Mg и K/Mg).

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5.3. Each table must begin with a caption. The caption must explain the contents of the table.

5.4. Footnotes to a table should be typed directly under the table.

5.5. The position of tables should be indicated at the left margin.

6. Units, names, formulas, and abbreviations.

6.1. Only SI quantities and units are to be used (SI = Systeme International d'Unit's); in exceptional cases, other officially accepted units may be used.

6.2. For molar concentration, an italicized M (underlined) should be used.

6.3. Biological names in Latin should be italicized (underlined).

6.4. Chemical structural formulas and equations should be drawn (not written or typed), ready for photographic reproduction.

6.5. Only standard abbreviations should be used. Where specialized abbreviations are used, the term should be given initially in full with the abbreviation indicated in parentheses.

6.6. Mathematical expressions should be written in such a way as to use the minimal number of lines, while retaining their clarity, for example: 2/3 instead of 2:3, exp (-ab) instead of  $e^{-ab}$ , etc.

7. Short Communications

7.1. Proceedings for Natural Sciences offers an opportunity to publish short communications on all aspects that are implied by the journal's title.

7.2. Short communications are limited to 4 typewritten pages including all illustrations.

7.3. The presentation and format of the short communications are similar to those of a normal paper, except for the list of references, in which the titles of the papers should be omitted.

8. Information of authors

8.1. When the manuscript has been accepted, the author will be informed of the approximate time of publication.

8.2. Corrections of the proofs should be restricted to printer's errors only. Other than these, substantial alterations will be charged to the author. Proofs should be handled promptly and returned to the Editorial Office.

8.3. Fifty offprints are supplied free of charge. Copies in addition to these may be ordered and paid for through the Editorial Office.

9. DISKETTES: After acceptance, the final revision should be submitted on disk. Include text, tables and figures on a double-density or high-density 3.5-inch diskette. An accompanying printout is needed to facilitate the incorporation of electronic tables and figures. Word for Windows (any version) is the preferred word-processing program. When copying the paper on disk, it is important to follow this procedure: *File>Save as>Options>Embed True Type fonts>ok>Save*.

# УПУТСТВО ЗА АУТОРЕ

1. Опште напомене

1.1. Зборник за природне науке прима оригиналне радове и прегледне чланке као и кратка саопштења из свих научних области које обухвата назив часописа. Прегледни радови се објављују само на позив редакције. Радови који су већ објављени или су послани за објављивање у другом часопису не могу бити прихваћени.

1.2. Прихватају се рукописи писани на енглеском језику. Језик мора бити исправан у погледу граматике и стила. Аутори треба да предају рукопис у три примерка (оригинал и две копије). Аутори чији матерњи језик није енглески такође треба да приложе и копију рада на изворном језику.

1.3. По примању рукописа, аутори ће добити редни број свога рада. Тај број треба наводити у даљој преписци. Редакција ће обавестити ауторе о приспећу рукописа и мишљењу рецензената у року од три месеца од пријема. Сваки рад рецензирају најмање два рецензента. Ако рад не буде прихваћен, рукопис се не враћа аутору.

1.4. Рукописе за објављивање треба слати на адресу редакције Зборника за природне науке, 21000 Нови Сад, Ул. Матице српске 1, Југославија.

2. Припрема рукописа

2.1. Рукописи се куцају са двоструким проредом у свим деловима текста (укључујући литературу, табеле итд.), на папиру формата А4. Све маргине треба да буду широке 2,5 сантиметра.

2.2. Рукопис треба поделити на: Сажетак, Кључне речи, Увод, Материјал и/или методе, Резултати испитивања, Расправа, Литература, Сажетак на српско-хрватском језику, Захвалност.

2.3. Назив рада треба да буде информативан, али не дужи од десет речи.

2.4. Кључне речи треба да указују на целокупну проблематику истраживања. Треба их навести абецедним редом и одвојити зарезима. Кључне речи не треба да пређу сто словних знакова.

2.5. Треба навести презимена, средње слово и имена аутора рада као и назив установе (без скраћеница) у којој је рад настао, заједно са пуном поштанском адресом. 2.6. Сажетак, на енглеском и српском, треба да буде информативан и да резимира садржај рада. Дужина енглеског сажетка треба да буде до 5%, а српског до 10% дужине укупног текста. Српски сажетак треба да садржи наслов рада, презимена и имена аутора и назив установе у којима су аутори запослени.

2.7. Податке о финансијској помоћи, саветима и другим врстама помоћи треба навести на крају рада, под насловом Захвалност.

2.8. Радови не смеју бити дужи од 12 куцаних страна, укључујући литературу, табеле, легенде и слике.

3. Литература

3.1. Литературу треба ограничити на неопходан број навода.

3.2. Литературне наводе треба сложити абецедним редом, на следећи начин:

а. Чланци из часописа Аутор CD, Аутор DC (1990) Назив рада. Име часописа 135: 102—134.

б. Чланци из књига

Аутор ED, Аутор SI, Аутор BB (1991) Назив цитираног дела књиге. У: А. Blom, B. Lindau, Eds., Назив књиге, Ed 3, Vol 2, Издавач, Град, 242—255.

в. Дисертације

Аутор VA (1989) Назив тезе. Докторска дисертација. Универзитет, Град.

г. Књиге

Аутор АЕ (1987) Назив књиге, Издавачи, Град, 237.

д. Публикације без аутора или уредника

Назив књиге, брошуре, итд. (1989) Издавач или установа, Град.

ђ. Необјављени радови

Навод "у штампи" треба да се односи само на прихваћене радове; навести име часописа у којем ће рад бити објављен.

3.3. Имена часописа треба скраћивати према "Bibliographic Guide for Authors and Editors" (BIOSIS, Chemical Abstracts Service and Engineerings Index, Inc., 1974).

3.4. Референце у тексту треба да укључе презиме аутора и годину издања. Ако има два аутора, треба навести обојицу, а у случају три или више аутора треба навести првог аутора и назначити "et al.".

3.5. Ако се наводе два или више радова истог аутора, објављених у истој години, потребно је у тексту и списку литературе ставити a, b, с итд. иза године објављивања.

4. Илустрације

4.1. За илустрације могу се користити црно беле фотографије и цртежи. Фотографије треба да имају добар контраст а цртежи треба да буду цртани тушем, на папиру доброг квалитета. Осим графикона, метаболичке шеме, компликоване формуле и велике или компликоване табеле такође треба третирати као слике.

4.2. Сва слова, бројке и симболи треба да буду довољно велики у оригиналу, тако да после смањивања не буду мањи од 1,5 mm. Текст на сликама и графиконима такође треба исписати тушем.

4.3. Илустрације треба приложити уз рад а не уметнуте у текст. По могућности, легенде треба назначити на илустрацијама.

4.4. Места илустрација треба означити на левој маргини, арапским бројевима.

4.5. Свака илустрација треба да има текст који објашњава садржај прилога. Текст за илустрације треба куцати на посебној страни.

5. Табеле

5.1. Табеле треба куцати на одвојеним странама (једна табела по страни) и приложити их на крају рада.

5.2. Табеле се означавају арапским бројевима.

5.3. Свака табела треба да почне насловом који објашњава њен садржај.

5.4. Напомене треба куцати одмах испод саме табеле.

5.5. Места табела у тексту треба означити на левој маргини.

6. Јединице, имена, формуле и скраћенице

6.1. Треба користити SI ознаке количина и јединица (SI Systeme International d'Unit's), изузетно се могу користити и друге званично прихваћене јединице.

6.2. Моларну концентрацију треба означити са М и подвући.

6.3. Биолошка имена на латинском треба подвући.

6.4. Хемијске структурне формуле и једначине треба нацртати (не исписивати или куцати) и припремити за фотографску репродукцију.

6.5. Прихватају се само стандардне скраћенице. При коришћењу специјалних скраћеница, пун термин треба навести приликом првог спомињања, а скраћеницу додати под наводним знацима.

6.6. Математички изрази треба да буду написани тако да се користи најмањи број редова, али да се сачува читљивост, нпр. 2/3 уместо 2:3, ехр (-ab) уместо a-ab, итд.

7. Кратка саопштења

7.1. Зборник за природне науке нуди могућност објављивања кратких саопштења о свим научним областима обухваћеним називом часописа.

7.2. Величина кратког саопштења је ограничена на 4 куцане стране, укључујући све илустрације.

7.3. Кратко саопштење се пише по упутствима за припрему рада нормалне дужине, сем што у литератури треба изоставити наслове рада.

8. Обавештавање аутора

8.1. Када рукопис буде прихваћен, аутор ће о приближном времену објављивања бити обавештен у писаној форми.

8.3. Исправљање текста припремљеног за штампу треба ограничити на штампарске грешке. Значајне промене текста ће се наплаћивати. Кориговани текст треба вратити уредништву у најкраћем могућем року.

8.3. Аутори добијају 50 бесплатних примерака сепарата. Ако аутор жели већи број сепарата може их наручити код издавача уз надокнаду.

## 9. КОПИЈА РАДА НА ДИСКЕТИ

После прихватања рада потребно је доставити дискету са коначном верзијом рада. Дискета треба да садржи текст рада, табеле и слике (прилоге) на DD или HD дискети од 3,5 инча. Приложите и једну копију одштампаног рада ради лакше обраде табела и слика. Молимо да обраду текста вршите програмом Word for Windows (било која верзија). Приликом копирања рада на дискету, придржавајте се следеће процедуре: File> Save as>Options>Embed True Type fonts>ok>Save.