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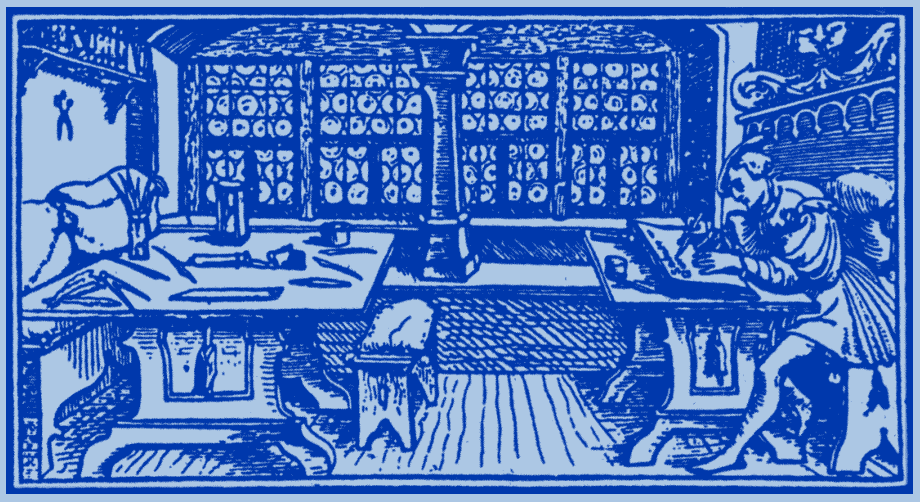

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DATA ON THE MICROBIOTA IN THE VADU CRIŞULUI CAVE

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SUMMARY. - The Vadu Crişului cave, located in the Pădurea Craiului Mountains (Romanian Western Carpathians) intensely visited by tourists, has been investigated from a microfloristic point of view. After a brief topographic and topoclimatic presentation of the cave, the paper provides a taxonomic approach to the troglophil and troglobiontic micromycetes and algae occurring in the cave. The investigation revealed the presence of 6 strains of micromycetes, a bacterial strain, 5 blue-green algae (*Cyanoprokaryota*), 4 taxa of xanthophytes (*Xanthophyta*) and 12 green algae (*Chlorophyta*) in the soil, on calcareous cave walls, in condensed water and limestone pool water.

Located at an altitude of 304 m a.s.l., on the left side of the Crişul Repede River, between the localities Şuncuiuş and Vadu Crişului, the Vadu Crişului cave is the main element of the "Defileul Crişului Repede" (Crişul Repede Strait) Nature Reserve and one of the most important habitats of the underground fauna in the Apuseni Mountains. The cave gallery has formed along a diaclase and it is penetrated by an underground water flow, except for an upper cavity, *i.e*. the balcony hall, which is the only part exhibiting outstanding concretions (Fig. 1). The first siphon occurs at 655 m from the entrance and may be penetrated only during dry periods, while the second siphon located at the end of the cave is always closed.

Although the cave is 1000 m long, it is not subjected to external meteorological influences except for the first 100 m. R a c o v i ț ă [10] described the cave as a cavity exhibiting permanent bidirectional ventilation. This topoclimatic type is in perfect agreement with the general topography of the cave, which is a practically horizontal one-entrance cavity. This sole opening concentrating all the aerodynamic exchanges with the outer world is the site of two air currents of opposite directions. The lower part of the gallery is affected by an ascending current during winter and a descending one during summer. The limits in time of the two ventilation types correspond to October and May, so that winter lasts for seven months and summer for five.

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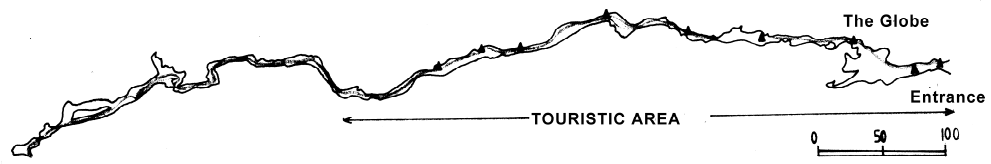
The annual ventilation-dependent variations in air temperature have two different characteristics: at floor level the thermic values are directly influenced by outer temperatures only during winter, while in summertime the mean thermic value remains constantly around 10.2° C. Annual relative humidity ranges between 98 and 100% [10].



F i g. 1. *The map of the Vadu Crişului cave*.
The sampling sites are indicated by ▲.

Ventilation speed varies according to season; yet the speed of ascending winter current resembles that of the descending summer one. Evapocondensation is strictly dependent upon ventilation: condensation typically occurs during summer, while the winter current change brings about an increasing evaporation intensity with ventilation speed.

Light intensity in the lower half of the gallery drops sharply along the first 20 m from 3200 lux (outside) to 0.1 lux (October 31, 1980).

Few data have been reported as regarding microbiota of the Vadu Crişului cave [5]; consequently, the aim of our study was to investigate from a taxonomic point of view the troglophil and troglobiontic micromycetes and algae occurring in the cave.

Materials and methods. The first studies were carried out in the cave (October 31, 1980) and comprised temperature, water pH and light intensity measurements (by means of field thermometer, pH-paper and luxmeter) and underground microscopic investigation (portable light microscope). As the resulting data were not conclusive, due to poor magnifying possibilities and scarce occurrence of organisms, samples were collected for further laboratory investigation (December 8, 1980) either in sterile test tubes or in tubes with culture media. The microclimatic measurements were repeated.

Micromycetes were isolated from samples obtained from cave sediments, limestone pool water and calcareous walls (pelicles, crusts or spots displaying different colours and consistency against the bearing rocks) and preserved in sterile test tubes. Sterility of tubes was achieved by maintenance in drying oven at 105° C for 48 h. Czapek medium solidified with agar was used for inoculation. Incubation took place in laboratory lockers in the absence of light at room temperature (19-22° C). The cultures were macro- and microscopically examined after 14 and 28 days of incubation.

Algae were collected from limestone pool water samples and the underground water flow, and subsequently transferred in sterile test tubes. These samples were imediately fixed in 4% formol. Other samples collected from limestone pools, soil and calcareous walls were inoculated on mineral culture media, such as Knop-Pringsheim (KP), Benecke (BK) or Watanabe (W) [9]. Incubation was carried out in light at room temperature (19-22^{o} C) for about 3 months, until colonies were clearly visible. The taxonomic identification of algae was carried out by light microscopy.

Results and discussion. The topoclimatic measurements were similar to those obtained by R a c o v i ţ ă [10]: the recorded temperature by the end of October was 10^{o} C at the entrance, subjected to relative variations, and 11^{o} C in the stable meroclimatic part. As mentioned before, light intensity drops below 1 lux after the first 20 m. The underground water has been found to be slightly acid (pH=5) without any variations.

Table 1

Micromycetes and bacteria isolated from the Vadu Crişului cave

Isolated strains	Sampling sites
Cladosporium herbarum (Person) Link	Aphotic area, calcareous wall
Scopulariopsis brevicaulis Bainier var. *glabra* Thom	Aphotic area, condensed water
Torula sp.	Aphotic area, calcareous wall
Verticillium lateritium Berkeley	Aphotic area, calcareous wall
Verticillium lateritium Berkeley, white mutant	Aphotic area, calcareous wall
Verticillium terrestre (Link) Lindau	Aphotic area, condensed water
Unidentified bacterium	Aphotic area, calcareous wall

The taxonomic data reveal the presence of bacteria, micromycetes and algae in the cave microflora. The micromycetes (Table 1) have been assigned to the *Fungi imperfecti* and most of them were previously isolated from other caves of the Crişul Repede Strait [4, 6, 11]. Most of the taxa isolated from the Vadu Crişului cave also occur in the soils of the Apuseni Mountains [6, 7], thus revealing their origin and high adaptability. While this is the first account of *Torula* sp. in the Apuseni Mountains, the species *Scopulariopsis brevicaulis* Bainier has been recorded in all the caves studied in the area [5]. Noteworty is the high morphological variability of the micromycete strains which makes their identification extremely difficult. For instance, *Verticillium lateritium* Berkeley, usually developing brownish colonies, produced a white mutant under laboratory conditions. All micromycete and bacterium strains were isolated from the aphotic part of the cave (Table 1).

The water samples collected in the sterile test tubes and fixed with formol were found to contain only the resistant forms. An unidentified viable blue-green alga also occurred. It seems [3] that formol is not suitable for fixing underground samples, as it easily destroys the few forms present in this biotope.

Table 2

Algal species isolated from the Vadu Crişului cave

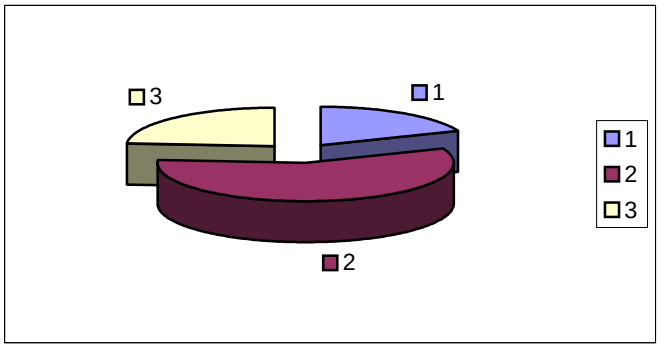
Algal taxa	Sampling sites	Culture media*
CYANOPROKARYOTA		
Aphanothece saxicola Naegeli	□ ▲ ▼ ● ◘ ○	KP, BK, W
Gloeocapsa montana (Kützing) Hollerbach	□ ▼ ●	KP, BK
Gloeocapsa sp.	⌂ ●	KP
Nostoc sp.	⌂ ▲ ● ◘ ○	KP, W
Symploca sp.	□ ▲ ▼ ● ○	KP, BK
XANTHOPHYTA		
Monodus dactylococcoides Pascher	□ ▲ ● ◘	BK
Monodus subterranea Boye-Petersen	⌂ □ ▲ ● ◘	KP, BK
Monodus sp.	▲ ○	KP
Pleurochloris sp.	□ ●	BK
CHLOROPHYTA		
Chlorella vulgaris Beijerinck	□ ▲ ▼ ● ◘ ○	KP, BK
Chlorella luteo-viridis Chodat	⌂ ●	KP
Chlorella zofingiensis Dönz	□ ▲ ● ◘	KP, BK
Chlorococcum sp.	⌂ □ ▲ ▼ ● ◘	KP, BK, W
Chlamydomonas rosae H. et O. Ettl	⌂ ▼ ●	KP
Chlamydomonas sp.	□ ▲ ▼ ● ◘	KP, BK, W
Gloeotila sp. Kützing	□ ●	BK
Gloeococcus mucosus Braun	▲ ◘	W
Hormidium flaccidum Braun	▲ ○	KP
Stichococcus bacillaris Naegeli	⌂ □ ▲ ▼ ● ◘ ○	KP, BK,W
Stichococcus minor Naegeli	▲ ▼ ● ◘	KP
Tetracystis dissociata Brown et Bold	⌂ ●	KP

▲ - Calcareous wall. □ – Soil. ▼ - Lignified plant. ⌂ - Water from cave pool.
● - Aphotic area. ◘ - Entrance area (low light). ○ - Photic area (external area).
* For the composition of culture media see [9].

The algae living in the cave (Table 2) were identified after incubating the sample on mineral media in the light. Being highly resistant, the algae can survive latently in a cave and recover under adequate conditions (*e.g.* in light). Such phenomenon also occurs in electrified caves and leads to the formation of the so called „lamp flora". This was the case in the Vadu Crişului cave too, where electricity was installed for a certain period of time.

The algal forms isolated are very similar to those recorded most often in underground environment [2, 8]. In the Vadu Crişului cave the most frequent ones are the green algae (12 taxa) as compared to the blue-green ones (*Cyanoprokaryota* – 5 taxa) (Fig. 2). The former ones are supposed to exist latently in the aphotic area, considering their presence in different cave microbiotopes as well as in the entrance

area and outside the cave. Of the 7 outer species isolated from the calcareous wall above the entrance, only two could not be identified inside the cave; all the others were found in different parts of the aphotic area suggesting a possible external source and dissemination through the cave *e.g*. water flux (Table 2).



F i g. 2. *The proportion of different algal groups in the Vadu Crişului cave*. 1 – *Xanthophyta*. 2 – *Chlorophyta*. 3 – *Cyanoprokaryota*.

Other 10 taxa were identified only in the aphotic area. These species are usually present in restricted microbiotopes, being sometimes isolated from a single site. This account for their ability to live in an underground environment, either by heterotrophy or by other mechanisms. Of course, these suppositions require experimental support.

Conclusions. The microbiota, which is considered to play a leading role in the caves [1], should be thoroughly investigated for a better understanding of the underground hollow genesis and ecology. Therefore, knowledge of the microbiota in the Vadu Crişului cave should also be completed by further investigations.

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ANALIZA FAUNISTICĂ, ECOLOGICĂ ŞI ZOOGEOGRAFICĂ A SUPRAFAMILIEI *PENTATOMOIDEA (INSECTA, HETEROPTERA)* DIN SECTORUL INFERIOR AL BAZINULUI RÂULUI ARIEŞ

PAUL-VASILE BELDEAN*

SUMMARY. - Faunistical, Ecological and Zoogeographic Analysis of the Suprafamily P e n t a t o m o i d e a (I n s e c t a, H e t e r o p t e r a) from the Inferior Area of the Arieş River Basin. The biological material was collected during 2001, 2002 and 2003. In the studied area we have revealed a number of 565 *Pentatomoidea*, belonging to 26 species, 19 genera and 4 family. The majority of the species (20) belongs to the family *Pentatomidae*. The researches were made in nine different types of ecosystems: mixed forest (beech and hornbeam), forest of hornbeam, cluster of osier, hay field with high humidity, hay field with medium humidity, rocky region, grassland, crop of alfalfa and roadside vegetation. Most species (17) live in the hay field with medium humidity. In the mixed forest 10 species of *Pentatomoidea* live (*Dolycoris baccarum, Pentatoma rufipes* and *Palomena prasina* have the highest values of numerical and relative abundance). In the hay field, the highest values of abundance were shown by *Eurydema oleraceum, Coptosoma scutellatum* and *Aelia acuminata*. In the crop of alfalfa *Piezodorus lituratus* and in the roadside vegetation *Coptosoma scutellatum* and *Graphosoma lineatum* are the most abundant species. The highest number of adults was recorded in June. A proportion of 69% of the species is polyphagous. From all species 50% belong to the Palearctic Region. *Sciocoris (Sciocoris) deltocephalus* is a new record for Transilvania.

Publicaţiile anterioare referitoare la fauna de *Pentatomoidea* din Transilvania au caracter faunistic, cuprinzând liste de specii din diferite zone [3, 5, 9, 11]. Unele lucrări prezintă speciile de *Pentatomoidea* considerate dăunătoare în diverse culturi agricole, în acest caz apărând şi informaţii sumare legate de mediul de viaţă al acestora [6, 7, 10].

Nu se cunosc publicaţii anterioare referitoare la întreg sectorul inferior al Arieşului, semnalări ale câtorva specii din această zonă fiind cuprinse în volumul VIII din Fauna R.S.R. [4]. În perioada 1974-1995 au fost colectate din Cheile Turenilor 13 specii de *Pentatomoidea* aparţinând la trei familii (*Cydnidae, Scutelleridae* şi *Pentatomidae*), datele nefiind însă publicate [14].

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Material şi metode. Materialul biologic analizat a fost colectat pe parcursul anilor 2001, 2002 şi 2003 în diferite perioade ale anului. Au fost colectate 4 probe din fiecare ecosistem prin metode specifice tipului de vegetaţie existent: cu fileul entomologic din vegetaţia ierboasă (50 cosiri/probă) şi prin scuturarea coronamentului arborilor şi arbuştilor în plasa umbrelă. Pentru fiecare probă a fost notată data şi punctul de colectare. Materialul colectat a fost omorât în alcool 70% şi păstrat în alcool cu aceeaşi concentraţie în tuburi de plastic, separat pe probe.

În sectorul inferior al Bazinului Arieşului au fost studiate următoarele tipuri de ecosisteme:

I. Făgeto-cărpinet în staţiunea Moldoveneşti situat pe malul stâng al Arieşului, cu expoziţie sud-estică, între 350-400 m altitudine**.** Alături de *Carpinus betulus* şi *Fagus sylvatica*, se mai întâlnesc: *Acer campestre, Corylus avellana, Betula pendula*. Stratul ierbos este bine dezvoltat, mai ales de-a lungul pârâului care trece prin pădure, fiind frecvente: *Mentha, Medicago, Trifolium pratense, Urtica dioica, Equisetum arvense, Carduus spinosus* şi *Carduus echinatus*.

II. Cărpinet situat pe versantul drept al Cheilor Turenilor, cu expoziţie nordică, între 550-600 m altitudine, în care alături de carpen (*Carpinus betulus*) este frecvent alunul (*Corylus avellana*).

III. Salicete în staţiunile Luna şi Gura Arieşului. Sunt formate predominant din specii de salcie (*Salix* sp.), la care se adaugă plopi şi anini (*Populus* sp. şi *Alnus* sp.). Stratul ierbos este format din *Ononis* sp., *Centaurea* sp., *Typha* sp., *Urtica dioica, Coronilla varia, Plantago lanceolata* etc.

IV. Fânaţe higrofile în Luna şi Gura Arieşului.

Fânaţul studiat la Luna este situat lângă zăvoi, pe malul drept al Arieşului, la 300 m altitudine. Alături de *Agrostis* şi *Festuca* se întîlnesc *Trifolium pratense, Lotus corniculatus, Coronilla varia, Ononis spinosa* şi *Plantago lanceolata*. Există şi porţiuni cu *Sambucus ebulus*.

Fânaţul de la Gura Arieşului este situat pe malul drept al Arieşului, în zonă de luncă, la 200 m altitudine. Este puternic higrofil, alături de poacee fiind bine reprezentate speciile de: *Juncus, Typha, Trifolium, Ononis, Linaria, Cirsium* şi *Polygonum*. Se întâlneşte şi *Caltha palustris*.

V. Fânaţe mezofile în Moldoveneşti şi Mărtineşti. Sunt formate predominant din specii de *Agrostis* (*Agrostis tenuis, Agrostis alba* etc.) şi *Festuca* (*Festuca rubra, Festuca pratensis* etc.) alături de care, la Moldoveneşti se întâlnesc *Trifolium pratense, Lotus corniculatus, Coronilla varia, Ononis spinosa* şi *Plantago lanceolata*. La Mărtineşti stratul ierbos este format predominant din *Trifolium pratense, Trifolium repens, Coronilla varia, Medicago* sp. şi *Cirsium* sp.

VI. Stâncării în Cheile Turenilor. Vegetaţia acestor stâncării se încadrează în asociaţiile *Seslerietum rigidae* şi *Spireetum ulmifolie* [1, 8].

VII. Păşuni în Moldoveneşti, Mărtineşti, Cheile Turenilor, Luna şi Gura Arieşului. Predomină speciile de *Festuca* şi *Agrostis*. Păşunea din Moldoveneşti este higrofilă, bogată în vegetaţie ierboasă, fiind întâlnite specii de *Trifolium, Linaria* şi *Polygonum*.

În Mărtineşti păşunea studiată are expoziţie sud-vestică, fiind predominante *Festuca sulcata* şi *Agrostis tenuis.* Sporadic se întâlnesc tufe de *Prunus spinosa, Crataegus monogyna* şi *Rosa canina*.

În Cheile Turenilor a fost studiată o păşune puternic xerofilă şi intens păşunată, fiind încadrată în asociaţia *Festucetum sulcatae calcophilum* [1,8].

În păşunea din Luna, situată în lunca Arieşului şi cu un grad relativ scăzut de exploatare, alături de poacee sunt întâlnite *Trifolium pratense* şi *Althea rosea*, precum şi specii de *Ononis, Medicago, Euphorbia* şi *Artemisia*.

La Gura Arieşului păşunea studiată este intens exploatată, situată pe malul drept al Arieşului, fiind reprezentată în general de poacee şi specii de *Medicago, Euphorbia* şi *Artemisia*. Izolat se întâlnesc tufe de *Crataegus monogyna, Syringa vulgaris, Prunus spinosa* şi *Rosa canina.*

VIII. Cultură de lucernă situată în staţiunea Moldoveneşti, înainte de intrarea în localitate, la aproximativ 350 m altitudine.

IX. Vegetaţie ruderală în 4 staţiuni: Moldoveneşti, Mărtineşti, Luna şi Gura Arieşului. Este formată predominant din *Artemisia* sp., *Matricaria* sp., *Trifolium* sp., *Linaria* sp., *Verbascum* sp., *Raphanus* sp., *Echium vulgre, Coronilla varia, Equisetum arvense, Urtica dioica, Lycopus europaeus, Capsella bursa-pastoris, Mentha longifolia, Carduus nutans* şi *Carduus acanthoides*.

În staţiunea Moldoveneşti au fost efectuate colectări lunare din mai până în octombrie, în vederea efectuării studiilor de dinamică a populaţiilor de Pentatomoidea.

Determinările au fost efectuate în laborator cu ajutorul stereo-microscopului, folosind diverse surse bibliografice [4, 12, 13]. Materialul determinat a fost ordonat pe familii, subfamilii şi specii conform sistemului taxonomic actual [2, 4, 12].

Rezultate şi discuţii. Analiza taxonomică şi faunistică. În urma studiilor efectuate în sectorul inferior al Arieşului am colectat 565 indivizi de *Pentatomoidea*, aparţinând la 26 de specii, 19 genuri şi 4 familii. Atât din punct de vedere al numărului de specii cât şi al numărului de indivizi, cel mai bine reprezentată este familia *Pentatomidae* (481 de indivizi din 20 de specii). Din familia *Scutelleridae* au fost colectaţi 36 de indivizi din 3 specii, familia *Plataspidae* este prezentă printr-o singură specie cu 45 de indivizi, iar familia *Cydnidae* este reprezentată prin 2 specii cu 3 indivizi (Tabel 1).

La nivelul teritoriului României, din suprafamilia *Pentatomoidea* cel mai bine reprezentată este familia *Pentatomidae*, urmată de familiile *Cydnidae, Scutelleridae, Acanthosomidae* (prezentă în fauna României, dar nesemnalată în sectorul inferior al Arieşului) şi *Plataspidae* [4].

Ambele specii de *Cydnidae* colectate sunt încadrate în subfamilia *Sehirinae.* Cele trei specii ale familiei *Scutelleridae* sunt distribuite în două subfamilii: *Odontotarsinae* cu o singură specie şi *Eurygasterinae* cu 2 specii. Din familia *Pentatomidae* cel mai bine reprezentată este subfamilia *Pentatominae* cu 16 specii, în timp ce subfamiliile *Podopinae* şi *Amyotinae* sunt prezente fiecare prin câte 2 specii.

Tabel 1

Speciile de P e n t a t o m o i d e a colectate din sectorul inferior al Bazinului Arieşului

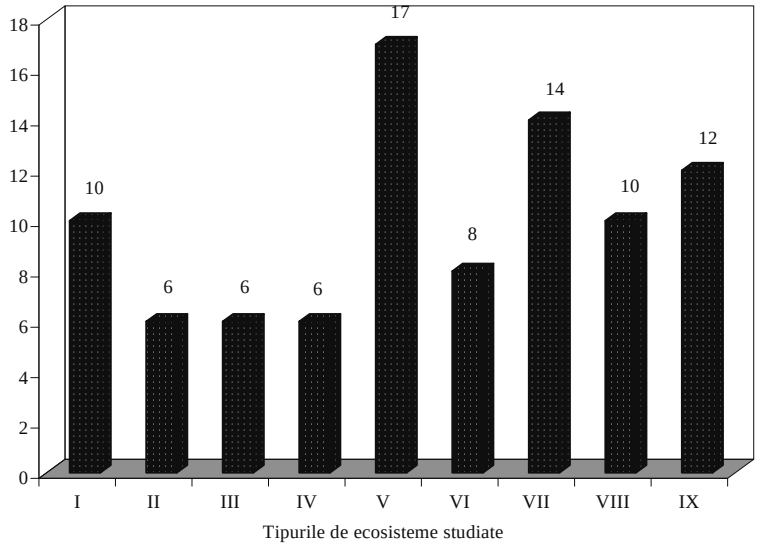
Familia, subfamilia, specia	Numărul de indivizi			Staţiuni cercetate					NTr. S.
	T	♂	♀	1	2	3	4	5	
Familia Plataspidae	**45**	**19**	**26**	**23**	**9**		**8**	**5**	
Coptosoma scutellatum (Geoffroy, 1785)	45	19	26	23	9		8	5	
Familia Cydnidae	**3**	**1**	**2**	**1**			**2**		
Subfam. Sehirinae	***3***	***1***	***2***	***1***			***2***		
Tritomegas bicolor (Linné, 1758)	2	1	1	1			1		
Tritomegas sexmaculatus (Rambur, 1842)	1		1				1		
Familia Scutelleridae	**36**	**18**	**18**	**25**	**7**	**4**			
Subfam. Odontotarsinae	***1***		***1***			***1***			
Odontotarsus purpureolineatus (Rossi, 1790)	1		1			1			
Subfam. Eurygasterinae	***35***	***18***	***17***	***25***	***7***	***3***			
Eurygaster maura (Linné, 1758)	21	13	8	13	5	3			
Eurygaster testudinaria (Geoffroy, 1785)	14	5	9	12	2				
Familia Pentatomidae	**481**	**231**	**250**	**251**	**52**	**56**	**105**	**17**	
Subfam. Podopinae	***25***	***13***	***12***	***12***	***5***	***4***	***1***	***3***	
Graphosoma lineatum (Linné, 1758)	24	12	12	12	5	4		3	
Podops inuncta (Fabricius, 1775)	1	1					1		S.
Subfam. Pentatominae	***432***	***206***	***226***	***227***	***37***	***52***	***102***	***14***	
Sciocoris (Sciocoris) deltocephalus Fieber, 1861	1		1	1					NTr.
Sciocoris (Aposciocoris) microphthalmus Flor, 1860	2	1	1		2				
Aelia acuminata (Linné, 1758)	54	28	26	28	9	7	8	2	
Aelia rostrata Boheman, 1852	2		2		2				
Eusarcoris aeneus (Scopoli, 1763)	11	6	5	8			3		
Stagnomus (Dalleria) pusillus (Herrich-Schäffer, 1830)	3	1	2			3			S.
Holcostethus (Holcostehtus) vernalis (Wolff, 1904)	31	15	16	16	2	10		3	
Carpocoris purpureipennis (De Geer, 1773)	22	10	12	15	1	6			
Carpocoris pudicus (Poda, 1761)	10	1	9	3	1		6		
Dolycoris baccarum (Linné, 1758)	101	49	52	57	11	19	11	3	
Palomena prasina (Linné, 1761)	11	5	6	9		2			
Piezodorus lituratus (Fabricius, 1794)	41	22	19	39			2		
Pentatoma rufipes (Linné, 1758)	12	7	5	12					
Eurydema ventrale Kolenati, 1846	2		2	1			1		S.
Eurydema ornatum (Linné, 1758)	3	1	2	2		1			
Eurydema oleraceum (Linné, 1758)	126	60	66	36	9	4	71	6	
Subfam. Amyotinae	***24***	***12***	***12***	***12***	***10***		***2***		
Picomerus bidens (Linné, 1758)	19	11	8	9	10				
Zicrona coerulea (Linné, 1758)	5	1	4	3			2		
Numărul total de indivizi	**565**	**269**	**296**	**300**	**68**	**60**	**115**	**22**	
Numărul total de specii		**26**		**20**	**13**	**11**	**12**	**6**	

Abrevieri: 1 - Moldoveneşti. 2 - Mărtineşti. 3 - Cheile Turenilor. 4 - Luna. 5 - Gura Arieşului. NTr. - Specie semnalată pentru prima dată în fauna Transilvaniei. S - Specie sporadică în fauna României.

Sciocoris (Sciocoris) deltocephalus este semnalată de noi pentru prima dată în fauna Transilvaniei, semnalările anterioare fiind din județele Dolj, Giurgiu, Galați, Constanța şi Tulcea. Este specie termofilă ponto-mediteraneană, întâlnită pe diferite plante ierboase (*Digitaria, Eragrostis, Filago, Salvia* etc.) în biotopuri nisipoase [4]. În urma studiilor noastre *Sciocoris (Sciocoris) deltocephalus* a fost colectată din stațiunea Moldoveneşti, de pe vegetația ierboasă din ecosistemul reprezentat de pășune situată pe malul stâng al Arieşului, pe substrat nisipos.

Podops inuncta, *Stagnomus (Dalleria) pusillus* şi *Eurydema ventrale* sunt considerate sporadice în fauna României [4].

Distribuția numărului de specii pe tipuri de ecosisteme. Din făgeto-cărpinetul studiat au fost colectate 10 specii (Tabel 2), fiind semnalate atât specii tipice pentru acest tip de ecosistem (*Palomena prasina, Pentatoma rufipes*) cât şi specii caracteristice vegetației ierboase (*Eusarcoris aeneus, Piezodorus lituratus*). Numărul cel mai mare de specii (17) a fost colectat din ecosistemele mezofile exploatate ca fânaț (Tabel 3), caracterizate printr-un număr mare de specii vegetale ierboase, urmate de pășuni cu 14 specii (Tabel 4), vegetație ruderală cu 12 specii şi cultura de lucernă cu 10 specii (Tabel 5). Din celelalte ecosisteme caracterizate prin vegetație lemnoasă (cărpinet, salicete) dar cu un număr de specii vegetale mai redus comparativ cu făgeto-cărpinetul studiat, au fost colectate câte 6 specii de *Pentatomoidea*. Tot 6 specii au fost colectate şi din fânațele higrofile cu suprafață redusă (aproximativ 500 m^2 fiecare) situate în vecinătatea culturilor agricole tratate cu ierbicide şi insecticide, în timp ce din ecosistemul reprezentat de stâncării au fost colectate 8 specii (Fig. 1).



F i g. 1. *Numărul de specii înregistrat în ecosistemele studiate.*

Numărul de specii scade odată cu scăderea cantității de vegetație ierboasă, reducerea numărului de plante prezente şi creşterea influențelor antropice în ecosistemelor studiate.

Analiza ecologică. În făgeto-cărpinetul din staţiunea Moldoveneşti (Tabel 2), din punct de vedere al abundenței numerice şi relative se remarcă *Dolycoris baccarum* (A = 0,5, Ar = 21,87) - specie polifagă larg răspândită în România, *Pentatoma rufipes* (A = 0,43, Ar = 18,75) şi *Palomena prasina* (A = 0,32, Ar = 14,06) - specii caracteristice ecosistemelor de tip forestier. Valorile abundenței numerice şi relative înregistrate în cărpinetul din staţiunea Cheile Turenilor sunt asemănătoare pentru majoritatea speciilor colectate, arboretul fiind slab închegat şi prezentând numeroase poteci şi luminişuri păşunate. Majoritatea speciilor colectate din salicetele situate în staţiunile Luna şi Gura Arieşului prezintă valori reduse ale abundenței numerice şi relative.

Tabel 2

Abundența numerică şi abundența relativă a speciilor de *Pentatomoidea* colectate din ecosistemele lemonase din sectorul inferior al Bazinului Arieşului

Familia, specia	I			II			III					
	1			3			4			5		
	N	A	Ar%	N	A	Ar%	N	A	Ar%	N	A	Ar%
Familia ***Pentatomidea***												
Graphosoma lineatum (Linné, 1758)	6	0,21	9,37	3	0,25	15						
Aelia acuminata (Linné, 1758)										1	0,08	33,33
Eusarcoris aeneus (Scopoli, 1763)	3	0,11	4,68				2	0,16	16,65			
Stagnomus (Dalleria) pustiim (Herrich-Schäffer, 1830)				3	0,25	15						
Holcostethus (Holcostehtus) vernalis (Wolff, 1904)	5	0,17	7,81	5	0,41	25				1	0,08	33,33
Carpocoris purpureipennis (De Geer, 1773)	2	0,07	3,12	4	0,33	20						
Carpocoris pudicus (Poda, 1761)							2	0,16	16,65			
Dolycoris baccarum (Linné, 1758)	14	0,5	21,87	3	0,25	15	2	0,16	16,65			
Palomena prasina (Linné, 1761)	9	0,32	14,06	2	0,16	10						
Piezodorus lituratus (Fabricius, 1794)	3	0,11	4,68									
Pentatoma rufipes (Linné, 1758)	12	0,43	18,75									
Eurydema oleraceum (Linné, 1758)	5	0,17	7,81				6	0,5	50	1	0,08	33,33
Picomerus bidens (Linné, 1758)	5	0,17	7,81									
Numărul total de indivizi/staţiune	**64**			**20**			**12**			**3**		
Numărul total de indivizi/ tip de ecosistem	**64**			**20**			**15**					
Numărul total de specii/staţiune	**10**			**6**			**4**			**3**		
Numărul total de specii/ tip de ecosistem	**10**			**6**			**6**					

Abrevieri: I - Făgeto-cărpinet. II - Cărpinet. III - Salicet. 1 - Moldoveneşti. 3 - Cheile Turenilor. 4 - Luna. 5 - Gura Arieşului. A - Abundența numerică. Ar - Abundența relativă. N - Numărul de indivizi.

În fânaţul higrofil din staţiunea Luna, *Eurydema oleraceum* prezintă cea mai mare abundenţă numerică pentru acest tip de ecosistem (A= 1,75) (Tabel 3). În ambele fânaţe higrofile studiate (Luna şi Gura Arieşului) este bine reprezentată *Coptosoma scutellatum*, singura specie din familia *Plataspidae* semnalată în fauna Transilvaniei, dar foarte frecventă în ecosistemele ierboase.

Tabel 3

Abundenţa numerică şi abundenţa relativă a speciilor de *Pentatomindea* colectate din ecosistemele ierboase cu intervenţie antropică moderată (fânaţe, stâncării) din sectorul inferior al Bazinului Arieşului

Familia, specia	IV						V						VI		
	4			5			1			2			3		
	N	A	Ar%	N	A	Ar%	N	A	Ar%	N	A	Ar%	N	A	Ar%
Familia *Plataspidae*															
Coptosoma scutellatum (Geoffroy, 1785)	6	0,5	15,38	5	0,62	55,55	9	0,32	8,1						
Familia *Cydnidae*															
Tritomegas bicolor (Linné, 1758)							1	0,03	0,9						
Familia *Scutelleridae*															
Odontotarsus purpureolineatus (Rossi, 1970)													1	0,08	3,57
Eurygaster maura (Linné, 1758)							6	0,21	5,4	5	0,31	20	3	0,25	10,71
Eurygaster testudinaria (Geoffroy, 1785)							7	0,25	6,3	1	0,06	4			
Familia *Pentatomidae*															
Graphosoma lineatum (Linné, 1758)							6	0,21	5,4						
Sciocoris (Aposciocoris) microphthalmus Flor, 1860										2	0,12	8			
Aelia acuminata (Linné, 1758)	4	0,33	10,25	1	0,12	11,11	22	0,78	19,82	7	0,43	28	6	0,5	21,42
Eusarcoris aeneus (Scopoli, 1763)							5	0,17	4,5						
Holcostethus (Holcostehtus) vernalis (Wolff, 1904)							6	0,21	5,4	1	0,06	4	4	0,33	14,28
Carpocoris purpureipennis (De Geer, 1773)							9	0,32	8,1				2	0,16	7,14
Carpocoris pudicus (Poda, 1761)	2	0,16	5,12				1	0,03	0,9						
Dolycoris baccarum (Linné, 1758)	4	0,33	10,25	1	0,12	11,11	17	0,6	15,31	5	0,31	20	9	0,75	32,14
Piezodorus lituratus (Fabricius, 1794)	2	0,16	5,12				8	0,28	7,2						
Eurydema ornatum (Linné, 1758)							2	0,07	1,8				1	0,08	3,57
Eurydema oleraceum (Linné, 1758)	21	1,75	53,84	2	0,25	22,22	5	0,17	4,5	4	0,25	16	2	0,16	7,14
Picomerus bidens (Linné, 1758)							4	0,14	3,6						
Zicrona coerulea (Linné, 1758)							3	0,1	2,7						
Numărul total de indivizi/staţiune	**39**			**9**			**111**			**25**			**28**		
Numărul total de indivizi/ tip de ecosistem	**48**						**136**						**28**		
Numărul total de specii/staţiune	**6**			**4**			**16**			**7**			**8**		
Numărul total de specii/ tip de ecosistem	**6**						**17**						**8**		

Abrevieri: IV - Fânaţ higrofil. V - Fânaţ mezofil. VI - Stâncării. 1 - Moldoveneşti. 2 - Mărtineşti. 3 - Cheile Turenilor. 4 - Luna. 5 - Gura Arieşului. A - Abundenţa numerică. Ar - Abundenţa relativă. N - Numărul de indivizi.

Aelia acuminata, specie polifagă caracteristică ecosistemelor ierboase însorite, a înregistrat valorile cele mai ridicate ale abundenței numerice şi absolute în fânațele mezofile studiate (A = 0,78, Ar = 19,82 în staţiunea Moldoveneşti, A = 0,43, Ar = 28 în staţiunea Mărtineşti). Valori ridicate ale acestor indici în fânațele mezofie au înregistrat şi speciile *Coptosoma scutellatum, Eurygaster maura, Carpocoris purpureipennis* şi *Dolycoris baccarum* (Tabel 3).

Pe stâncăriile din Cheile Turenilor, cel mai bine reprezentată este *Dolycoris baccarum* (A = 0,75, Ar = 32,14) urmată de *Aelia acuminata* (A = 0,5, Ar = 21,42) şi *Holcostethus vernalis* (A = 0,33, Ar = 14,28) (Tabel 3).

În păşunile studiate, valorile cele mai ridicate ale abundenței numerice şi relative au fost înregistrate de către *Eurydema oleraceum* în staţiunea Luna (A = 2,31, Ar = 80,43) (Tabel 4). Printr-un număr mare de indivizi au fost prezente şi speciile *Picomerus bidens* (A = 0,62, Ar = 50 în Mărtineşti) - una dintre puținele specii de pentatomoidee zoofage, adulții apărând în lunile de toamnă, *Dolycoris baccarum* (A = 0,58, Ar = 58,33 în Cheile Turenilor) şi *Coptosoma scutellatum* (A = 0,5, Ar = 35,29 în Moldoveneşti).

Din cultura de lucernă, a fost colectat un număr mare de indivizi de *Piezodorus lituratus* (A = 1,16, Ar = 40), specie frecventă în culturile de leguminoase din Transilvania [6]. Atât din cultura de lucernă, cât şi de pe vegetația ruderală au fost colectate în număr relativ mare *Dolycoris baccarum* şi *Eurydema oleraceum*. În vegetația ruderală din staţiunea Mărtineşti, valori ridicate ale abundenței numerice şi relative au prezentat şi *Coptosoma scutellatum* (A = 0,75, Ar = 39,13) şi *Graphosoma lineatum* (A = 0,41, Ar =21,74) (Tabel 5).

Diversitatea cea mai ridicată s-a înregistrat în fânațul mezofil din staţiunea Moldoveneşti urmat de făgeto-cărpinetul studiat în aceeaşi staţiune şi de stâncăriile studiate în Cheile Turenilor. Diversitatea cea mai scăzută s-a înregistrat în păşunile din Gura Arieşului şi Luna (Tabel 6).

Dinamica populațiilor. În vederea efectuării studiilor de dinamică a populațiilor de *Pentatomoidea*, în staţiunea Moldoveneşti au fost efectuate colectări lunare. Atât numărul de indivizi cât şi numărul de specii variază pe parcursul anului (Tabel 7). Numărul maxim este atins în luna iunie, când sunt prezenți atât indivizi ai unor specii la care generația ieşită din diapauză se menține până în această perioadă, cât şi indivizi ai unor specii la care a apărut noua generație. În iulie, atât datorită atingerii unui grad ridicat de exploatare al păşunii studiate, cât şi datorită cosirii fânațului şi culturii de lucernă, numărul de specii şi de indivizi a scăzut. În luna august, când vegetația ierboasă din ecosistemele antropizate s-a refăcut parțial, numărul de indivizi şi de specii de *Pentatomoidea* a înregistrat o uşoară creştere comparativ cu luna iulie. Valorile înregistrate în luna mai (situația populațiilor la ieşirea din diapauză) sunt asemănătoare cu valorile înregistrate în lunile septembrie şi octombrie (situația populațiilor la intrarea în diapauză) (Fig. 2).

Spectrul trofic. În urma analizei spectrului trofic al pentatomoideelor semnalate se remarcă speciile polifage (18), acestea reprezentând 69%. Nu a fost semnalată nici o specie monofagă. Dintre cele 26 de specii colectate 8 sunt oligofage, 4 specii fiind întâlnite pe graminee, 3 pe crucifere, iar una pe leguminoase (Fig. 3).

Tabel 4

Abundența numerică şi abundența relativă a speciilor de *Pentatomoidea* colectate din ecosistemele ierboase intens exploatate (pășuni) din sectorul inferior al Bazinului Arieşului

Familia, specia	VII														
	1			2			3			4			5		
	N	A	Ar%	N	A	Ar%	N	A	Ar%	N	A	Ar%	N	A	Ar%
Familia *Plataspidae*															
Coptosoma scutellatum (Geoffroy, 1785)	12	0,5	35,29							2	0,12	4,34			
Familia *Cydnidae*															
Tritomegas bicolor (Linné, 1758)										1	0,06	2,17			
Familia *Scutelleridae*															
Eurygaster testudinaria (Geoffroy, 1785)	4	0,16	11,76	1	0,06	5									
Familia *Pentatomidae*															
Graphosoma lineatum (Linné, 1758)							1	0,08	8,33						
Podops inuncta (Fabricius, 1775)										1	0,06	2,17			
Sciocoris (Sciocoris) deltocephalus Fieber, 1861	1	0,04	2,94												
Aelia acuminata (Linné, 1758)	2	0,08	5,88	1	0,06	5	1	0,08	8,33						
Aelia rostrata Boheman, 1852				2	0,12	10									
Holcostethus (Holcostehtus) vernalis (Wolff, 1904)	1	0,04	2,94				1	0,08	8,33				2	0,25	66,67
Carpocoris pudicus (Poda, 1761)										1	0,06	2,17			
Dolycoris baccarum (Linné, 1758)	10	0,41	29,41	5	0,31	25	7	0,58	58,33	3	0,18	6,52			
Eurydema ventrale Kolenati, 1846										1	0,06	2,17			
Eurydema oleraceum (Linné, 1758)	4	0,16	11,76	1	0,06	5	2	0,16	16,65	37	2,31	80,43	1	0,12	33,33
Picomerus bidens (Linné, 1758)				10	0,62	50									
Numărul total de indivizi/ stațiune	**34**			**20**			**12**			**46**			**3**		
Numărul total de indivizi/ tip de ecosistem	**115**														
Numărul total de specii/ stațiune	**7**			**6**			**5**			**7**			**2**		
Numărul total de specii/ tip de ecosistem	**14**														

Abrevieri: VII - Pășune. 1 - Moldovenești. 2 - Mărtinești. 3 - Cheile Turenilor. 4 – Luna. 5 - Gura Arieşului. A - Abundența numerică. Ar - Abundența relativă. N - Numărul de indivizi.

Tabel 5

Abundența numerică şi abundența relativă a speciilor de *Pentatomoidea* colectate din ecosisteme ierboase (cultură de lucernă, vegetație ruderală) situate în zone puternic antropizate din sectorul inferior al Bazinului Arieşului

Familia, specia	VIII			IX											
	1			1			2			4			5		
	N	A	Ar %	N	A	Ar %	N	A	Ar %	N	A	Ar %	N	A	Ar %
Familia *Plataspidae*															
Coptosoma scutellatum (Geoffroy, 1785)	2	0,08	2,85				9	0,75	39,13						
Familia *Cydnidae*															
Tritomegas sexmaculatus (Rambur, 1842)										1	0,08	5,56			
Familia *Scutelleridae*															
Eurygaster maura (Linné, 1758)	7	0,29	10												
Eurygaster testudinaria (Geoffroy, 1785)	1	0,04	1,42												
Familia *Pentatomidae*															
Graphosoma lineatum (Linné, 1758)							5	0,41	21,74				3	0,37	42,85
Aelia acuminata (Linné, 1758)	1	0,04	1,42	3	0,12	14,28	1	0,08	4,35	4	0,33	22,22			
Eusarcoris aeneus (Scopoli, 1763)										1	0,08	5,56			
Holcostethus (Holcostehtus) vernalis (Wolff, 1904)	3	0,12	4,28	1	0,04	4,76	1	0,08	4,35						
Carpocoris purpureipennis (De Geer, 1773)	2	0,08	2,85	2	0,08	9,52	1	0,08	4,35						
Carpocoris pudicus (Poda, 1761)	2	0,08	2,85				1	0,08	4,35	1	0,08	5,56			
Dolycoris baccarum (Linné, 1758)	11	0,45	15,71	5	0,2	23,8	1	0,08	4,35	2	0,16	11,11	2	0,25	28,57
Piezodorus lituratus (Fabricius, 1794)	28	1,16	40												
Eurydema ventrale Kolenati, 1846				1	0,04	4,76									
Eurydema oleraceum (Linné, 1758)	13	0,54	18,57	9	0,37	42,85	4	0,33	17,39	7	0,58	38,88	2	0,25	28,57
Zicrona coerulea (Linné, 1758)										2	0,16	11,11			
Numărul de indivizi/ stațiune	**70**			**21**			**23**			**18**			**7**		
Numărul de indivizi/ tip de ecosistem	**70**								**69**						
Numărul de specii	**10**			**6**			**8**			**7**			**3**		
Numărul de specii/ tip de ecosistem	**10**								**12**						

Abrevieri: VIII - Cultură de lucernă. IX - Vegetație ruderală. 1 - Moldovenești. 2 - Mărtinești. 4 - Luna. 5 - Gura Arieşului. A - Abundența numerică. Ar - Abundența relativă. N - Numărul de indivizi.

Tabel 6

Diversitatea şi echitabilitatea ecosistemelor studiate în funcţie de populaţiile de *Pentatomoidea*

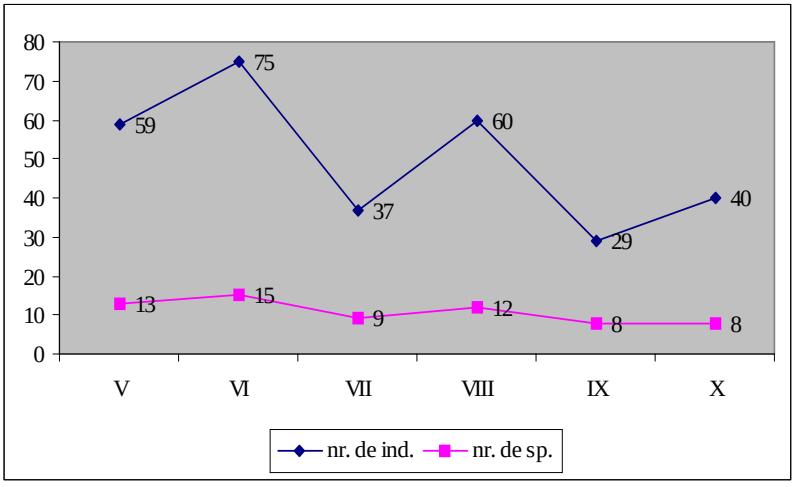
	Ecosistemele şi staţiunile studiate																		
	I	II	III		IV		V	VI		VII				VIII		IX			
	1	3	4	5	4	5	1	2	3	1	2	3	4	5	1	1	2	4	5
H’	0,92	0,76	0,53	0,47	0,60	0,49	1	0,76	0,79	0,69	0,59	0,53	0,35	0,27	0,76	0,65	0,73	0,72	0,46
E	0,92	0,97	0,89	0,99	0,77	0,82	0,83	0,9	0,87	0,82	0,76	0,76	0,42	0,91	0,76	0,83	0,80	0,85	0,98

Abrevieri: E - Indicele de echitabilitate. H’ - Indicele de diversitate Shanon-Wiener. I-IX - Tipurile de ecosisteme studiate. 1 - Moldoveneşti. 2 - Mărtineşti. 3 - Cheile Turenilor. 4 - Luna. 5 - Gura Arieşului.

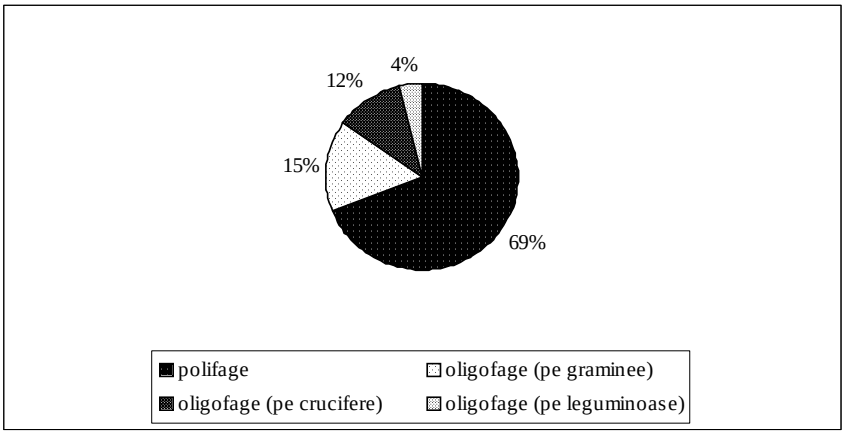
Tabel 7

Dinamica populaţiilor de *Pentatomoidea* în ecosistemele studiate în zona localităţii Moldoveneşti

Perioada colectării	Făgeto-cărpinet		Fânaţ mezofil		Păşune		Cultură de lucernă		Vegetaţie ruderală		Numărul total	
	nr. ind.	nr. sp.	nr. ind.	nr. sp.	nr. ind.	nr. sp.	nr. ind.	nr. sp.	nr. ind.	nr. sp.	ind.	sp.
Mai	3	3	40	11	2	1	13	7	1	1	59	13
Iunie	19	6	16	7	15	4	16	4	9	4	75	15
Iulie	15	4	11	4	2	2	5	3	4	2	37	9
August	13	4	20	5	5	3	19	5	3	2	60	12
Septembrie	4	2	9	4	8	3	7	3	1	1	29	8
Octombrie	10	3	15	7	2	2	10	4	3	2	40	8

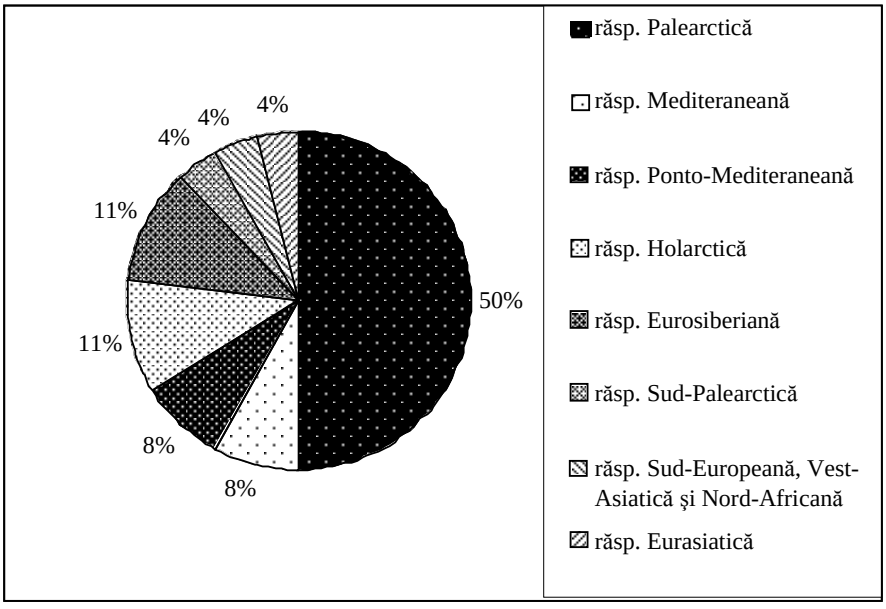


F i g. 2. *Dinamica suprafamiliei* Pentatomoidea *în zona localităţii Moldoveneşti.*



F i g. 3. *Spectrul trofic al pentatomoideelor semnalate în sectorul inferior al Bazinului Arieşului.*

Analiza zoogeografică. Din punct de vedere al spectrului zoogeografic cel mai bine reprezentate sunt speciile cu răspândire Palearctică (13). Au fost semnalate şi specii cu răspândire Mediteraneană (2), Ponto-Mediteraneană (2), Holarctică (3), Eurosiberiană (3), Sud Palearctică (1), Sud Europeană, Vest Asiatică şi Nord Africană (1) şi Eurasiatică (1) (Fig. 4).



F i g. 4. *Spectrul zoogeografic al pentatomoideelor semnalate în sectorul inferior al Bazinului Arieşului.*

Concluzii. 1. Din sectorul inferior al Bazinului Arieşului am colectat 26 specii de *Pentatomoidea* aparţinând la 19 genuri şi 4 familii. Atât din punct de vedere al numărului de specii, cât şi al numărului de indivizi, cel mai bine reprezentată este familia *Pentatomidae*.

2. Specia *Sciocoris (Sciocoris) deltocephalus* este citată pentru prima dată în fauna Transilvaniei, iar trei specii citate sunt sporadice în fauna României: *Podops inuncta, Stagnomus (Dalleria) pussilus* şi *Eurydema ventrale*.

3. Numărul cel mai mare de specii s-a înregistrat în ecosistemele ierboase şi scade odată cu scăderea cantităţii de vegetaţie ierboasă, reducerea numărului de plante prezente şi creşterea influenţelor antropice în ecosistemele studiate.

4. Cele mai ridicate valori ale abundenţei numerice şi relative au fost înregistrate în ecosistemele ierboase de către speciile *Coptosoma scutellatum, Aelia acuminata, Dolycoris baccarum* şi *Eurydema oleraceum.*

5. Diversitatea cea mai ridicată s-a înregistrat în fânaţul mezofil din staţiunea Moldoveneşti, iar cea mai scăzută în păşunile din Gura Arieşului şi Luna.

6. În dinamica populaţiilor de *Pentatomoidea* s-a înregistrat o variaţie a numărului de indivizi şi de specii pe parcursul anului, atingând maximul în luna iunie.

7. Din cele 26 de specii, 69% sunt polifage, iar 31% sunt oligofage. Nu a fost semnalată nici o specie monofagă.

8. Din punct de vedere zoogeografic, predomină speciile cu răspândire Palearctică, urmate de speciile cu răspândire Holarctică şi de cele cu răspândire Eurosiberiană.

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BREEDING BIOLOGY OF THE GOLDEN EAGLE (*AQUILA CHRYSAETOS CHRYSAETOS* L. 1758*)* IN THE GORGE OF TURDA NATURE RESERVE

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SUMMARY. - The paper presents the results of the research regarding the biology and ethology of the Golden Eagle (*Aquila chrysaetos chrysaetos)* during the breeding season. The Golden Eagle is an extremely rare species in Romania as well as in its whole spreading area, due to the loss of its specific habitat and excessive hunting. Protecting this species requires, apart from the legislative measures, the knowledge of its biological features. The present study took place between 2000 and 2003 in the Gorge of Turda Nature Reserve and its main objective was to analyse breeding biology of this species. The observations were carried out in 140 days (70 days in the year 2000, 50 days in 2001 and 10 days in 2002 and in 2003). In 2000, the behaviour of the adults as well as that of the hatchlings were monitored during the whole breeding season. In 2001, great attention was paid to the brooding biology and in 2002 and 2003, the main objective of the investigations was the breeding success. In each of these 4 years, two eggs were laid at 3-day intervals. Both partners take part in the brooding, but in different proportions, the main role belonging to the female (67.53% in 2000 and 70.7% in 2001). The incubation lasted for 42 days. In 2000, in the fledging period, the male provided food for the family in the proportion of 85.24%. As a result of the competition between the hatchlings, in three of the four seasons the younger hatchling died about one month after hatching. The duration of fledging period was 65 days in 2000 and 64 days in 2001. Totally, the breeding success was of 62.5%. Knowledge of the ecological requirements and of the reproductive features of the Golden Eagle is a very important step for working out the long-term conservation strategies that are so important for the survival of this species.

The Golden Eagle is the biggest *Aquila* species and has the largest spreading area among the species of this genus. This area comprises the whole Holarctic, where the species is represented by 6 subspecies. The nominate *Aquila chrysaetos chrysaetos* (L i n n a e u s, 1758) is spread in the whole Europe, except for the Iberian Peninsula (where it is replaced by *A. c. hommeyery* (S e v e r t z o v, 1888), which also reaches into North Africa, Asia Minor and Iran) and it reaches, in the East, well into Siberia and the Altai Mountains. In Turkestan, Manchuria, south-western China, northern India and Pakistan *A. c. daphnea* (S e v e r t z o v, 1888) can be found, and in the western part of Siberia, of the Altai Mountains and in Kamtchatka the

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subspecies *A. c. kamtchatica* (S e v e r t z o v, 1888) is present. The smallest of the subspecies, *A. c. japonica* (S e v e r t z o v, 1888) lives in the Korean Peninsula and the Archipelago of Japan, while *A. c. canadensis* (L i n n a e u s, 1758) is present on the North American continent[**].

The mountainous regions represent the most typical habitat of this species; it can rarely be found in depressionary forests and in wet lowlands. For nestling, it needs inaccessible places and uses cliffs and sea walls for this purpose. In Great Britain, this species was identified brooding between the altitudes of 16 and 900 m [20]. In the Alps, the nestling territories are situated between 1000 and 2460 m, exceptionally at 3000-m altitude [7] and in the Caucasus Mountains, nests were seen at altitudes between 1200 and 2400 m [8].

The trophic range is very varied, the diet of the Golden Eagle containing small and middle-sized mammals (*Lepus, Capreolus, Sus scrofa* or *Alces alces* youngs), as well as middle-sized and large birds (*Tetrao, Lyrurus, Phasianus*) and even reptiles (tortoises). Studies carried out in captivity have shown that the food requirements for an adult Golden Eagle is around 309 g of food/day for a female and 260 g of food/day for a male [11]. Such studies have also been done in Scotland, involving free individuals. These consume, on the average, 200 g of food/day, resulting that the annual necessity is around 84 kg/individual. By considering the losses too, it has been estimated that an adult Golden Eagle captures 214 kg of prey each year [5].

So, the Golden Eagle needs a large amount of food, when the efficiency of prey capturing does not exceed 30% [1]. Any qualitative and quantitative changes in the structure of their specific habitat can lead to the decrease of their chance of survival and breeding success.

In the first seven decades of the last century the number of individuals of this species has decreased at an alarming rate in the whole spreading area, mainly due to anthropic causes, such as habitat loss, excessive hunting and the use of intended carcasses poisoned with strychnine for the control of carnivorous mammals. In France, in this period, the number of Golden Eagle has decreased by about 70%, only 60 breeding pairs remaining [31]. A similar situation was recorded in Germany, where at the beginning of the seventies only 15 pairs were still breeding [12] and also in Great Britain, where in the sixties about 3000 pairs were breeding [9].

As a result, this species was put under protection. At this moment, due to the Appendix no. 3 of the Bern Convention, the Golden Eagle is a strictly protected species and the Appendix no. 1 of the Birds Directive [32] shows that the Golden Eagle is a priority species for conservation in the whole Europe. Under these conditions in some of the countries of Europe, the species effective became stable and even started to grow. For example, in France, the present number of Golden Eagle is estimated to be around 288 breeding pairs, in Germany 50 breeding pairs and 422 pairs breed in Great Britain [15]. The most important effectives are presently in Turkey, where 3000 pairs are breeding, in Spain (1100 breeding pairs) and in Norway, where the effective of this species is estimated to be about 1000 pairs [15].

[**] L i n n a e u s (1758) and S e v e r t z o v (1888) are cited based mainly on the review published by [7].

At the beginning of the last century, in Romania, the Golden Eagle was a widespread species. D o m b r o w s k y used to say ”in the Carpathians of Romania the Golden Eagle is a frequent species” [10], but in the 50’s the situations appeared to have changed: “the king of the birds and of the sky has become such a rare bird that I would be optimistic to estimate to about 50 the number of breeding pairs of Golden Eagle on the territory of R.P.R.” [17]. The present data regarding the situation of this species in Romania are relatively few. At the beginning of the 90’s, the whole effective of this species was estimated to be around 10 pairs [6], and 10 years later, the effective was estimated to be around 30-40 breeding pairs [21].

By joining the Bern Convention [33] and by issuing Law no. 462 [34], Romania made important steps towards the conservation of this species in its wild fauna. Although the anthropic factors have played and are still playing a decisive role, the decline of the Golden Eagle can not be explained only from this point of view. It is possible that the bahavioural particularities of the species combined with these anthropic factors could have lead to the present situation. For the application of protective measures, it is necessary to know the ecology and ethology of the species involved.

The Golden Eagle, heraldic symbol of the Romanian State, has not yet been studied in Romania from a behavioural point of view, mostly due to the difficulties raised by this kind of study. By taking advantage of the specific conditions of the Gorge of Turda, favourable for this purpose, the present study is a first step in this direction. The data about the presence of the Golden Eagle in this region are sporadic. N y á r á d y mentioned it for the first time in 1937 [23]. One year later, Ş e n c h e a reports once again the Golden Eagle [26], then K o h l in 1969 [16] and R ă ş i n a r u in 1995 [24]. None of these authors dealt with the behavioural aspects of the life of Golden Eagle.

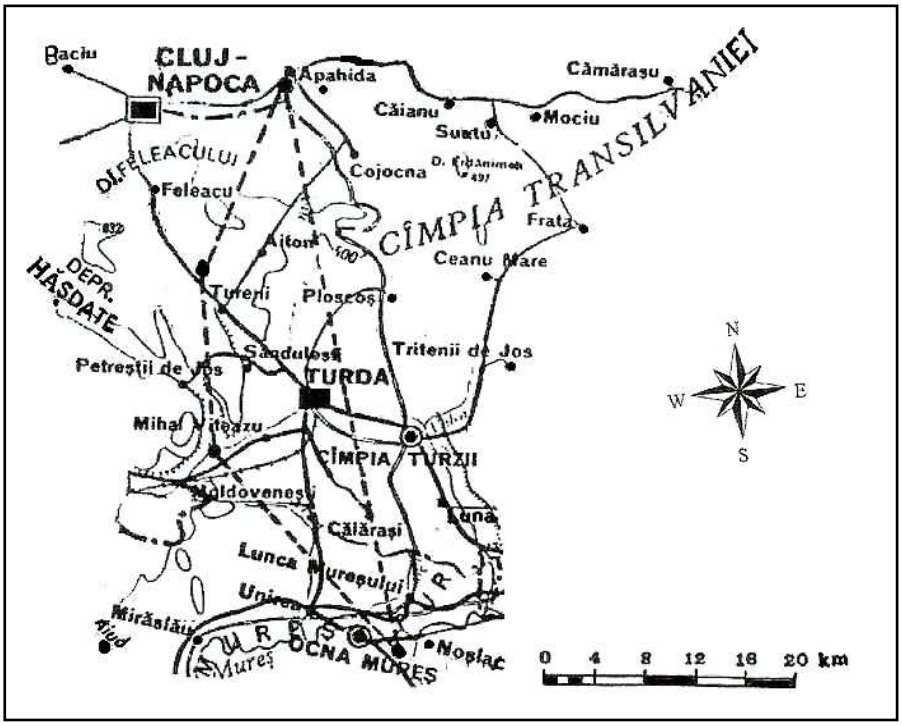
Methods. The observations were carried out in The Gorge of Turda Nature Reserve between the years 2000 and 2003. The one Golden Eagle (*Aquila chrysaetos chrysaetos*) pair that living here, was monitored by the direct observation method using for this goal a Nikon 26X45 field scope.

In 2000, the observations covered the entire breeding season. They began on 18 of February and continued weekly until 1 of July, totalling 70 days. In 2001, the observations began on the 15 of February and followed the same pattern until 28 of May (31 days) focusing upon the brooding biology. Also, during the 19 days, observations were done upon fledging period and the relationship between hatchlings until the death of the younger one. In the years 2002 and 2003, the breeding success was the main aspect studied.

Results and discussions. *The territory.* L o r e n t z defined territory as “the space protected by an individual, into which other individuals of the same species are not allowed to enter” [18]. The existence of the territory confers maximal advantages to the species. The chance for the congeners to meet is reduced to the

minimum, so that the intraspecific competition is attenuated, the efficiency of space usage is increased, the reproductive chance is also increased and the energy of the couple is economised in order to be channeled strictly towards breeding success.

The surface of the territory varies much among the different species. The existing trophic resources condition it. Some observations carried out during 2000 and 2001 allow the estimation of the territory surface of the studied Golden Eagle pair. Thus, on the day of 19th of April 2000, in full breeding season, the male was seen in a tree at the skirt of a forest close to the village of Micesti. Eight days later, on the 27th of April 2000 the same male was seen flying above the grazing grounds near the village of Apahida. Characteristic of this male in the mentioned period was the lack of the third secondary remige. On the 20th of June 2000 the female was seen on the Ciucaş Hill, near Cornești village (about 12 km downstream on the Hăşdate, towards southeast). The last observation of this kind was done on 18th of May 2001 at Ocna-Mureş, where the male was sighted flying above the moorland of the Mureş, which accounts for a surface of about 300 km^2 (Fig. 1).



F i g. 1. *Presumptive limits of the Golden Eagle pair territory.*

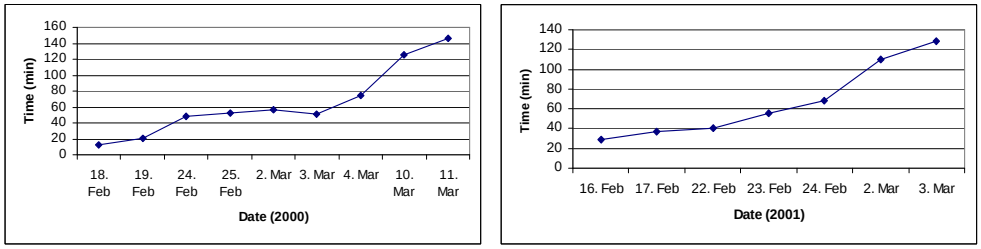
Being top predators, the Golden Eagles have very large territories, the surface of which is between 50 and 200 km^2 and in some regions that are less suitable for the species like the Apennine Mountains this surface can exceed 300 km^2 [14].

This kind of territory in which Golden Eagles perform all their vital activities is called reproduction territory [3] and it is made up of two components: the vital range and the feeding range and there is contiguity between these two components. The establishment in time of the two components is done gradually and it is achieved in the moment when the eggs are laid and the nest becomes the biological centre of the territory.

Between November and January 2000–2002, the Golden Eagles were missing from the Gorge of Turda. Fidelity towards the territory is a characteristic feature of this species. They leave it only when food becomes insufficient. Starting from the middle of February the couple comes back into the territory. This is the moment when they have to consolidate their position as owners of the territory and to decide upon the nest that they would use.

The most usual way for marking the territory is based on optic signals, given the extraordinary eyesight of the eagles. These signals are represented by the presence of one or both partners flying above the territory.

In order to investigate the couple's return to the nest, observations were done for a period of 9 days in 2000 and 7 days in 2001. The increasing periods of time spent by the partners flying above the territory was done gradually (Fig. 2).



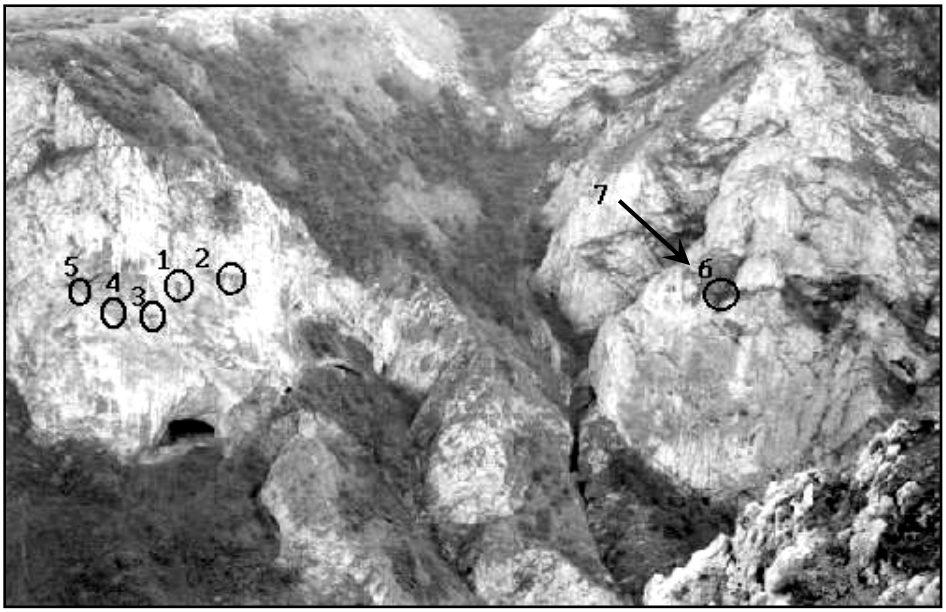
F i g. 2. *Dynamics of the presence of Golden Eagle in the Gorge of Turda before the laying period.*

Preparing the nest for laying the eggs. In the Gorge of Turda, the zone of minimal altitude in Romania (about 500m) where the Golden Eagle breeds, there are 7 nests, all of which belong to the studied pair. Five of them are situated on the "Peretele Vulturilor" and other two on the "Colțul cel Lat" (Fig. 3).

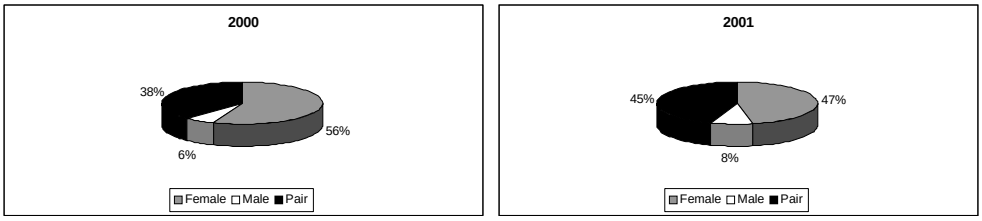
Supernumerary nests are a characteristic of the species; the average being of 5.3-nests/pair [28] and the reported maximum number is 12-nests/pair [27].

The nests are used in turn. The Golden Eagles do not hatch two consecutive years in the same nest. This is an efficient adaptation for diminishing the attack of ecoparasites (*Mallophaga*), the reproductive cycle of these being synchronised with emergence of the hatchlings [30].

After the adults return to the territory an extremely important first step is choosing the nest into which the eggs are to be laid, the decisive role belonging to the female. This aspect result from the longer time spent by the female at the nests (Fig. 4).



F i g. 3. *Position of the nests of the studied Golden Eagle Pair.*



F i g. 4. *Proportion of the presence of partners at the nests before the laying period.*

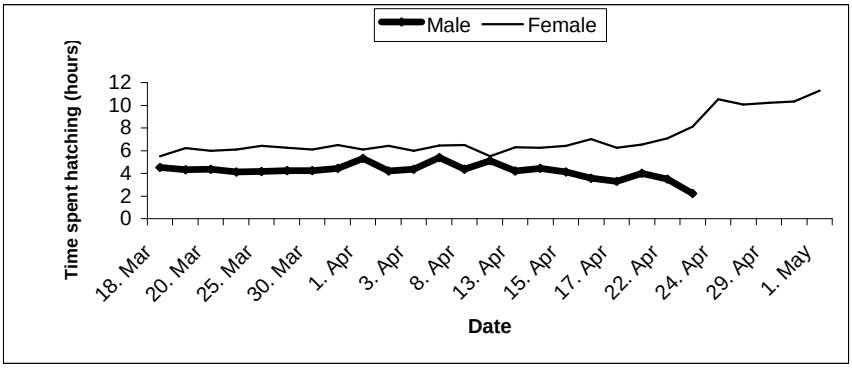
In each year, before laying the eggs, the couple rebuilt two or three nests and finally one of them was chosen. Thus, by the multi-year use of the nest, some of them acquired impressive dimensions. The dimensions of nest no. 1, the oldest in the area, can be estimated to 4 m in height and 2.5 m in diameter, with a volume of about 7 m^3. Nests with a diameter varying between 1.5 and 3 m and 2 to 4 m in height, having a volume of up to 6 m^3 are reported in the literature [4]. Probably, these are used in successive generations (the nest no. 1 was photographed by N y á r á d y in 1939 [23]).

From the seven existing nests, in the year 2000 the female rebuilt nests no. 1, 2 and 3, finally choosing nest no. 2 and in 2001 fresh vegetal materials (pine and spruce branches) was seen in nests no. 3 and 6, the eggs being laid in nest no. 3.

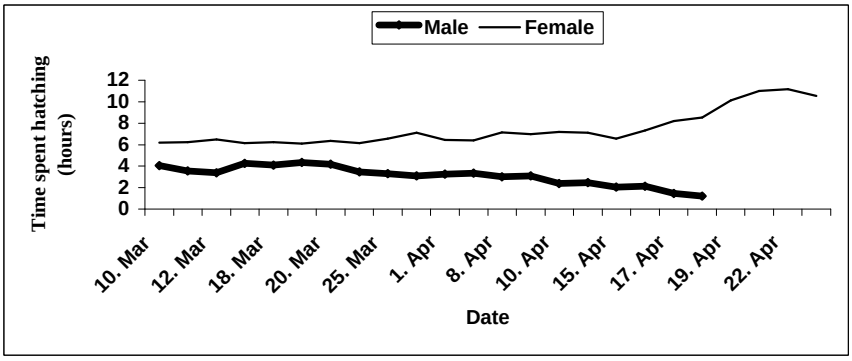
Laying and brooding biology. In each of the studied four breeding seasons the female laid two eggs. In the year 2000, the first egg was laid on the 18th of March and in 2001 on the 10th of the same month. This 8-day delay probably was due to higher temperatures in 2001 as compared to 2000. In 2000, the second egg was laid on the 21st of March; between the layings of the two eggs there was a delay of about 76 hours. In 2001, the second egg was laid on the 13th of March at an interval of 73 hours after laying of the first egg. The laying of the Golden Eagle is most often composed of two eggs, rarely one egg and only in exceptional cases, of three eggs. In Scotland, from 82 studied nests, 18% contained one egg, 72% contained two eggs and only 10% of them containing three eggs [13]. Between the laying of the first egg and that of the second there is a delay of three or even of four days [2].

Brooding began with the laying of the first egg, the incubation period lasting for about 42 days both in 2000 and in 2001. Observations upon this aspect have been done for a period of 27 days in 2000 and for a period of 24 days in 2001.

Both parents took part in brooding, but in different proportions, the main role belonging to the female (Figs. 5 and 6).



F i g. 5. *Time spent brooding by the two partners in 2000.*



F i g. 6. *Time spent brooding by the two partners in 2001.*

Thus, during the daytime, the female insured brooding in the proportion of 67.53% in 2000 and 70.7% in 2001. The observations have shown that during the night only the female spent the night at the nest brooding. The time spent by male brooding decreased gradually toward the moment of hatching. In 2000, the male was not seen brooding for about 40 hours before the hatching of the first young and in 2001 for about 35 hours before hatching. Also, the male no longer brooded in the interval between the hatching of the first and of the second young. During the whole period of brooding both adults got their food independently. The participation of both partners to brooding is common to many species of eagles and hawks, including the representatives of the genus *Aquila* [22], but in that case the female's contribution to brooding is more significant than that of the male [2, 20].

The ethogram of the shifts of partners at the nest. The partners shift is done in a ritual manner and it is a consequence of the rythmicity of behavioural acts. In order to know when the partner who is to be changed is prepared to do it, the other partner provokes an answer on the question: will it take over the brooding or not. In getting close to the nest and sitting onto the eggs, the Golden Eagle makes a series of characteristic movements (Table 1).

Table 1

Ethogram of the shifts of partners at the nest

Male		Female	
1.	Appearance closer to the nest.	2.	Optically received signal, change of position form normal to attentive (stretched neck).
3.	Flying in the front of the nest and emitting the sound signal.	4.	Acts of shifting the surroundings objects. Usually, branches are taken out from one part of the nest and put in another part. Response to the sound signal.
5.	Landing on the edge of the nest.	6.	Cleaning the feathers by means of the beak, followed by the repeated shakings of the feathers and stretching of the wings.
7.	Getting close to the eggs, bowing of the head and pulling it between the shoulders.	8.	Taking off in flight.
9.	Rotating the eggs.	10.	Flying in large circles in front of the nest.
11.	Binding of the legs and sitting on the eggs.	12.	Flying in larger and larger circles and away to the feeding range.
13.	The normal brooding position, followed or not by sleep.		

Note: in the case of changing the male by the female the procedure is reversed.

The ritual of the shifting of partners at the nest is a mixt one, a combination of clock, optical and sound ritual [25]. The most important stimuli that unleash the ceremony shifting are the optical ones. Being birds with small chances of obtaining their food in equal time limits (the smaller size of the male confers greater agility and thus greater chances to capture the prey) and in short time (usually these last

for hours, in which smaller or greater distances are covered), they would shift at the nest only when the partner in search for food, appears around the nest. The clock ritual, characteristic to grain feeding birds, for example, cannot be respected due to these reasons. Nevertheless, the time allocated by the individual for brooding (with few exceptions in unfavorable weather conditions) is relatively well delimited and respected as it is.

As a result of the observations in 2000 and 2001, an average of about 3 hours and 10 minutes elapsed until the replacement of the female by the male and an average of about 1hour 20 minutes was necessary until the replacement of the male by the female. The attempts to replace the brooding partner under the mentioned time limit, that is, without having spent a certain number of hours, did not have positive results in replacement of the partner.

These data only account for the rigid mathematical value of the process. In reality, there have been situations in which, because of the unfavorable weather conditions, the period allocated to brooding by one individual was prolonged to 5 hours, in the case of both partners.

There is also the possibility of replacing the partners at the nest without any ceremony. This happens when one of the partners delays its arrival with much time over the average allocated to brooding by its partner, in favorable weather conditions. So, the time factor has deeper implications in the ethology of the Golden Eagle for the given sequence, probably with more complex origins in the phylogeny of the *Falconiformes* order itself. The acoustic signals seem to be of secondary importance.

The hatching. In 2000, the hatching of the first young took place on the 28th of April and that of the second on the 1st of May, at an interval of about 72 hours. The same interval was kept in 2001, when the first hatchling emerged on the 20th of April and the second on the 23rd of April.

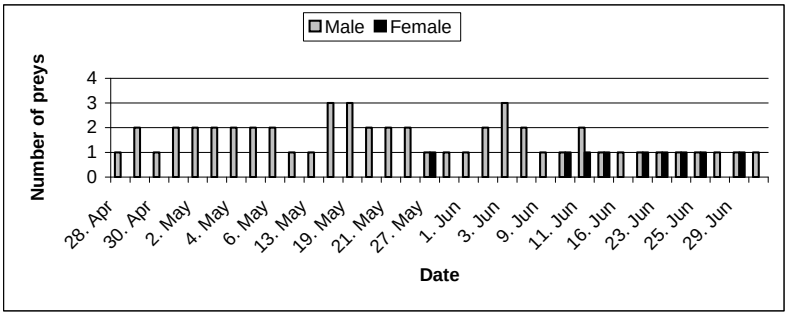
Rotating the eggs stopped completely with about two days before the hatching. In this period of time the male was not observed brooding, although this made short visits to the nest, bringing fresh vegetal material, which the female arranged in the nest. In all the cases in the moment of hatching only the female was present to the nest. It does not have a typical bahaviour during the hatching, although the phenomenon is indicated in its phenology by its almost imperceptible rising from the eggs and the shift stretching of the wings. The hatchlings emerged by themselves, without help of the female.

In many of the species, the shells of eggs are immediately removed after hatching [25], because the intense heat leads to the drying and intense whitening of these and, thus, the presence of the nest may be revealed. In the animal world, white as well as red has signaling significance. This behaviour was not observed in this case. The female has consumed most of the shells in order to restore the calcium level in blood.

Roles of the parents during the fledging period. In 2000, this period lasted for 65 days and for 64 days in 2001. Regular observations were done only in 2000 for a period of 34 days (53.30% of the total). Depending on the conditions the duration of fledging period can vary between 64 and 70 days [2, 7, 20].

In this period the roles of the two adults became distinct, being superposed only to a small extent. The male provided food for the entire family in the proportion of 85.24%. The female hunted for the first time 35 days after hatching, her contribution to providing food being of 14.76%. Warming and feeding the hatchlings were provided only by the female.

Most of the preys were captured and brought to the nest in the first half of the day and their number varied between one and three preys/day. The maximum number of preys was brought to the nest in the middle of the fledging period (Fig. 7).



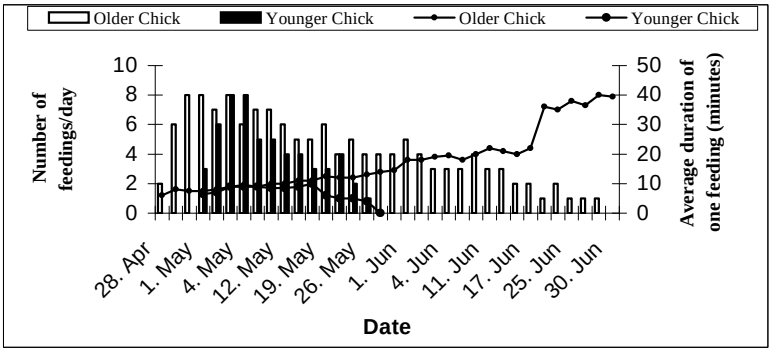
F i g. 7. *Number of preys captured by the adults in 2000.*

The female participated actively to providing food only after the hatchling has achieved the age of 35 days, when the food brought by the male was not satisfying any more the nutritional requirements of the hatchling. In this period the hatchling had its plumage already formed so it was not necessary any more to be warmed by the female. Until this moment, but to same, even later, the female fed from the prey captured and brought by the male.

The active participation of the female to providing food in the second stage of fledging period also explains the accentuated dimorphism in favour of the female. The smaller size of the male confers him greater agility, so that he is capable of capturing more abundant small-sized prey. The larger size of the female deprives her of the agility of the male and does not confer her the possibility of capturing this type of prey, but she is capable of hunting large-sized prey, which can provide food to her and hatchling for a longer period [29].

As for the male, the time not allocated to hunting was used in the proportion of about 70% for the grooming behaviour.

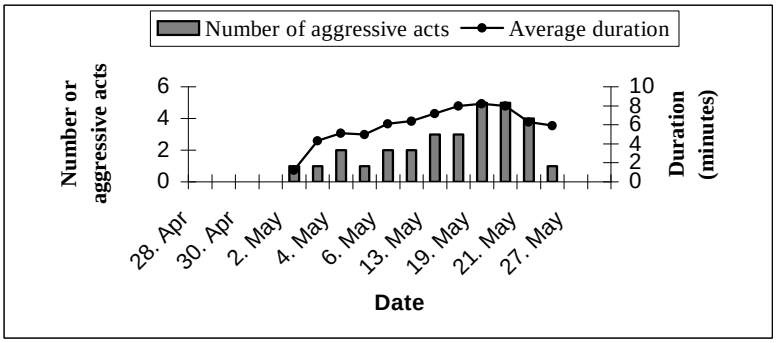
The active feeding for the hatchlings were exclusively the duty of female. They were fed for the first time about seven hours after hatching. There is a correlation of inverse proportionality between the number of daily feedings and the average duration of one feeding (Fig. 8).



F i g. 8. *Number of daily feedings and the average duration of one feeding.*

Thus, in the first phase of fledging period, the hatchlings were feed many times (up to 8 feedings/day) and as they grew up, the number of feedings decreased (to 1 feeding/day). On the other hand, the average duration of one feeding was short (6 minutes) at the beginning of the fledging period and it was increasing gradually (until 40 minutes). Thus, one may conclude that as the hatchling advanced in age, the amount of consumed food increased. This aspect was valid for both of the hatchlings until a certain moment. The situation changed about three weeks after hatching of the younger chick. This died of hunger even in the period when the male brought the greatest number of preys to the nest, which means that the principal cause of its death was the strong competition exercised by its older brother. Although the older hatchling was capable of feeding itself about 30 days from hatching, the female continued to feed it until its leaving the nest.

The relationship between the brothers. Right after the hatching of the younger chick, an intense competition begins between the two brothers, materialised by aggressive acts of the older one upon its younger brother. These manifestations were observed for the first time in 2000, on the 2nd of May, when the older hatchling had the age of four days and the younger of one day. The aggressive acts continued with increased frequency close to the moment of the death of the younger hatchling (Fig. 9).



F i g. 9. *The relationship between number of aggressive acts and their average duration in 2000.*

It has been observed that with ageing the older hatchling manifestations of aggressivity upon the younger one became more and more frequent. The increase in the number of these manifestations was accompanied by the increase of their average duration.

The aggressive acts were observed before as well as during after feeding. The reaction of the younger hatchling always consisted in adopting a state of total immobility. After finishing the acts of aggression the older hatchling was always feed by the female. The younger chick was fed only during the sleeping period of the older one. In many cases its feeding was interrupted by a new series of aggressive manifestation of the older hatchling, which, in order to celebrate its "victory", consumed some more pieces of meat, after which it slept again. The female never intervened in the relationship between the youngs.

Starting with the 21st of May, about six days before the moment of death of the younger hatchling (the 27th of May), the number as well as the average duration of the aggressive manifestations decreased. On the 26th of May, the older hatchling was at the last capable of feeding itself. Although the difference of age between the hatchlings was only three days, the older hatchling was almost double in size.

Although between the years 2001-2003 no more regular observations were made, the aggressive manifestations of the older hatchling were present and they lead to the same final result: the death of the younger hatchling. The exception took place in 2003 when the younger one passed over the critical period and managed to survive.

The result of the aggressions upon the younger hatchling was its gradual physical exhaustion, which lead to its death. The death was caused by starvation, due to the competition exerted by the older brother and not due to lack of food. These results, evidentiate the reproductive strategy of the Golden Eagle. This produces two hatchlings every year only as a measure of safety for the case the older hatchling dies from different reasons (strong attack form various ectoparasites, its falling from the nest) [7, 12, 20].

Breeding success. In each of the four years of observations, the Golden Eagle pair produced 2 hatchlings. Due to the fact that in 2003 both hatchlings survived, the breeding success was of 62.5%. The survival of both hatchlings is extremely rare in the Golden Eagle, few such cases having been cited in literature [19, 30]. In Scotland, between 1964 and 1968, the breeding success was studied for 64 pairs of Golden Eagles and an average of 1.22 hatchlings/nest was fund, which means 61% breeding success.

About 100 days after leaving the nest the hatchlings become fully independent. After this period no juvenile or sub-adult has been seen at the Gorge of Turda.

Conclusions. 1. The breeding season for the Golden Eagle begins at the end of February by the gradual return of the couple to the territory, to which they show a great fidelity, the same couple being studied in each of the four seasons.

2. The vital space of the couple is located in the Gorge of Turda, where the seven nests have been identified. The total surface of the territory, including the two components, was estimated to be about 300 km^2.

3. After the couple's return to the territory, three nests were rebuilt in 2000 and two in 2001. The role of choosing and rebuilding the nests belonged to the female.

The nests were used in turn; the couple did not brood in two consecutive years in the same nest.

4. Two eggs were laid in each of the four years. In 2000, between the moment of laying the first egg and the second one there was a period of 76 hours, and in 2001 a period of 73 hours.

5. Brooding the eggs started with the laying of the first egg and was provided by both partners, but in different proportions. In 2000, during the day, the female did the hatching in the proportion of 67.53% and in 2001 in the proportion of 70.7%. At night, the female was always the one who hatched. The brooding period has lasted for 42 days in both 2000 and in 2001.

6. Replacing the partners at the nest is ritualized and it is based mainly on optical signals. On the average, the female was replaced at brooding after a period of about 3 hours and 10 minutes and the replacement of the male occurred after 1 hour and 20 minutes.

7. The hatching was phased, the young chick hatched 72 hours after the older one. The fledging period lasted for 65 days in 2000 and 64 days in 2001.

8. During the fledging period the roles of the adults became distinct. The male got involved in providing food for the entire family and the female warmed and fed the hatchlings. The female hunted for the first time only one month after the hatching. The male was never seen feeding or warming the chicks.

9. In the first stage of the fledging period the hatchlings were feed often (8 feedings/day) and for short periods (about 6 minutes/meal). As they progressed in age, they were fed more and more seldom (1 meal/day) for longer and longer periods (up to 40 minutes/meal).

10. Right after hatching of the younger chick, a relationship of competition developed between the two brothers, materialised in aggressive manifestations of the older chick upon the younger one. These manifestations were seen mainly during feeding. Their frequency and duration increased in time resulting finally in the death of the younger hatchling.

11. In three of the four investigated seasons only the older hatchling survived. In the year 2003 both hatchlings survived. The breeding success during the four seasons was 62.5%.

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PEROXIDASE ACTIVITY, STOMATAL CONDUCTANCE AND CARBOHYDRATE METABOLISM IN SOYBEAN PLANTS UNDER HEAVY METAL STRESS

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SUMMARY. – The influence of heavy metals from mine spoils (Cavnic, Baia Mare mining area, Romania) on the guaiacol peroxidase (GPOD) activity, stomatal conductance and the content of carbohydrates in soybean leaves was studied. Soybean plants (*Glycine max* cvs. Agat and Diamant) were grown under field conditions in the following variants: control (unpolluted soil), 50% spoils+50% unpolluted soil, 100% spoils. The activity of GPOD increased in the leaves of both cvs. of soybean plants grown on 100% spoils and decreased in the plants of both cvs. cultivated on 50% spoils. Plants grown in spoil variants showed a decrease in stomatal conductance. The decrease was higher in plants from 100% spoil variant for both cultivars. For both cvs., the content of reducing carbohydrates and disaccharides increased in plants cultivated on 50% spoils and decreased in plants from 100% spoils, as compared to control, whereas the amount of starch diminished in plants from 50% spoil variant and increased in plants from 100% spoils, as compared to control. The results obtained suggest that heavy metals from mine spoils can induce oxidative stress in soybean plants, affecting the plant metabolism.

Reactive oxygen species (ROS) are generated in normal cell metabolism and their regulation is a common cellular event, but under stress conditions (high and low temperatures, high light intensities, UV irradiation, drought and salt stress, exposure to pollutant gases, herbicides, metal toxicity, mechanical and physical stress, pathogen attack, senescence) their production is increased, resulting in oxidative damage [8, 21]. Besides exacerbating cellular damage, ROS can act as signal molecules during the acclimation of plants to stress, mark the formation of tracheary element, lignification and other cross-linking processes in the cell wall [8, 34, 40]. At low concentrations, ROS induce defence genes and adaptative responses, but at high concentrations, cell death is initiated [40].

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One of the causes of heavy metals toxicity in plants is the production of ROS [2, 9, 15, 19, 35, 44], which can react with lipids, proteins, pigments and nucleic acids. ROS are involved in senescence of cells and organs such as leaves and the appearance of chlorosis and necrosis of leaves as visual symptoms of heavy metal toxicity is often related to elevated levels of harmful oxygen species, which inhibit the export of photosynthates and cause the accumulation of large amount of starch and sucrose in leaves [20].

Heavy metals can induce oxidative stress directly (Cu, Fe) by generating ROS via the Fenton-type reactions or indirectly (Cd, Zn) by producing the degradation of chlorophyll and carotenoids or the inhibition of their biosynthesis [28], leading to disturbances in the electron transport rates of photosystems and, consequently, to the generation of ROS [33], or by inducing lipoxygenase, which produces $O_2^{\bullet -}$ by oxidising NADPH [31].

H_2O_2 is generated in various cellular compartments: chloroplasts, mitochondria, peroxisomes, cytoplasm, at the plasma membrane level or extracellularly in the apoplast [8, 12, 40]. Unlike other reactive oxygen species, H_2O_2 is relatively stable and able to diffuse across cell membranes, allowing the interaction between different scavenging systems, even those located in separate organelles [10]. The toxicity of H_2O_2 itself is relatively weak compared with that of other reactive oxygen species, but in the presence of superoxide, H_2O_2 can generate highly reactive hydroxyl radicals ($OH^{\cdot}$) via the metal-catalysed Haber-Weiss reaction. $OH^{\cdot}$ can potentially react with all biological molecules, and because cells have no enzymatic mechanism to eliminate it, its excessive production leads ultimately to cell death [40, 43]. Thus, the scavenging of H_2O_2 in cells is critical to avoid oxidative damage [43].

Plants possess a range of antioxidative defense systems for detoxification of ROS, including several enzymes and metabolites: superoxide dismutases, which catalyse the dismutation of $O_2^{\bullet -}$ to H_2O_2, as well as catalases and peroxidases, which convert H_2O_2 to H_2O and O_2, ascorbate, glutathione, tocopherol, carotenoids, flavonoids etc. [8, 9, 20, 34, 43].

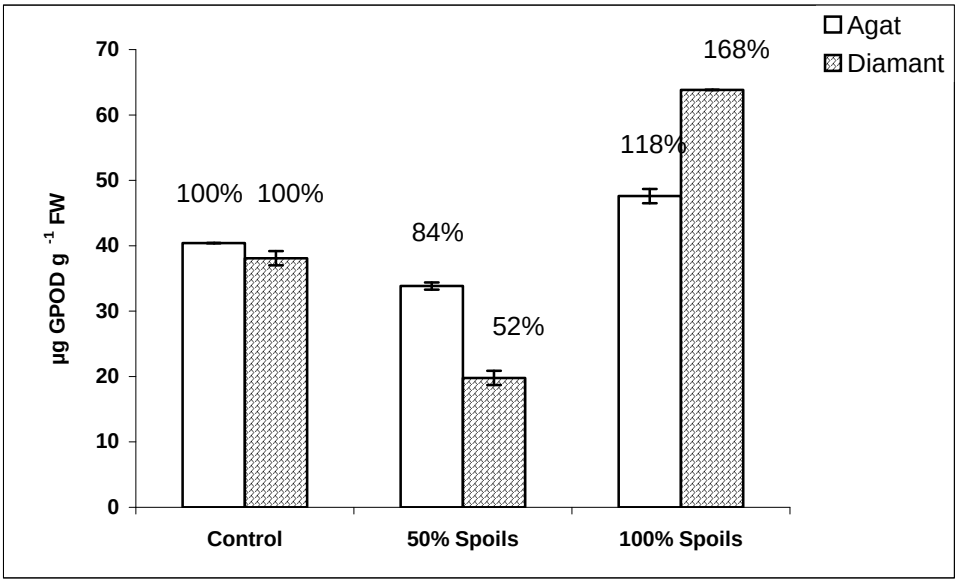
Peroxidases are oxidoreductases that catalyse the oxidation of a diverse group of organic compounds using hydrogen peroxide as the ultimate electron acceptor [32]. They are widely distributed in the plant kingdom, highly specific for H_2O_2 as electron acceptor and less specific for the donors, which can be ascorbate, pyrogallol, guaiacol etc. [24]. Peroxidases are generally considered to be the most sensitive indicators of pollutants in the absence of visible injury. The most commonly observed response of peroxidases to pollution is an increase of their activity [14].

Heavy metals can affect stomatal behaviour, that controls both transpiration and photosynthesis in plants, indirectly by generating ROS, which are involved in the regulation of stomatal movement [17, 21, 33, 45], or directly by the action of toxic metals at the guard cell level [30].

The purpose of this study was to investigate the effects of the heavy metals from mine spoils on the peroxidase activity, stomatal conductance and the content of carbohydrates in the leaves of soybean plants.

Materials and methods. The plant used in the present study was soybean, *Glycine max* (L.) Merrill. The plants of two soybean cultivars, Agat and Diamant, were cultivated under field conditions. The experiment was described in a previous paper [29]. The activity of guaiacol peroxidase (GPOD) was determined according to B o u c h e t *et al.* [3]. The extraction of peroxidase was carried out by grounding 500 mg of fresh leaves with quartz sand, in the presence of K-phosphate buffer (pH 6.1). The extract was centrifuged for 30 minutes at 5000 rpm. For the enzymatic determinations, the supernatant was used. The formation of tetraguaiacol was monitored spectrophotometrically (UNICAM SP1800), at 420 nm and 45 seconds after the initiation of reaction. The activity of GPOD was expressed as μg g^{-1} fresh weight. The stomatal conductance was measured with a porometer (AP4 Delta T, Cambridge) and expressed as mmol water vapour $m^{-2}s^{-1}$. Carbohydrates were determined spectrophotometrically (UNICAM SP1800), at 660 nm [26, 36]. Reducing carbohydrates were determined from an aqueous extract and disaccharides were determined by the hydrolysis of this extract with HCl 2% for 5 minutes at 68-70°C. Starch content was obtained by the hydrolysis of dried vegetal material with HCl 1N for one hour at 100°C. The results were expressed as g 100 g^{-1} dry weight.

Results and discussion. The results presented in Fig. 1 show that the activity of guaiacol peroxidase (GPOD) increased in the leaves of soybean plants grown on 100% spoils by 18 and 68% of control in cv. Agat and Diamant, respectively, whereas in the plants of both cvs. cultivated on 50% spoils it decreased by 16 and 48% of control in cv. Agat and Diamant, respectively.



F i g. 1. *Guaiacol peroxidase activity in leaves of two soybean cvs., Agat and Diamant, as influenced by the heavy metals from spoils* (mean values ± SD, n=3).

With reference to an increase or a decline in the activity of GPOD in plants treated with heavy metals, variable responses have been reported [6, 19, 33, 38], likely due to the experimental conditions, which strongly differ from one to another. W e c k x and C l i j s t e r s [41] found that the activity of peroxidase was enhanced as a result of Cu toxicity, with a parallel decrease in the H_2O_2 level in the leaves of *Phaseolus vulgaris*. Z a c c h i n i *et al*. [44] reported an increase of peroxidase activity in maize callus treated with lead. H_2O_2 can induce defence or stress-related genes [10] and may play a signalling role during the acclimation of plants to stress [8, 25, 40]. The stimulation of peroxidase capacity in soybean plants grown on 100% spoils indicates increased levels of H_2O_2 generated by heavy metal toxicity and an attempt of plants to protect themselves against oxidative stress. However, the cellular antioxidative system of these plants might not have been completely successful since their growth and production of pods and seeds were reduced [27].

The increased activity of GPOD in plants cultivated on 100% spoils could be an indication of enhanced senescence caused by heavy metals [18, 33]. The decreased activity of GPOD in plants grown on 50% spoils might suggest not a weak antioxidative system, but the production of small amount of H_2O_2 in these plants, because their growth was stimulated compared to the plants cultivated on 100% spoils and even to control [27]. However, this is only a speculation, since the amount of H_2O_2 was not determined in our present study.

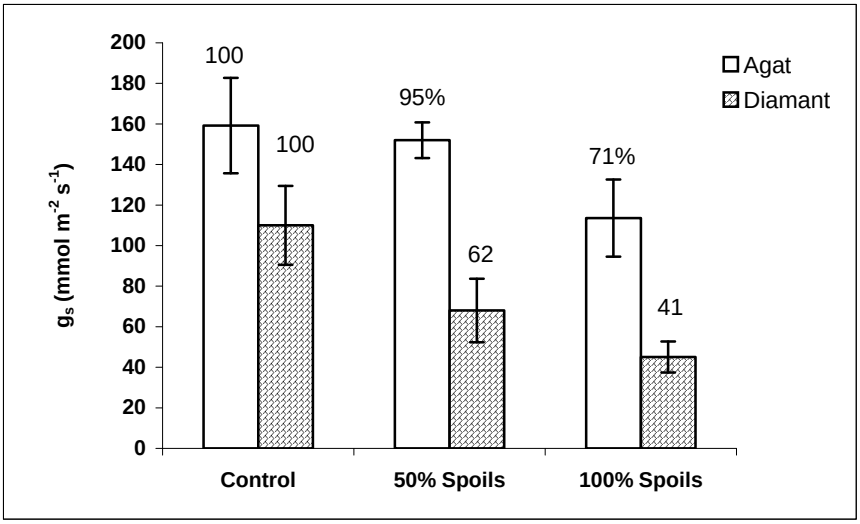
The peroxidase activity is higher in the leaves of cv. Diamant on 100% spoils as compared to cv. Agat and this could represent an appropriate protection against overproduction of peroxides [22] caused by the higher accumulation of heavy metals [29]. As in the case of other studied parameters [27], cv. Diamant appeared to be more tolerant to heavy metal toxicity and/or nutrient deficiency from mine spoils.

Y a m a s a k i and G r a c e [42] suggested that cations like Zn, Al and Cd could promote oxidative damage due to stabilization of phenoxyl radicals, which are formed by peroxidase-catalysed reaction between phenolics and H_2O_2 [37] and are toxic to living systems.

Heavy metals affect the stomatal physiology. Plants grown in spoil variants showed a decrease in stomatal conductance (Fig. 2). The decrease was higher in plants from 100% spoil variant for both cultivars. The leaf conductance in plants of Diamant cv. was lower as compared to that of cv. Agat in all the cultivation variants.

S a n d a l i o *et al.* [33] found that Cd stimulated stomata to close. Stomatal conductance may be decreased by a direct interaction of the toxic metals at the guard cell level [30]. P e r f u s - B a r b e o c h *et al*.[30] reported that Cd reduced stomatal conductance, leading to the decrease of CO_2 uptake, and did not significantly affect the photosynthetic apparatus, suggesting that Cd perturbed stomatal regulation and limited photosynthesis by CO_2 diffusion at low leaf conductance resulting from stomatal closure. The same authors concluded that the stomatal closure induced by Cd was independent of the abscisic acid (ABA)-related process and Cd did not block the K^+ channels in guard cells, but the Ca^{2+} channels in the plasma membrane of the

guard cells are highly permeable to Cd, suggesting that Cd could enter guard cells through Ca^{2+} channels, resulting in a perturbation of the intracellular calcium level and calcium signalling, a key element in guard cell osmoregulation.



F i g. 2. *Stomatal conductance in leaves of two soybean cvs., Agat and Diamant, as influenced by the heavy metals from spoils* (mean values ± SD, n=3).

Stomatal closure may be a consequence of toxic effects of heavy metals in various plant tissues, leading to decreased water availability in leaves and, finally, to decreased stomatal regulation [30]. Heavy metals inhibit the water transport to shoots, causing a water deficit in plants [7]. Under water stress conditions, ABA accumulates in leaf tissues, generating a net loss of guard cell turgour that leads to stomatal closure, thus reducing water loss through transpiration [13]. Z h a n g *et al.* [45] reported that H_2O_2 was involved in the stomatal closure induced by abscisic acid. The findings in our study, namely the increased activity of guaiacol-peroxidase, indicating a high amount of H_2O_2, and the decrease of stomatal conductance in plants grown on 100% spoils, suggest that ABA, the production of which is increased in plant tissues during heavy metal exposure [11], could have induced the stomatal closure in the leaves of soybean plants.

An increase in ABA content alters the metabolism of indole-3-acetic acid (IAA) and greatly decreases the content and proportion of free IAA, leading to a decrease in cell wall extension [20]. Guaiacol peroxidase is inactivated by IAA [1]. Enhanced synthesis and higher levels of ABA in roots and shoots are a typical response to water deficiency and also to nitrogen and phosphorus deficiency [20]. In our study, the activity of GPOD increased in plants grown on 100% spoils and also P [29] and N (data not shown) levels in 100% spoils were very low, suggesting that ABA might be involved in decreasing the stomatal conductance and also in the reduction of leaf expansion.

M c A i n s h *et al.* [21] and Z h a n g *et al.* [45] reported that H_2O_2 inhibited stomatal opening and promoted stomatal closure, in a concentration-dependent manner. At low concentrations, H_2O_2 induced changes in stomatal aperture that were reversible and due to increases in cytosolic free Ca^{2+} from guard cells induced by the oxidative stress. At high concentrations, the changes in stomatal aperture were irreversible, indicating a reduction in membrane integrity and guard cell viability. An increase in cytosolic free Ca^{2+} concentrations depolarises the plasma membrane, decreasing membrane potential and enhancing K^+ efflux, thus leading to loss in turgour of the guard cells and stomatal closure [20].

Stomatal closure is associated with K^+ efflux across the plasma membrane, which is preceded by an increase in cytosolic free Ca^{2+} and cytosolic alkalinisation in the guard cells, which occur in response to ABA [16]. Heavy metals and peroxide induce K^+ leakage which may occur through K^+ channels or through nonspecific lesions in the cell membrane caused by lipid peroxidation [23, 41]. However, K ö h l e r *et al.* [17] reported that H_2O_2 and ABA activated the same Ca^{2+} channel and in a similar manner, increasing the cytosolic free calcium, but H_2O_2 inhibited the outward-rectifying K^+ channels, while ABA activated them, suggesting that ABA and H_2O_2 pathways diverge further downstream in their actions on the K^+ channels and, thus, on stomatal control.

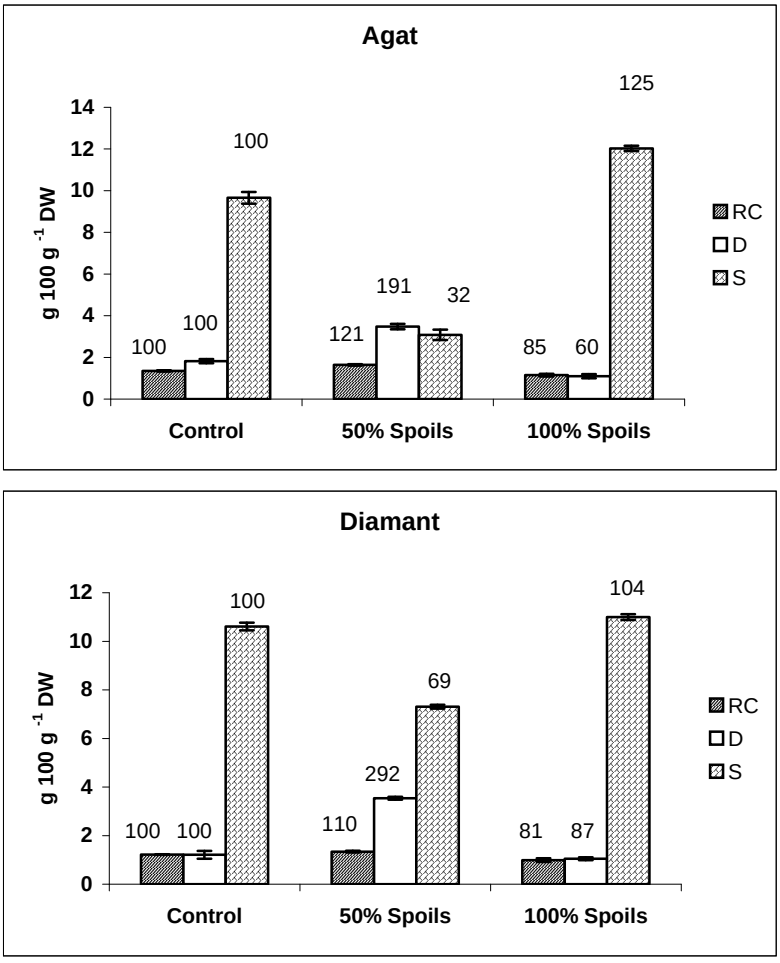
Stomatal closure inhibits the ability of plants to carry out photosynthesis; therefore a tolerance of plants to water stress can be an important factor affecting growth. The reduction in stomatal conductance accompanied by decreased levels of chlorophyll content in soybean leaves grown on 100% spoils [28] could cause a reduction of leaf photosynthesis and, consequently, a decrease in plant growth [27].

Heavy metals could also affect carbohydrate metabolism in plants. Carbohydrates are the primary products of biosynthesis processes in plants, out of which all the other substances are synthetised. They represent the source of energy and carbon needed for adaptative and/or defensive responses of plants to stresses. Sucrose is the main form in which the carbohydrates are transported in most species of vascular plants. It has an important role in protecting cells from water stress, participating in osmoregulation or protecting the macromolecules or membranes [4, 5, 39].

The content of carbohydrates in the leaves of soybean plants (Fig. 3) presented a similar evolution in both cvs., having close values for all the variants, except for the starch in plants of 50% spoil variant, the level of which decreased in a higher percentage in cv. Agat as compared to cv. Diamant. For both cvs. the content of reducing carbohydrates and disaccharides increased in plants cultivated on 50% spoils and decreased in plants from 100% spoils, as compared to control, whereas the amount of starch diminished in plants from 50% spoils and increased in plants from 100% spoils, as compared to control.

The increase of the amount of disaccharides (sucrose) in leaves of soybean plants grown on 50% spoils could be ascribed to the adaptation tendency of carbohydrate metabolism to the stress caused by heavy metals present in the cultivation substratum. Plants on 50% spoils tried to cope with this stress by compensatory,

energy-consuming mechanisms, the increase of the required energy inducing the synthesis of sucrose. Plants grown on 100% spoils had a decreased content in soluble sugars as compared to the control, being unable to adapt to the unfavourable growth medium. The results regarding the carbohydrate metabolism are in accordance with the morphological observations. The growth of soybean plants from 100% spoils was strongly inhibited, whereas the growth of plants from 50% spoils was stimulated [27]. As for other studied parameters [27], the higher disaccharide content in the leaves of Diamant cv. plants grown on 50% and 100% spoils, as compared to cv. Agat, indicates a better resistance of this cultivar to the stress represented by heavy metals.



F i g. 3. *The content of reducing carbohydrates (RC), disaccharides (D) and starch (S) in leaves of soybean plants, Agat and Diamant cvs., grown on mine spoils* (mean values ± SD, n=3).

High levels of toxic oxygen species cause premature leaf senescence by inhibiting the export of photosynthates, leading to the accumulation of large amounts of starch and sucrose in the source leaves [20].

Reduction of root growth strongly reduces shoot growth by limiting the leaf expansion and lateral stem formation. Simultaneously, carbohydrates (starch in particular) accumulate in the leaves and the carboxylation efficiency of photosynthesis decreases [20]. This could be one of the explanations for the high levels of starch in the leaves of soybean plants grown on 100% spoils, where the elongation of roots was reduced by half when compared to the control [27] and the leaf expansion was decreased (data not shown). The content of phosphorus in 100% spoils is also very low [29] and one of the typical features of phosphorus deficiency is the accumulation of large amounts of starch in the chloroplasts [20].

Conclusions. 1. The activity of guaiacol peroxidase increased in the leaves of soybean plants, Agat and Diamant cvs., grown on 100% spoils and decreased in the plants of both cvs. cultivated on 50% spoils.

2. Soybean plants grown in spoil variants showed a decrease in stomatal conductance. The decrease was higher in plants from 100% spoil variant for both cultivars.

3. Heavy metals from mine spoils affect carbohydrate metabolism in soybean plants, causing an increase in the content of reducing carbohydrates and disaccharides in plants cultivated on 50% spoils and a decrease in plants from 100% spoils, as compared to control, whereas the amount of starch diminished in plants from 50% spoil variant and increased in plants from 100% spoils, as compared to control.

4. By mixing the mine spoils with soil in equal percentages, the noxious action of heavy metals on soybean plants was diminished.

5. The Diamant cv. plants appeared to be more resistant to the stress represented by heavy metals from spoils, when compared to the Agat cv. plants.

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UPTAKE OF HEAVY METALS BY MAIZE (*ZEA MAYS*) PLANTS CULTIVATED ON MINE SPOILS

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SUMMARY. – The uptake of heavy metals (Cd, Cu, Pb and Zn) by maize (*Zea mays*) plants cultivated, under field conditions, on mine spoils from Cavnic (Baia Mare) was studied. The experiment, consisting of 4 treatments: control (agricultural soil) and 3 mixtures of agricultural soil with contaminated spoils from Cavnic, in the following proportions: 10, 20 and 30% spoils, was repeated for three years. The concentration of Cd in the maize organs was under the detection limit of the Atomic Absorption Spectrometry (AAS). The accumulation of Zn and Cu in stems and leaves of maize was in the range of normal values: 20-150 mg Zn kg^{-1} d. wt. and 5-20 mg Cu kg^{-1} d. wt., whereas the concentrations of Pb in the leaves of plants treated with 20 and 30% spoils were between 1.2 to 2.2 fold higher than the upper normal limit: 6-8 mg Pb kg^{-1} d. wt. Lead accumulated mainly in roots, whereas Zn and Cu accumulated both in roots and leaves. In all treatments, the concentrations of Cd, Cu, Pb and Zn in grains of maize were in the range of normal values. The above-ground "green" biomass of maize plants was not severely reduced by the heavy metals from spoils, but the grain yield was significantly diminished, especially in treatments with 20 and 30% spoils. The climatic factors are also able to modify the influence of heavy metals from spoils on the growth and yield of maize plants.

Heavy metals present in the edible seeds may potentially enter the human food chain. These elements retained in the stems, leaves and other parts of the crops are either used as fodder or recycled when the inedible crop residues are returned to the soil. The mobilisation of heavy metals from storage sites in the vegetative organs and their transfer to deposition sites in the reproductive organs (seeds) during plant maturation have been intensively studied [9, 15]. Since the heavy metals rise a serious health risk to living organisms, much research has been centred on how to clean up the heavy metals from the soil and how to eliminate the threat.

The aim of our present study was to investigate whether maize, cultivated on soils contaminated with heavy metals, is capable to accumulate metallic elements under the conditions of maintaining the productivity and reducing the toxicity index to a level that is not affecting the health of human and/or animal consumers.

For this study, mine spoils from Cavnic were selected. At Cavnic, there is a large centre of extraction and processing of the metallic ore, which is a very important source of pollution of the environment, including, of course, the soil, too, due to

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the storing process of the mine spoils in the area [1]. Leaching of heavy metals from the spoils and from the polluted soils into the ground water by rainfall makes the contaminants available for being incorporated into the human food chain. In the Cavnic area, such studies are also important due to the fact that people use the spoils for planting vegetables on rocky substrates. So it is important to establish the amount of heavy metals that the plant species accumulates and the way in which the phytoremediation of the polluted soils may be achieved. Using the plants in the phytoextraction-like activities represents a very convenient way to phytoremediate the soils, especially when the plants have also an economic importance, and the heavy metals do not accumulate in toxic quantities in the edible organs.

Materials and methods. The experiment was carried out using maize plants (*Zea mays* L. c.v. Turda 200), because of its ability to accumulate relatively high levels of toxic metals [11, 12, 14, 19] and also because it has a large biomass and it is one of the most important crops used for human and animal consumption in Romania. The intention was to establish the conditions under which maize can be used as a productive plant and as a plant also involved in the phytoremediation process of the soil.

The experiment was carried out in Turda and consisted of 4 treatments: control (agricultural soil) and 3 mixtures of agricultural soil with spoils in the following proportions: 10, 20 and 30%. These percentages of spoils in the substrates were chosen based on a preliminary laboratory experiment which had shown that they do not significantly affect the above-ground biomass of maize and, therefore, the total uptake of heavy metals from soil would not be reduced because of a low biomass. A treatment with 100% spoils was not performed for this study, because the preliminary experiment showed a 60% inhibition of plant growth and very high heavy metal content in maize organs, which would reduce the phytoremediating potential and/or the possibility of using the above-ground organs for human and/or animal consumption.

The spoils from Cavnic were collected from 0-50 cm depth. The mixed treatments were prepared by removing the agricultural soil to 30 cm depth and mixing it with the appropriate quantity of spoils. After thorough mixing in a cement blender, the mixture obtained was placed back into the field. Each treatment plot was established on 2 m^2 land. The same experiment was repeated for three years: 2000, 2001 and 2002. The pH of the soil was measured in water (1:2.5), each year, at the beginning of the experiment (Table 1). The total heavy metal concentrations in the agricultural soil and in the mixed treatments are specified in Table 2. Length of shoots of plants was measured at 20, 30, 45, 60 and 75 days after germination. Two plants per treatment were harvested each month for heavy metal content determination. The plants were separated into roots, shoots, leaves and grains. A composite sample was taken after thorough mixing of each organ from the two plants collected for each treatment. Before drying, roots were carefully washed with deionised water to remove any soil adhering to their surface.

Table 1

pH of the substrates, measured in each year at the beginning of the experiment (mean ± SE, n = 3)

Treatment	pH
Control (agricultural soil)	7.27 ± 0.05
10% Spoils	7.68 ± 0.05
20% Spoils	7.64 ± 0.05
30% Spoils	7.78 ± 0.05

Table 2

Concentrations of heavy metals (Zn, Pb, Cu and Cd) (mg kg^{-1} d. wt.) **in the substrates compared to the reference values [18]**

Metal	Treatment	Measured values (mg kg^{-1} d. wt.) 2000 (ICP-AES) n = 1	2001 ± SD (AAS) n = 4	2002 ± SD (AAS) n = 4	Normal values	Alert value Standard land-use A[a)]	Alert value Standard land-use B[b)]	Intervention value Standard land-use A[a)]	Intervention value Standard land-use B[b)]
Zn	Control	92.32	86.65 ± 3.90	89.43 ± 5.47	100	300	700	600	1500
	10% Spoils	208.43	228.19 ± 8.93	239.56 ± 14.42					
	20% Spoils	314.90	322.89 ± 7.12	335.28 ± 24.56					
	30% Spoils	400.10	415.70 ± 6.53	418.20 ± 7.93					
Pb	Control	20.73	22.72 ± 2.35	23.83 ± 1.97	20	50	250	100	1000
	10% Spoils	88.34	83.45 ± 9.31	81.46 ± 6.67					
	20% Spoils	181.70	172.62 ± 4.94	174.52 ± 5.21					
	30% Spoils	289.60	265.50 ± 6.76	279.40 ± 6.56					
Cu	Control	22.24	23.82 ± 2.40	26.82 ± 4.24	20	100	250	200	500
	10% Spoils	36.85	37.17 ± 3.33	34.67 ± 3.68					
	20% Spoils	58.13	53.18 ± 5.18	51.44 ± 5.97					
	30% Spoils	71.59	69.98 ± 4.97	68.21 ± 3.67					
Cd	Control	0.50	0.49 ± 0.13	0.47 ± 0.17	1	3	5	5	10
	10% Spoils	1.27	1.31 ± 0.05	1.37 ± 0.03					
	20% Spoils	1.72	1.80 ± 0.07	1.83 ± 0.04					
	30% Spoils	2.44	2.39 ± 0.12	2.36 ± 0.11					

A[a)] – Residential with plant uptake and/or allotment land-use.
B[b)] – Commercial/industrial land-use.

On each plot, fifty seeds of maize were sown in two rows. After germination, the seedlings were thinned to 24 on each plot. No fertilizers, pesticides or additional water were added. In each year, the plants were grown in the field, from May to October for an entire vegetative cycle.

Samples of soil and plant material were oven-dried at 75^0C to constant weight and then were ground in a stainless steel mill. The concentrations of heavy metals were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES) in 2000 and by flame atomic absorption spectrometry (AAS – AAnalyst Perkin Elmer 100) in 2001 and 2002. For ICP-AES, the soil and plant samples were digested with concentrated nitric acid and hydrogen peroxide and for AAS, their digestion was carried out with concentrated nitric and perchloric acids [23]. Duplicate analysis, reagent blanks and certified reference materials (U.S. National Institute of Standards and Technology- NIST SRM (Standard Reference Material) 2709, 2710 and 2711 – for soil, and spinach leaves – NIST SRM 1570a for plant material) were used for analytical quality. Analytical bias and precision for Cu, Pb and Zn were estimated as being <10%.

Tukey tests were used to compare the differences between mean values.

Results and discussion. The concentrations of heavy metals (Zn, Pb and Cu) in roots, shoots and leaves of maize plants are presented in Table 3. Concentration of Cd was also measured but it was under the detection limit of the AAS - 0.015 mg Cd kg^{-1} d. wt. The concentrations of heavy metals in maize organs increased with the increase of the level of elements in the substrates (Table 3). The concentrations of metals in the organs of plants cultivated on substrate containing 30% spoils were higher than in those of the control plants. The concentrations were between 1.2-2.7 fold higher at 20 days and between 1.2-3.8 fold higher at the end of the experiment for Zn, between 1.4-13 fold higher at 20 days and between 3-9 fold higher at the end of the vegetative cycle for Pb and between 1.2-2.5 fold higher at 20 days and between 1.3-3.4 fold higher at the end of the vegetative cycle for Cu (Table 3).

Normal concentrations of metals in plants show a large variation between plant species, cultivars, plant tissues and age of plants. However, levels of 20-150 mg kg^{-1} d. wt. for Zn [6, 13, 20], 5-20 mg kg^{-1} d. wt. for Cu [1, 17, 19], 0.05-2 mg kg^{-1} d. wt. for Cd [8] and 0.2-8 mg kg^{-1} d. wt. for Pb [8, 9, 13] are considered normal. According to these values, the concentrations of metals measured in our study, in the vegetative organs of maize, were all in the range of normal values for Zn and for Cu, except for the roots of plants cultivated on substrate with 30% spoils (Table 3). With regard to Pb, although this element is considered less bioavailable than other heavy metals (Cd, Zn), its concentrations in the roots of plants cultivated on substrates containing spoils were up to 4.5 fold higher than the upper limit considered normal. Moreover, the concentrations of Pb in the leaves of plants from treatments with 20 and 30% spoils were up to 2.2 fold larger than the normal values during 2001 and 2002 (Table 3). These results disagree with those of C a r l s o n *et al.* [4], who found that the amount of Pb in leaves of sunflower and corn treated with up to 500 mg L^{-1} Pb, as lead chloride, averaged 4 mg L^{-1} Pb and was not statistically different from the Pb concentration in the control (untreated) plants. The relative high concentrations of Pb found in the leaves of maize plants corroborate with findings of H u a n g *et al*. [11, 12], who concluded that maize could be used for phytoremediation of Pb-contaminated soils.

Table 3

Heavy metal concentrations (mg kg^{-1} d. wt) **in the vegetative organs of maize plants cultivated on substrates containing different percentages of mine spoils**

Metal	Age of plants (days)	Year	Treatment											
			Control			10% Spoils			20% Spoils			30% Spoils		
			R	S	L	R	S	L	R	S	L	R	S	L
Zn	20	2000	30	54	30	44	100	39	59	92	46	78	107	47
		2001	36	62	36	48	61	39	57	55	40	73	53	43
		2002	48	42	35	86	51	37	114	58	43	130	89	51
	40	2000	24	32	28	29	36	29	35	38	29	41	43	29
		2001	19	63	31	30	69	36	32	71	37	40	78	38
		2002	20	24	31	29	28	33	33	32	38	43	39	38
	70	2000	23	16	30	55	17	32	57	26	36	87	33	39
		2001	17	11	26	24	9	40	27	19	42	32	50	47
		2002	20	11	23	23	15	23	32	27	23	52	32	25
	100	2000	-	-	-	-	-	-	-	-	-	-	-	-
		2001	18	8.0	11	28	14	18	37	36	29	48	40	33
		2002	20	8.0	23	31	10	25	32	12	31	45	165	38
	130	2000	-	-	-	-	-	-	-	-	-	-	-	-
		2001	16	10	28	22	11	29	30	11	31	31	15	31
		2002	17	8.0	28	25	11	37	42	13	40	46	24	56
Pb	20	2000	0.9	0.0	0.0	6.5	1.4	0.6	8.8	1.9	1.2	11	2.9	3.5
		2001	4.3	3.3	6.3	17	4.4	7.0	18	5.5	8.3	22	7.5	9.0
		2002	4.6	1.6	2.1	22	2.6	2.7	25	2.8	5.7	35	4.3	6.0
	40	2000	1.3	0.0	0.0	5.0	1.1	1.1	8.2	1.3	1.7	14	1.4	2.1
		2001	5.0	3.0	4.4	12	2.7	5.2	15	3.9	5.6	23	5.0	7.1
		2002	4.3	1.6	5.0	9.6	2.5	2.3	12	3.8	4.8	21	5.0	5.0
	70	2000	2.6	0	0	11	1.8	2.4	14	2.0	2.9	16	3.0	3.1
		2001	4.7	1.1	2.8	14	1.5	4.0	15	1.5	4.7	19	1.7	4.4
		2002	4.6	1.9	2.5	6.2	2.0	2.8	14	3.0	3.0	23	3.6	3.1
	100	2000	-	-	-	-	-	-	-	-	-	-	-	-
		2001	4.4	0.4	4.0	16	3.8	6.1	23	4.1	13	37	5.8	14
		2002	5.7	1.6	6.8	18	2.4	7.2	17	2.8	8.1	29	3.1	12
	130	2000	-	-	-	-	-	-	-	-	-	-	-	-
		2001	4.6	1.0	2.9	15	4.2	5.2	21	4.8	8.8	28	5.1	9.6
		2002	3.5	0	2.0	8.2	0.8	6.6	12	0.9	14	26	1.0	18
Cu	20	2000	5.4	4.7	4.9	7.5	5.1	5.4	8.2	5.9	5.4	9.7	6.0	7.0
		2001	9.7	6.8	9.5	15	7.5	11	19	9.2	11	20	11	15
		2002	8.8	5.0	7.0	18	7.0	9.3	19	8.0	9.3	23	8.5	11
	40	2000	6.3	3.6	5.6	6.6	3.7	5.6	8.9	4.4	8.2	10	5.6	10
		2001	9.0	6.6	11	15	7.0	11	19	9.4	13.1	20	11	16
		2002	8.7	3.8	11	10	3.9	10.5	12	4.6	14.8	16	5.2	17
	70	2000	4.5	2.3	9.7	10	2.4	13	14	2.8	13	15	4.2	14
		2001	7.4	3.7	14	14	5.9	14	14	7.9	17	18	10	18
		2002	7.6	2.3	6.2	8.5	2.4	6.2	9.3	5.7	6.3	12	7.0	6.7
	100	2000	-	-	-	-	-	-	-	-	-	-	-	-
		2001	12	4.2	12	16	7.3	13	20	9.6	16	25	11	19
		2002	6.4	2.2	6.0	9.8	2.4	7.4	12	2.5	11	16	3.5	11
	130	2000	-	-	-	-	-	-	-	-	-	-	-	-
		2001	8.2	5.7	10	12	6.6	12	16	6.2	15	16	7.4	17
		2002	13	1.8	7.7	17	1.9	15	20	2.8	17	21	3.8	19

R – Roots. S – Shoots. L – Leaves.

Depending on the metal, year-to-year variations in the accumulation of heavy metals in the maize parts were registered. These variations from one year to another were probably caused by the climatic factors, as the same soil, treatments, plant species and location of the experiment were used during each year. Year-to-year variations, due to climate variations, were also found by B r o w n *et al*. [3] for lettuce. In our experiment, the concentration of Zn in roots and shoots of maize tended to decrease to the end of the vegetative cycle; the most evident decrease was, in general, registered between day 20 and 40 of the experiment (Table 3) This trend was observed previously for maize [25] and was probably due to the diluting effect of the enlargement of plant biomass. In our study, it could also be due to the expansion of the roots down, in the uncontaminated agricultural soil, situated beneath the 30-cm layer of substrate containing spoils.

The decrease in the Zn concentration with the increase of plant age was on average by a factor of 2 and 3 for roots, in 2001 and 2002 and by a factor of 3.0, 5.0 and 4.5 for shoots, in 2000, 2001 and 2002, respectively (Table 3). However, the concentration of Zn in maize leaves, although it followed some ups and downs during the vegetative cycle, was similar at the end of the vegetative cycle comparing to that of 20 days old seedlings and, moreover, it was similar in the three years of the experiment. For Cu and Pb, although their concentrations in the maize parts also tended to decrease between day 20 and 40, but to a lower extent than for Zn, they, in general, increased again after day 70 of the experiment (Table 3).

The results showed that Pb, a non-essential element, was accumulated mainly in roots, whereas Zn and Cu, essential microelements, were accumulated in both roots and leaves (Tables 3 and 4). Previous experiments showed that most plants appear to have natural barriers to translocation of lead from root into stem, leaves and edible fruiting parts [4, 9, 24]. Lead retention within the roots of plants was also observed in our experiment, in which more than 50% of the Pb taken up by maize was found at root level. With regard to Zn, the percentage of this element in roots increased, whereas in leaves it decreased at higher amounts of spoils in the substrates. The increase of the percentage of Zn in roots (up to 21, 9 and 12% in year 2000, 2001 and 2002, respectively) was equivalent with the decrease of its percentage in leaves (up to 20, 9 and 9% in 2000, 2001 and 2002, respectively), showing that in highly contaminated soils, the plants tend to protect themselves by retaining the metals in the roots system [15], the xylem translocation occurring at a small degree (Table 4). The results of our study showed that Cu was relatively equally distributed in both roots and leaves of maize plants, which agrees with the results of C h l o p e c k a [5] and M a n t o v i *et al*. [17], who also found that this element was intensively translocated from the roots to the above-ground tissues. Depending probably on the climatic factors, the percentage of Cu found in leaves was either not significantly different or it was slightly higher than the percentage of Cu found in the roots (Table 4).

Table 4

Heavy metal concentrations (% of the total amount taken up by plants) **in the vegetative organs of maize plants cultivated on substrates containing different percentages of mine spoils**

Metal	Age of plants (days)	Year	Treatment: Control			10% Spoils			20% Spoils			30% Spoils		
			R	S	L	R	S	L	R	S	L	R	S	L
Zn	20	2000	26	48	26	25	53	22	30	47	23	34	46	20
		2001	27	46	27	33	41	26	38	36	26	44	31	25
		2002	39	33	28	50	29	21	53	27	20	48	33	19
	40	2000	28	39	33	31	38	31	34	37	29	36	38	26
		2001	16	56	28	22	51	27	23	50	27	26	50	24
		2002	27	32	41	32	31	37	32	31	37	36	32	32
	70	2000	33	23	44	53	16	31	48	21	31	54	21	25
		2001	31	20	49	33	12	55	31	22	47	25	38	37
		2002	37	20	43	38	24	38	39	33	28	48	29	23
	100	2000	-	-	-	-	-	-	-	-	-	-	-	-
		2001	48	22	30	46	24	30	36	35	29	40	33	27
		2002	39	16	45	47	15	38	43	16	41	46	16	38
	130	2000	-	-	-	-	-	-	-	-	-	-	-	-
		2001	30	18	52	35	18	47	41	16	43	39	19	42
		2002	32	14	54	34	15	51	44	14	42	36	19	45
Pb	20	2000	100	0	0	76	16	7	74	16	10	64	16	20
		2001	46	19	35	60	15	25	57	17	26	57	20	23
		2002	55	20	25	80	10	10	75	8	17	84	7	9
	40	2000	100	0	0	68	16	16	73	12	15	80	8	12
		2001	41	24	35	60	14	24	61	16	23	66	14	20
		2002	52	20	28	60	15	25	59	18	23	68	16	16
	70	2000	100	0	0	73	12	15	74	11	15	72	14	14
		2001	56	13	32	72	8	20	71	7	22	76	7	17
		2002	52	21	27	57	18	25	70	15	15	77	12	11
	100	2000	-	-	-	-	-	-	-	-	-	-	-	-
		2001	51	4	45	63	14	23	58	10	32	63	13	24
		2002	41	11	48	65	9	26	61	10	29	67	7	26
	130	2000	-	-	-	-	-	-	-	-	-	-	-	-
		2001	55	11	34	61	17	22	61	14	25	66	22	22
		2002	64	0	36	53	5	42	45	3	52	58	2	40
Cu	20	2000	36	31	33	42	28	30	39	29	32	43	27	31
		2001	37	26	37	46	23	32	48	23	29	44	24	32
		2002	42	24	34	52	21	27	52	22	26	54	20	26
	40	2000	41	23	36	37	20	43	42	20	38	39	22	39
		2001	34	25	41	43	21	36	46	23	31	43	23	34
		2002	38	16	46	38	14	48	39	14	47	42	14	44
	70	2000	27	14	59	40	10	50	46	10	44	46	13	42
		2001	30	15	55	40	17	43	36	21	43	38	22	40
		2002	47	14	39	50	14	36	44	26	30	47	27	26
	100	2000	-	-	-	-	-	-	-	-	-	-	-	-
		2001	44	15	41	44	20	36	45	21	34	45	20	35
		2002	44	15	41	50	12	38	48	10	42	54	12	34
	130	2000	-	-	-	-	-	-	-	-	-	-	-	-
		2001	34	24	42	39	22	39	43	17	40	40	18	42
		2002	56	9	35	49	6	45	50	7	43	48	9	43

R – Roots. S – Shoots. L – Leaves.

Highly significant correlation coefficients were obtained between the total concentration of metals (Zn, Pb and Cu) in soil and their concentration in roots (Table 5). For shoots, no significant correlations between the metal concentrations in plants and soil were found, for any of Zn, Pb or Cu, except for Cu in 2002. Similar significant positive correlation between total Cu in soil and its concentration in maize shoots was found by C h l o p e c k a [5]. In disagreement with the data of C h l o p e c k a [5], we found significant positive correlation between the total concentration of Pb in substrate and in the leaves of maize plants (Table 5). However, this positive correlation for Pb might have been falsely significant because the concentrations of Pb in leaves were under the detection limit for control, 10 and 20% spoils (Table 6). The concentrations of metals in grains were positively correlated with their total concentration in soil for Zn and Cu, but not for Pb, which agrees with previous observations on maize [5] and *Silene vulgaris* [7]. Other authors found that effects of soils on the concentrations of Cd, Cu, Pb and Zn were more evident in the leaves than in the grains [15], whereas in a study performed with rice, H e r a w a t i *et al*. [10] observed no correlation between the content of Cd, Cu and Zn in plant tissues and the amount of these metals found in soil.

Table 5

Correlations between the metal concentrations (mg kg^{-1} d. wt.) **in maize organs and in soil at the end of the vegetative cycle**

Plant parts	Zn			Pb			Cu		
	2000	2001	2002	2000	2001	2002	2000	2001	2002
Roots		0.98*	0.96*		0.98*	0.97*		0.95*	0.95*
Shoots		0.89	0.90		0.82	0.82		0.87	0.99*
Leaves		0.91	0.93		0.96*	0.99*		0.99**	0.87
Grains	0.97*	0.97*	0.98*	no Pb	0.81	0.97*	0.99**	0.99*	0.98*

* and ** indicate that the correlation coefficients are significant at $P < 0.05$ and 0.01, respectively.

Table 6

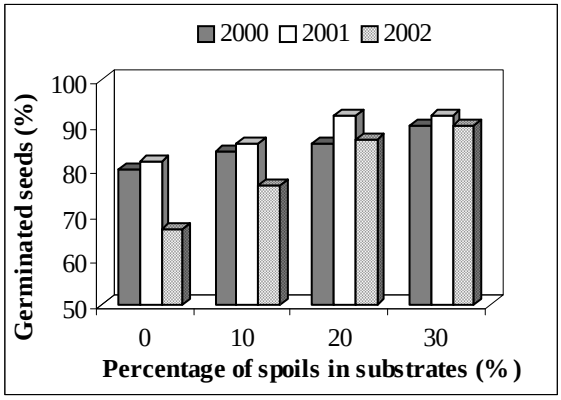
Concentrations of Cu, Pb and Zn in grains of maize plants (mg kg^{-1} d. wt.) **at the end of the vegetative cycle**

Treatment	Zn			Pb			Cu		
	2000	2001	2002	2000	2001	2002	2000	2001	2002
Control	23.5	26.1	22.4	0.0	0.0	0.0	1.6	1.5	1.6
10% Spoils	28.7	31.7	27.8	0.0	0.0	0.0	2.1	1.9	2.0
20% Spoils	29.4	33.5	28.7	0.0	0.0	0.1	2.6	2.9	2.3
30% Spoils	33.6	35.6	32.9	0.0	0.4	0.2	3.0	3.3	2.8

Distribution of Cd, Cu, Pb and Zn in the seeds and other above-ground parts of crops is of nutritional importance to humans. The grains of crops represent the part most likely to be eaten. The apparent ease with which Zn moves into the grains and the apparently restricted movement of Cd and Pb into seeds could both be important to human health [15].

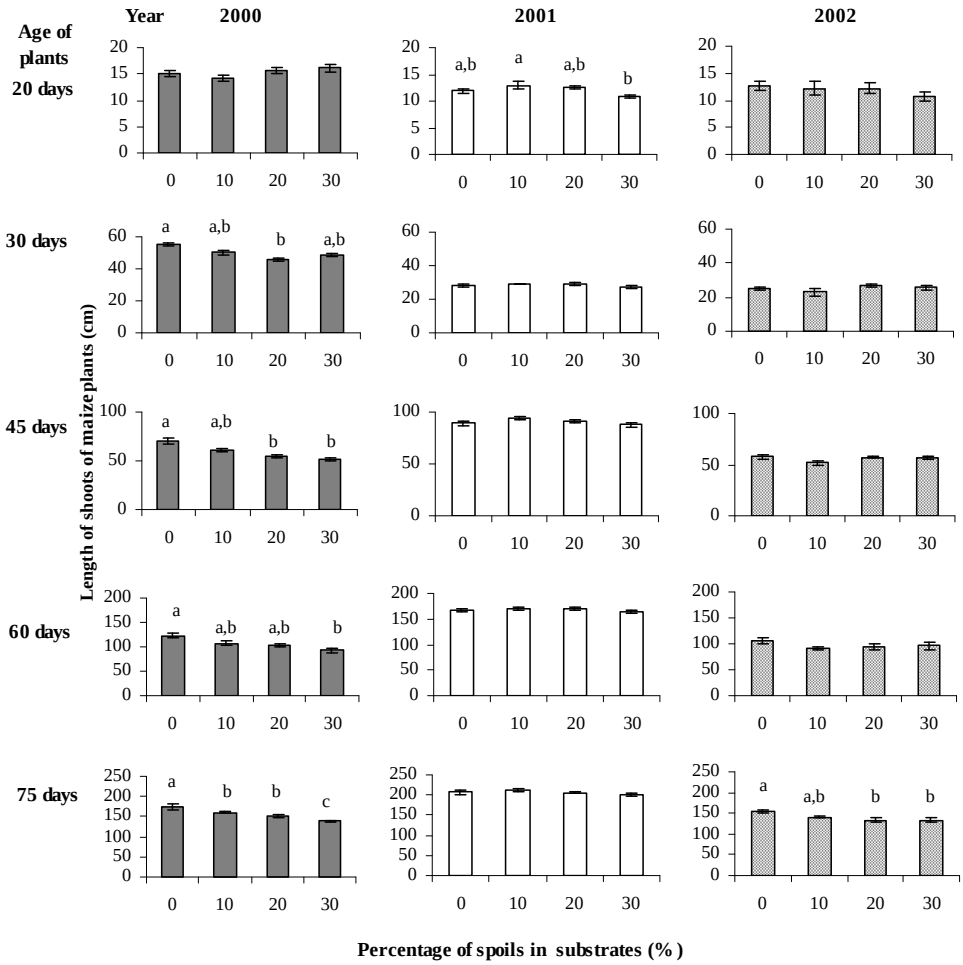
Grain and seed crops serve as natural barriers to the movement of potentially toxic heavy metals, such as Cd and Pb, into the animal/human food chain, minimising their transfer from soils, while conserving Zn and Cu levels in the edible parts of these crops (K u b o t a *et al*. [15], working on maize, wheat and soybean). The copper content of maize grains was below the lowest limit for copper contents in plants (5-20 mg kg^{-1} d. wt., which is considered the critical value for plant growth [2, 17, 19]).

The presence of spoils in the substrates had a positive influence on the germination of maize seeds (Fig. 1), slightly stimulating this process. Similar results were also obtained for maize [13] and soybean [21].



F i g. 1. *Germination capacity of maize seeds sown on substrates containing different percentages of mine spoils.*

The results of our study are in agreement with previous findings which showed a reduced biomass of plants cultivated on polymetal-contaminated substrate [5, 16, 22]. However, the reduction in the length of the shoots of maize plants grown on contaminated substrates compared to control plants, depended largely on the climatic conditions. Thus, during 2000, the driest and the warmest among the three years (data not shown), the length of shoots was significantly smaller in all treatments containing spoils compared to control plants, whereas during 2001, the coolest and the wettest year, there was no difference in the growth of maize plants. Moreover, length of shoots in 2001 was significantly higher than in 2000 and 2002 (Fig. 2). This was due to lower-normal temperatures and to a “normal” regime of rainfalls during 2001, compared to 2000 and 2002. In 2000, stunted growth became obvious in the case of 30 days old plants and it heightened with increasing plant age, whereas in 2002, the differences in the length of shoots were significant only at the end of the vegetative growth period (Fig. 2). In 2000, the length of shoots was reduced by a factor of 1.12 for plants grown on 10 and 20% spoils and by a factor of 1.25 for plants on 30% spoils as compared to control plants, whilst in 2002, the length of shoots of plants from treatments with 20 and 30% spoils was by 1.15 fold smaller than that of the control

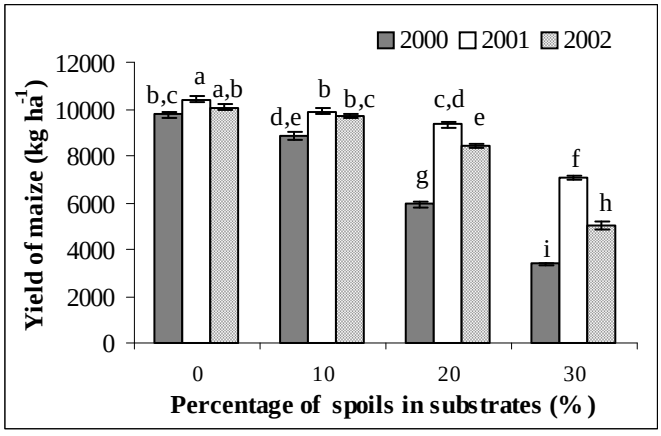


F i g. 2. *Length of shoots of maize plants(cm)* (mean ± SE, n = 15). Bars with different letters are significantly different (confirmed by Tukey test). Tukey test was performed separately for each graph; therefore, letters from different graphs are not comparable. When no letters are attached, the means are not significantly different (confirmed by Tukey test).

plants. The statistically significant reduction of growth of maize plants registered in our study was, however, much lower than in other experiments, probably because of the capacity of maize to tolerate relatively high concentrations of heavy metals [11, 12, 14]. Shoot length of alfalfa (*Medicago sativa* L. c.v. Mesa) grown on soil contaminated with 50 mg of each Cd, Cu, Ni and Zn kg^{-1} d. wt. was on average 2 fold lower than that of the control plants [22]. The authors concluded that the low growth of plants was due to the combined stress caused by the mixture of heavy metals, because the alfalfa

plants have demonstrated the ability to grow well in soils individually contaminated with more than 50 mg kg^{-1} d. wt. of heavy metals [22]. Similar results were obtained by C h l o p e c k a [5], who showed that Cd, Cu, Pb and Zn added jointly as carbonates, at a rate of 50, 500, 500 and 1500 mg kg^{-1} d. wt., respectively, reduced the biomass of shoots and leaves of maize by a factor of 2 and the grain yield by a factor of 2.5. L u o and R i m m e r [16] also obtained an average of 10% decrease in the shoot biomass of barley when grown in substrate containing all four metals (Cd, Cu, Pb and Zn) than in treatments with only one, two or three of these metals.

Despite the fact that the growth of shoots was not severely inhibited and that the concentrations of heavy metals in the maize parts were in general in the range of normal values, the grain yield of maize was significantly affected in each year of the experiment. Similarly to the length of the shoots, the grain yield was also influenced by the climatic factors, being more severely reduced under drier and hotter weather (Fig. 3). The reduction in the grain yield in 2000, 2001 and 2002, respectively, expressed as a percentage of the control, was 9.3, 4.9 and 3.8% for the treatment with 10% spoils, 39.3, 10.4 and 16.7% for the treatment with 20% spoils and 65.5, 32.1 and 50.1% for the treatment with 30% spoils. Outstandingly, the concentrations of heavy metals in grains of maize were in the range or even below the normal values (Table 6). Due to the complexity of the soil composition and of the climatic conditions, it was difficult to establish the factors that influenced the grain yield the most. Probably, the high pH of soil and the complex interactions between elements in soil and in plants affected the uptake of macro- and micronutrients, which might have been the cause for the low yield obtained for the treatments containing spoils.



F i g. 3. *Grain yield of maize plants (kg ha^{-1})* (mean ± SE, n = 7).
Bars with different letters are significantly different (confirmed by Tukey test).

Conclusions. 1. The accumulations of Zn and Cu in stems and leaves of maize grown, under field conditions, on mixtures of agricultural soil with 10, 20 and 30% of spoils from Cavnic, were in the range of normal values: 20-150 mg Zn kg^{-1} d. wt. and 5-20 mg Cu kg^{-1} d. wt. Despite this, the above-ground organs of maize plants could not be used to feed the animals because the concentration of Pb in the leaves of treated plants was between 1.2 to 2.2 fold higher than the upper normal limit: 6-8 mg Pb kg^{-1} d. wt.

2. The concentrations of Cd, Cu, Pb and Zn in the grains of maize were in the range of normal values in all treatments used in our study. Therefore, the grains of maize could be used for animal and/or human consumption.

3. Lead, a non-essential element, accumulated mainly in roots, whereas Zn and Cu, essential microelements, accumulated in both roots and leaves.

4. Mixtures of agricultural soil with 10, 20 and 30% spoils from Cavnic did not severely reduce the above-ground "green" biomass of maize plants, but they significantly diminished the grain yield, especially in the treatments with 20 and 30% spoils. The influence of heavy metals found in the spoils on the above-ground biomass and grain yield of maize was modified by climatic factors, too (*i.e.* by high temperatures and low humidity in soil).

5. Maize can be used for phytoremediation of polymetal-contaminated soils, but its phytoremediating efficiency is relatively low, because of the reduced uptake and translocation of heavy metals from soil to the above-ground organs. Caution should be taken if the above-ground maize material is to be used for animal consumption.

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ASPECTE HISTOLOGICE ŞI ULTRASTRUCTURALE ALE TIMUSULUI ÎN INTOXICAȚIA ACUTĂ CU VENIN DE ALBINE

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SUMMARY. – Histological and Ultrastructural Aspects of the Thymus in the Acute Intoxication with Honey Bee Venom. In this study the histological and ultrastructural modifications in the rat thymus were investigated after the experimental administration of very high doses of the bee venom water-soluble fraction. We have taken into account this organ since the honey bee (*Apis mellifera L.*) venom is well known to be a powerful immuno-stimulating agent. First of all, a massive lymphocyte migration in the blood flow was recorded after two hours of the acute treatment. The presence of a high amount of exogenous, toxic substances, as well as the severe alterations occurred in the structure of the most organs and tissues are mainly responsible for this effect. On the other hand, we have also recorded ultrastructural alterations in all cell populations, many cells being totally destroyed; the number of apoptotic cells is increased and the number of mitotic cells is decreased. At the subcellular level, in most of the cells the nuclear envelope was deeply affected and vacuolisations of endoplasmic reticulum were observed. Mitochondria also respond strongly to the bee venom: they appeared to be swollen and with a rarefied matrix. These results indicate both a specific response of the thymus against the action of bee venom molecules, and a direct, unspecific effect of the venom upon all cell membranes leading to the death of many cells.

Timusul este un organ limfoid, de origine epitelială infiltrat cu limfocite şi alte celule mezenchimale. El îndeplineşte un rol esențial în dezvoltarea aparatului imun, dar şi în dobândirea şi întreținerea imunității organismului prin formarea, diferențierea şi maturarea limfocitelor T la acest nivel. Deşi producerea limfocitelor T este specifică şi zonelor periferice ale nodulilor splenici sau din alte organe limfo-poietice, timusul reprezintă principala sursă pentru acest tip de celule, fiind deci principalul factor responsabil pentru apărarea organismului. Scopul studiului nostru a fost de a urmări reacțiile provocate la nivel structural şi ultrastructural în acest țesut la administrarea experimentală a veninului de albine în doze foarte mari, fiind bine cunoscută capacitatea acestui produs biologic de a dezvolta diverse reacții imunologice [2, 8, 17].

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Materiale şi metode. *Animale.* Experimentele s-au realizat în două etape pe 4 loturi de *şobolani albi Wistar* (câte 3 animale în fiecare lot), crescuți în biobaza U.M.F. "Iuliu Haţieganu", fără restricții în ce priveşte hrana, apa sau mişcarea.

Veninul de albine. Am utilizat venin de albine pur cristalizat, recoltat de noi prin metoda stimulării abinelor cu impulsuri electrice [15, 33]. Cu 24 ore înainte de administrarea experimentală, veninul a fost resuspendat într-o soluție izotonă (150 mM NaCl, 5,5 mM glucoză, 5 mM HEPES, la pH 7,4) şi păstrat la frigider, ferit de acțiunea luminii.

Tratament. Animalelor din două loturi – *TL1 şi TL2* – li s-a injectat subcutan, dorso-lateral posterior o doză unică, foarte mare de venin de albine (DL_{50} – letală pentru 50% din subiecți), echivalentă cu cantitatea eliberată la un număr de 100 înțepături de albine (62 mg/kg corp). Probele de țesut recoltate au fost prelucrate pentru realizarea de preparate histologice (*Lotul TL1*), respectiv pentru microscopia electronică de transmisie (*Lotul TL2*). Doza semiletală a fost stabilită în jurul valorii de 60 mg venin/kg corp pe baza datelor din literatură [16, 19]. Pentru evidențierea eventualelor efecte, în paralel s-au recoltat şi prelucrat fragmente de timus de la animale din 2 loturi martor – *ML1 şi ML2* – corespunzător fiecărui lot de animale tratate.

Histologie. Pentru realizarea preparatelor histologice, piesele biologice recoltate au fost fixate în fixator Bouin, deshidratate, incluse în parafină şi apoi secționate la microtom. După etalarea pe lame şi deparafinare, colorarea s-a făcut prin metoda Hurduc (pe bază de orange G, albastru de metil, xilidină şi acid acetic glacial) [27]. Examinarea s-a realizat la un microscop Olympus BX51.

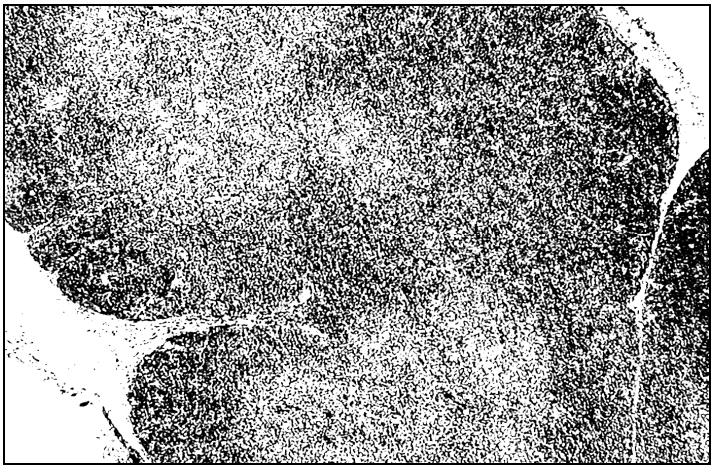
Microscopie electronică de transmisie. Probele de timus recoltate în vederea studierii la microscopul electronic de transmisie au fost prefixate în soluție de glutaraldehidă 2,7%, postfixate în acid osmic 2%, spălate şi deshidratate în acetonă, apoi incluse în Epon 812 şi secționate la un ultramicrotom LKB-III. Secțiunile preluate pe grile electrolitice au fost contrastate cu acetat de uranil şi apoi cu citrat de plumb [32]. Examinarea s-a realizat la un microscop electronic de transmisie Jeol JEM 1010.

Rezultate. Examinarea la microscopul optic (Fig. 1), respectiv la microscopul electronic (Fig. 3) a probelor de timus de la loturile martor a relevat structura normală a lobulilor timici, aspectul caracteristic al timocitelor (limfocite T) şi al altor tipuri celulare prezente: polimorfonucleare, macrofage, rare monocite, în corticală, respectiv plasmocite, macrofage, puține eozinofile, numeroase celule reticulare şi corpusculii Hassal în zona medulară [1, 5, 14, 25, 26, 28, 34-37].

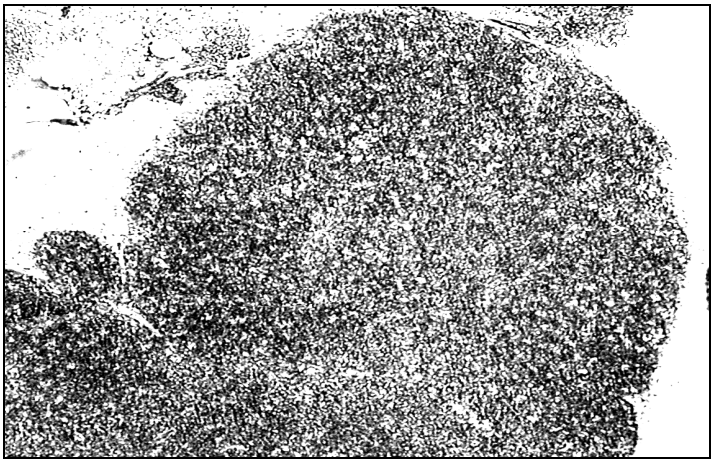
Capilarele sanguine sunt concentrate în special la nivelul medularei, fiind rar observate în corticală. Perivascular se poate întâlni un spațiu în care ocazional se află fibre de colagen; vasele de sânge nu sunt în contact cu timocitele, între aceste elemente structurale fiind interpusă o barieră formată din celulele reticulare [7, 25, 35].

La examinarea probelor prelevate de la *lotul TL1*, s-a remarcat histologic o reactivitate puternică a glandei, manifestată prin dispariția totală a delimitării între zonele corticală şi medulară. Aceste două componente ale timusului se întrepătrund

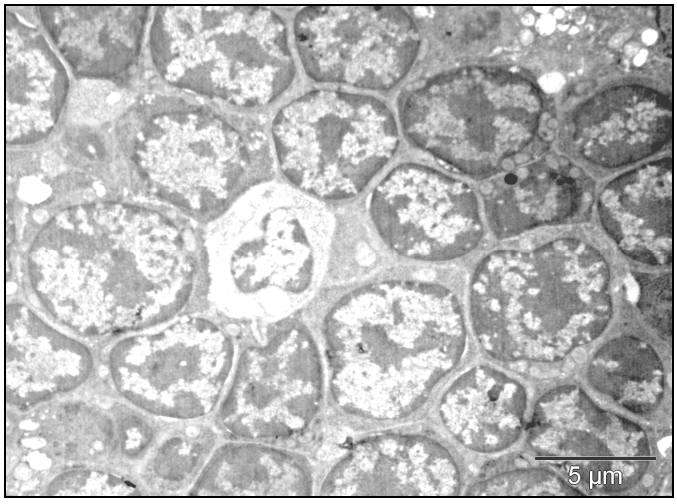
şi confluează. Aspectul morfologic cel mai pregnant îl reprezintă hipoplazia marcată a limfocitelor timice din parenchimul timic, atât din regiunea corticală, cât şi din cea medulară. Se remarcă prezența a numeroase spații clare, acelulare, de diferite dimensiuni, rezultate ca urmare a migrării timocitelor din aceste zone ale timusului. Toate aceste modificări contribuie la apariția aspectului de „cer înstelat" al timusului (Fig. 2) care este caracteristic în stările de stres acut sau în unele infecții ale organismului. În medulara lobulilor timici se observă o uşoară hiperplazie a corpusculilor Hassal de formă rotundă sau ovoidală.



F i g. 1. *Structura normală a lobulului timic – lotul ML1* (ob. 10 ×).

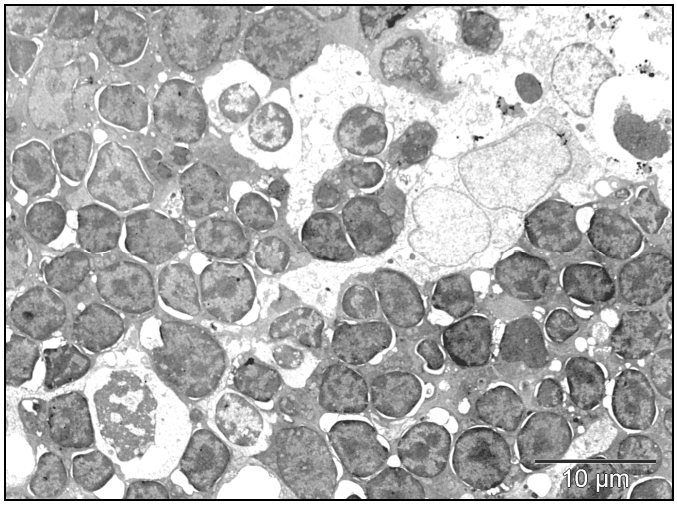


F i g. 2. *Aspectul de "cer înstelat" al lobulului timic – lotul TL1* (ob. 10 ×).



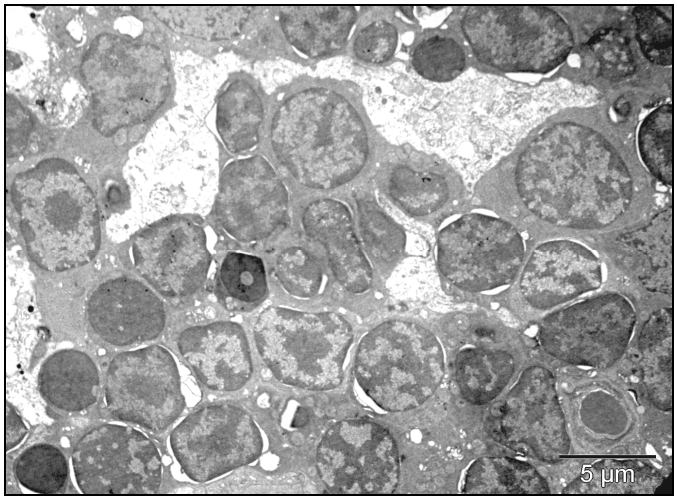
F i g. 3. *Timocite dens împachetate, grupate în „cuiburi" – lotul ML2.*

Electronomicroscopic, studiul secţiunilor ultrafine, prin timusul animalelor din *lotul TL2*, evidenţiază şi în acest caz o densitate scăzută a timocitelor şi vacuolizări citoplasmatice de amploare ce privesc în primul rând spaţiul perinuclear, dar şi profilele reticulului endoplasmic (Fig. 4-7). Pe de altă parte, există în numeroase zone celule în care afectarea citoplasmei este totală – aici celulele apar fără conţinut citoplasmatic perinuclear (Fig. 4, 6, 7). Acest aspect este observat şi în cazul unor celule surprinse în timpul mitozei (Fig. 4), sau chiar în cazul unor celule apoptotice (Fig. 4 şi 7).

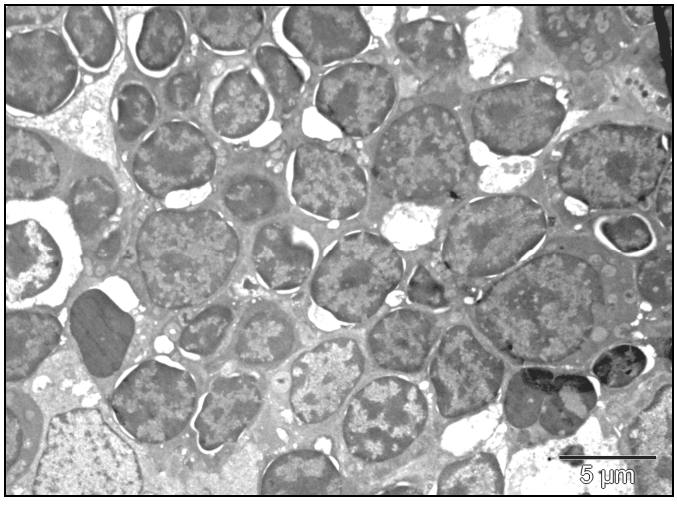


F i g. 4. *Migrarea timocitelor evidenţiază reticulocitele rarefiate; rarefiere şi liză în timocitele aflate în diverse etape ale ciclului celular – lotul TL2.*

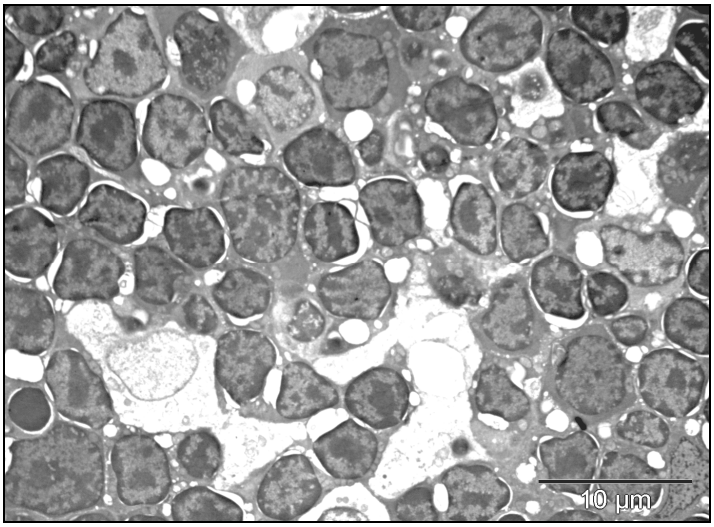
În timocitele ce prezintă un grad de afectare mai redus, totuşi mitocondriile au matricea rarefiată (Fig. 4-6). În condițiile prezenței acute a veninului în cantitate mare, apare un număr sporit de timocite surprinse în diferite etape ale proceselor de apoptoză comparativ cu loturile martor (Fig. 4-7). În acelaşi timp, numărul mitozelor înregistrate este mai scăzut.



F i g. 5. *Densitate celulară redusă; număr mare de timocite apoptotice – lotul TL2.*



F i g. 6. V*acuolizări în toate celulele; spațiul perinuclear dilatat; mitocondrii cu matricea rarefiată – Lotul TL2.*



F i g. 7. S*pațiul perinuclear dilatat; reticulocite electronotransparente – Lotul TL2.*

Discuții. Efectele produse asupra timusului în urma administrării veninului de albine în doze mari indică faptul că în această variantă experimentală răspunsul acestui organ este, în principal, unul imunologic. Astfel, timusul răspunde acut la prezența şi acțiunea substanțelor străine care atacă structurile altor țesuturi, în primul rând prin punerea masivă a limfocitelor în circulație. Complementar cu acest mecanism, migrarea timocitelor din întreaga structură a timusului este modulată şi indirect.

Pe de o parte, Lundin [citat în 29] arată că există o strânsă interrelație între activitatea timusului şi cea a adenohipofizei, iar pe de altă parte, s-a demonstrat că eliberarea timocitelor este stimulată şi ca urmare a prezenței unei cantități crescute de glucocorticoizi circulanți (răspunsul neuro-endocrin clasic la stres), organele limfopoietice fiind deosebit de sensibile la steroizi [21]. S-a raportat că acțiunea acestor hormoni (de exemplu a cortizonului) poate fi în multe situații răspunzătoare de depopularea şi involuția rapidă a timusului [25].

În acelaşi context, Oancea şi Cojocea [29] au descris experimente efectuate de mai mulți cercetători care au arătat că extirparea experimentală a suprarenalelor a fost urmată de creşterea în greutate a timusului, cu accentuarea imediată a activității limfocitoproliferative timice. Din contră, administrarea de prednisolon a indus o creştere imediată a numărului de limfocite eliberate de timus, pentru ca apoi, în scurt timp, numărul acestora să fie din ce în ce mai scăzut, asociat cu scăderea în greutate a timusului [29]. Această situație este similară cu efectul obținut în experimentul nostru, când, la administrarea acută de venin de albine se înregistrează, la un interval de două ore de la administrare, o scădere a limfocitelor circulante cu 30% față de martor (date preliminare încă nepublicate).

Rezultatele noastre sunt asemănătoare cu cele descrise de Hermenean şi colaboratorii [20], care au înregistrat o reducere a populațiilor de timocite din zona corticală în urma administrării pe o perioadă scurtă a unor doze mari dintr-o altă substanță cu efect citotoxic pronunțat (ciclofosfamidă). De altfel, acestea se corelează şi cu modificările observate la nivelul splinei după administrarea aceleiaşi substanțe într-o singură doză terapeutică, când s-a observat o inducție a fenomenelor apoptotice la nivelul limfocitelor [31].

Al doilea efect important al veninului de albine evidențiat prin experimentul nostru este cel timotoxic. Acesta este, de asemenea, foarte pronunțat fiind exercitat direct asupra tuturor categoriilor celulare din structura organului. Veninul a ajuns în cantități mari în proximitatea celulelor ca urmare a dilatărilor de amploare ale capilarelor, proces însoțit de diminuarea fluxului sanguin, respectiv de stagnarea sângelui în aceste zone. A urmat rapid traversarea sau, în unele situații, distrugerea pereților capilarelor, ceea ce a permis moleculelor din compoziția veninului de albine să se infiltreze pe calea spațiilor intercelulare spre toate tipurile de celule, până în profunzimea organului, respectiv să-şi exercite efectul citolitic la nivelul structurilor timusului. Modificările structurale şi ultrastructurale observate de noi au fost determinate, prin acțiunea directă a componentelor veninului, mai ales a melitinei şi fosfolipazei A_2 asupra tuturor categoriilor de membrane celulare [4, 6, 18, 19, 22-24, 30, 38]. Vacuolizările citoplasmatice afectează întreaga populație celulară; observarea lor şi la nivelul celulelor endoteliale reticulare sugerează ideea că aceste alterări au contribuit decisiv la scăderea coeziunii timocitelor şi au favorizat migrarea lor.

Modificările survenite la nivel ultrastructural sunt deci nespecifice şi extinse, de aceeaşi natură cu cele înregistrate în alte țesuturi investigate de noi în condiții similare [9-11]. Leziunile multiple produse în timus nu sunt însă la fel de ample şi generalizate ca cele observate în cazul administrării experimentale a veninului de albine pentru o perioadă mai îndelungată de timp [12].

Rezultatele prezentate de noi sunt în concordanță cu cele raportate de alți cercetători care au abordat studiul efectelor veninului de albine, prin alte metode sau tehnici de investigare [3, 6, 16]. De asemenea, rezultatele noastre experimentale concordă cu cele raportate în cazul unor accidente soldate cu multiple înțepături de albine în urma cărora s-au efectuat variate investigații clinice, inclusiv examinarea unor preparate prin microscopie electronică de transmisie [13, 30].

Concluzii. În urma analizei rezultatelor obținute, putem afirma că dozele foarte mari de venin de albine influențează semnificativ structura şi ultrastructura timusului. Pe de o parte, efectele produse la nivelul întregului organism au contribuit la depopularea bruscă a timusului, care încearcă astfel să facă față invaziei acute a moleculelor bioactive străine. Alterările profunde survenite la nivelul diferitelor țesuturi, inclusiv al sângelui sunt deci şi ele reflectate, în morfologia şi fiziologia timusului, fiind, în ansablul lor responsabile pentru colapsul organismului. Pe de altă parte, componentele veninului au provocat, prin acțiunea lor directă asupra timusului,

leziuni majore la nivelul tuturor categoriilor de celule, dar în primul rând la nivelul limfocitelor T. Ca şi în cazul altor ţesuturi studiate de noi, consecutive administrării experimentale de venin de albine, efectele veninului la nivelul timusului sunt direct dependente de doză.

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ASPECTE HISTOPATOLOGICE ALE TOXICITĂŢII PANCREATICE A UNUI AGENT ALCHILANT ANTITUMORAL (CISPLATIN) LA ŞOBOLANII ALBI WISTAR DE VÂRSTE DIFERITE

CRISTINA PAŞCA[*] şi VICTORIA-DOINA SANDU*

SUMMARY. - Histopathological Aspects of the Toxicity of an Antitumour Alkylating Agent (Cisplatin) on the Pancreas of White Wistar Rats of Different Ages. Cisplatin is an anticancer drug, more exactly an alkylating agent belonging to the family of platinum-containing products, widely used in the chemotherapy of many types of malignant diseases in humans. Unfortunately, according to the previous studies concerning its side effects, this drug has an antitumour activity correlated with a significant toxicity on different vital organs, consisting of the appearance of many and serious structural and functional alterations, which, sometimes, can endanger the life of the patient. Cisplatin has a significant toxicity on the whole digestive system (digestive tube and glands), which, so far, has been just tangentially investigated. Therefore, our researches intended to evaluate the histological and functional alterations induced by a single therapeutic dose of Cisplatin on the pancreas in puberal and mature white Wistar rats.

Our results demonstrated that this antitumour drug, in monochemotherapy, has a moderate toxic effect on the rat pancreas, depending on the age of the rats, the most sensitive being the puberal individuals, in which the destructive processes appeared and got worse more quickly, and affected more seriously the structure of the pancreas. The toxic effect consisted of the appearance of certain histological modifications which affected both the cellular and vascular components of the organ. But, the histopathological alterations, both at the level of the exocrine and endocrine secretory units, seem to be due (induced and aggravated owing) to the grave circulatory disturbances (blood stasis, congestion, oedemas, intravascular coagulation phenomena) and thrombocytopenia, which determined a deficient supply with oxygen and nutritive substances, and an accumulation of some toxic metabolic products.

The exocrine units (or acini) seem to be more sensitive than the islets of Langerhans. They appeared affected by an obvious diffuse oedema correlated with a serious perturbance of the intraacinar and intraductal transit of the pancreatic fluid. Besides, many and abundant lymphocyte and granulocyte infiltrations could be observed, especially perivascularly, around the pancreatic acini.

The endocrine units of the pancreas (islets of Langerhans) were a little more resistant, the toxic action of Cisplatin determining only a transitory and discrete oedema and intravascular coagulation phenomena correlated with a moderate granulo-vacuolar dystrophy, and zonal necrosis processes. In addition, the destructive processes at the level of the islets of Langerhans were not associated with serious perturbation of the secretions of insulin and/or glucagon and, thus, the level of glucose in the blood was not significantly affected.

Fortunately, both in the puberal and mature rats, the histological alterations induced by this dose of Cisplatin, administered in monochemotherapy, although perturbed both the exocrine and endocrine units of the pancreas, had no irreversible character, as at the end of the experimental period, without any protective or regenerative treatment, an obvious natural recovery process could be observed.

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Extinderea chimioterapiei antineoplazice din ultimii ani, precum şi folosirea citostaticelor ca imunosupresoare în pregătirea organismelor pentru realizarea transplantelor de grefe şi organe impun cu necesitate studierea şi cunoaşterea cât mai completă a acţiunii acestor medicamente nu numai asupra structurilor tumorale (cărora le sunt în principal destinate), dar şi asupra ţesuturilor şi organelor sănătoase, la nivelul cărora ele exercită anumite efecte secundare datorită acţiunii selective extrem de reduse pe care o au.

În plus, literatura de specialitate denotă clar faptul că mecanismul de acţiune al citostaticelor în general şi, implicit, şi al Cisplatinului se bazează pe capacitatea acestor medicamente de a forma legături ireversibile cu ADN-ul şi de a interfera cu sinteza ARN-ului şi a proteinelor [6, 8, 10]. În consecinţă, administrarea de citostatice blochează atât diviziunea celulară, cât şi sinteza proteică, ambele esenţiale pentru conservarea structurii tisulare, pentru buna desfăşurare a activităţii ţesuturilor, organelor şi, în ultimă instanţă, a întregului organism. Datorită acestui mecanism de acţiune al citostaticelor, structurile cel mai drastic afectate în condiţii de chimioterapie vor fi acelea, la nivelul cărora indicele mitotic este ridicat şi/sau acelea care sunt implicate în desfăşurarea unor procese de sinteză proteică mai intense.

Toate aceste considerente justifică pe deplin cercetările noastre, care şi-au propus depistarea unor aspecte inedite ale toxicităţii pancreatice exercitate la nivel structural, la şobolanii albi Wistar de vârstă diferită, de către Cisplatin (citostatic frecvent utilizat în terapia antineoplazică şi într-o oarecare măsură şi în transplantul de grefe şi organe), cunoscut fiind că acesta este un organ vital, cu o activitate de sinteză proteică constantă şi extrem de intensă. În urma parcurgerii unui material bibliografic destul de bogat, am putut constata că aceste aspecte sunt doar tangenţial şi, implicit, insuficient abordate la ora actuală pe plan mondial.

Material şi metodă. Cercetările s-au efectuat pe şobolani albi Wistar, masculi, juvenili (cu o greutate medie de 100 $\pm$ 10 g) şi adulţi (cu o greutate medie de 190 $\pm$10 grame). Animalele de experienţă au fost întreţinute în condiţii optime de laborator, asigurându-li-se îngrijirea şi hrana corespunzătoare şi apă *ad libitum*. S-a lucrat pe două serii experimentale (una constituită din juvenili şi alta din adulţi), fiecare serie incluzând câte 8 loturi a câte 8 indivizi fiecare:

-loturile martor M_1-M_4 – constituite din şobolani sănătoşi, netrataţi cu medicament;

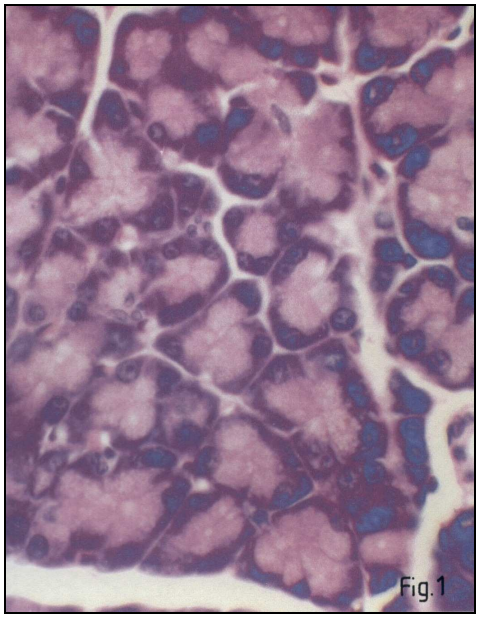
-loturile C_1-C_4 - constituite din şobolani sănătoşi, trataţi cu o doză terapeutică unică de 13 mg Cisplatin/kg corp, administrată intravenos în vena codală şi sacrificaţi la 24 ore, 4, 11 şi, respectiv, 18 zile de la tratament în vederea realizării unor dozări biochimice sanguine şi a unor investigaţii structurale.

Sacrificarea animalelor s-a făcut dimineaţa, la 16 ore de inaniţie, după o anestezie profundă cu eter etilic, prin decapitare şi exsangvinizare. Imediat după sacrificare s-au recoltat probe de sânge şi fragmente de ficat, care au fost imediat prelucrate conform tehnicilor uzuale, astfel încât să fie posibilă dozarea glicemiei şi realizarea în condiţii optime a investigaţiilor de microscopie optică [11, 13].

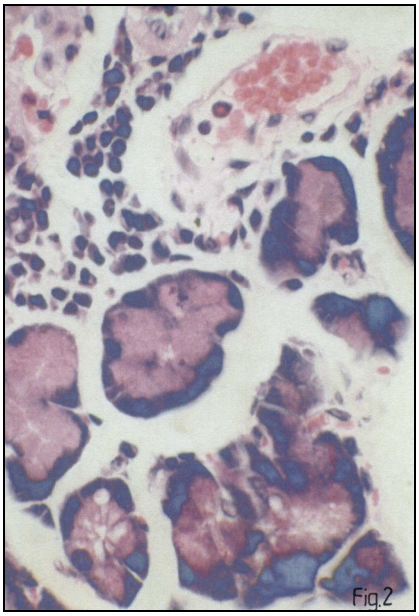
Rezultate şi discuţii. Cu toate că în cadrul experimentului nostru am administrat doar o singură doză terapeutică de Cisplatin, în condiţii de monochimioterapie, rezultatele investigaţiilor structurale şi biochimice reflectă cu certitudine faptul că acest citostatic exercită un anumit efect toxic asupra pancreasului de şobolan, atât asupra componentei exocrine, cât şi endocrine. Gravitatea, amploarea şi extinderea proceselor tisulare distructive par a fi strâns corelate cu vârsta organismului supus tratamentului, precum şi cu intervalul de timp scurs de la administrarea citostaticului.

Cele mai pregnante şi extinse modificări histopatologice au fost înregistrate la ***juvenili***, la care efectul toxic se instalează rapid după administratea medicamentului, se accentuează progresiv şi semnificativ în prima jumătate a perioadei experimentale, după care începe să scadă atât ca intensitate, cât şi ca extindere. La finele celor 18 zile ale experimentului se ajunge la o refacere structurală avansată a ambelor componente pancreatice, deşi, zonal, se mai păstrează încă amprenta acţiunii toxice exercitate de acest xenobiotic.

Astfel, comparativ cu lotul de control, cu o structură histologică normală a pancreasului (Fig.1), am constatat că primele aspecte histopatologice sunt deja decelabile microscopo-optic la doar *24 ore* de la tratament şi se materializează în prezenţa unor acini pancreatici tumefiaţi, cu celule acinoase supraîncărcate cu produşi de secreţie concentraţi apical, care concură la deplasarea nucleului mult înspre polul bazal al celulelor. În plus, apare şi un edem difuz intraacinar şi diminuarea până la dispariţie a lumenului adenomerului (Fig. 2).



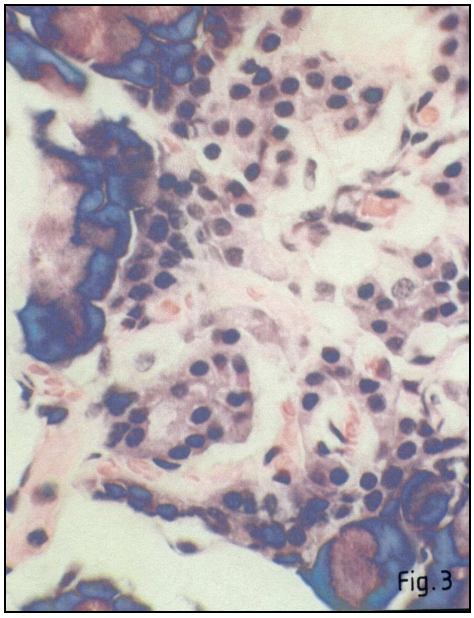
F i g. 1. *Aspectul histologic al pancreasului la lotul martor (x 1280).*



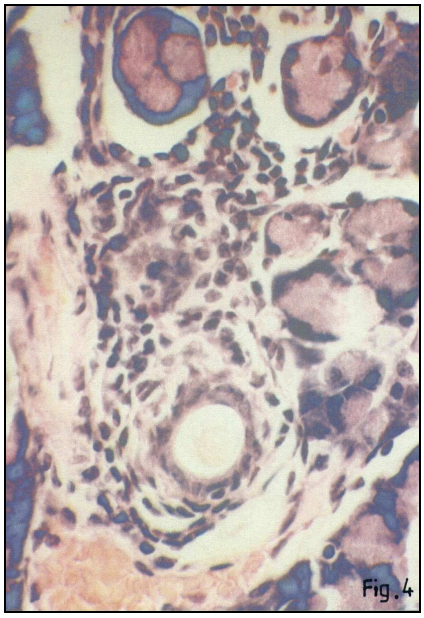
F i g. 2. *Acini pancreatici uşor tumefiaţi, cu celule supraîncărcate cu produşi de secreţie (x 1280).*

Componenta endocrină este moderat afectată, la nivelul ei înregistrându-se o stază uşoară a reţelei vasculare corelată cu procese evidente de coagulare intravasculară diseminată şi un edem în insulele Langerhans (Fig. 3).

După *4 zile* de la tratament, staza şi edemul acinar înregistrează o uşoră intensificare, aspectul tumefiat al acinilor accentuându-se. Totodată, în canalele pancreatice intra- şi interlobulare se constată prezenţa unei secreţii abundente de material oxifil, granular. Deci, caracteristic pentru componenta exocrină a acestui lot este perturbarea activităţii secretorii acinare, cu stagnarea produsului de secreţie atât intracelular, cât şi intraductal. În plus, la nivel interstiţial se constată prezenţa unui bogat infiltrat limfogranulocitar (Fig. 4).

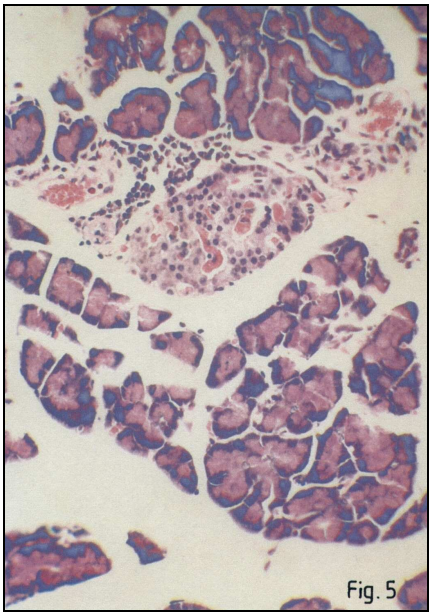


F i g. 3. *Stază sanguină şi fenomene discrete de coagulare intravasculară diseminată, edem accentuat în insulele Langerhans (x 1280).*

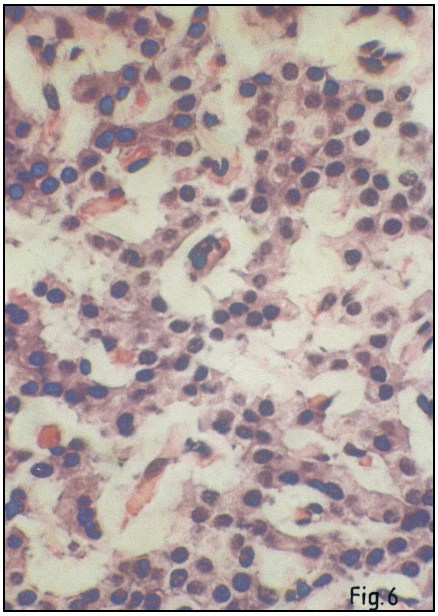


F i g. 4. *Infiltrat limfogranulocitar interstiţial abundent (x 1280).*

Insulele Langerhans continuă să prezinte tulburări vasculare de tipul perturbărilor de permeabilitate a peretelui vascular, care justifică edemul moderat existent la acest nivel. De asemenea, persistă şi procesele de coagulare intravasculară diseminată, ele fiind ceva mai accentuate decât la 24 ore. Nici dimensiunea şi nici densitatea celulară a majorităţii insulelor Langerhans nu par a fi modificate comparativ cu lotul de control (Fig. 5). Însă, unele insule apar mai puternic alterate, în sensul că ele se remarcă printr-o stază sanguină mai pronunţată, prin distrofia granulo-vacuolară şi chiar necrozarea unor celule endocrine (Fig. 6).



F i g. 5. *Congestie vasculară, stază sanguină şi edem moderat în insulele Langerhans (x 512).*



F i g. 6. *Distrofie granulo-vacuolară şi chiar necroza unor celule ale insulelor Langerhans (x 1280).*

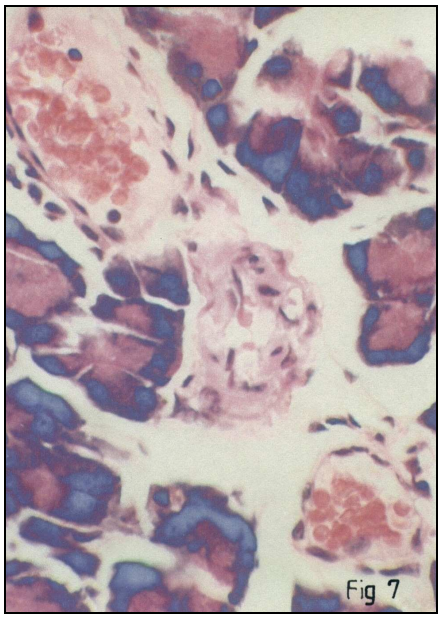
La *11 zile*, acinii pancreatici manifestă o tendință de normalizare sub aspectul dimensiunii şi structurii lor. Staza sanguină şi edemul, deşi persistente şi destul de extinse, sunt mai reduse, la fel şi staza intracelulară şi intracanaliculară (Fig. 7). Infiltratul limfogranulocitar interstițial (cu preponderența eozinofilelor) poate fi încă sesizat, ceea ce denotă că citostaticul continuă să exercite un efect iritativ pancreatic semnificativ (Fig. 8).

Componenta endocrină pancreatică apare şi ea mai puțin afectată, staza vasculară fiind mai uşoară, iar edemul mai discret, în timp ce fenomenele de coagulare intravasculară diseminată, deşi prezente, au doar un caracter zonal (Fig. 9).

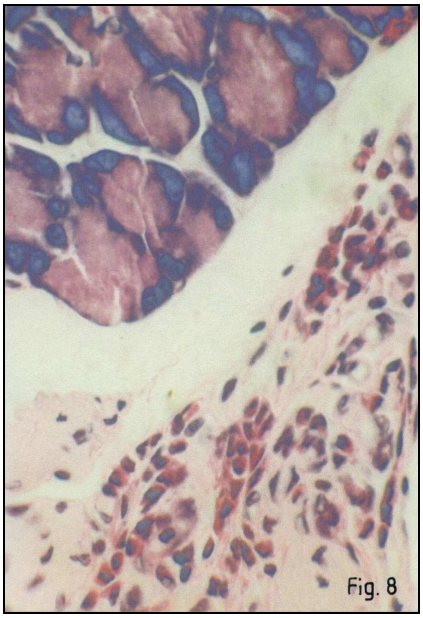
La *18 zile* de la administrarea citostaticului, aspectele histopatologice înregistrate la 11 zile sunt mai discrete şi se mai păstrează doar într-un număr redus de zone pancreatice, în timp ce fenomenele de coagulare intravasculară diseminată nu mai sunt deloc semnalate. Pe ansamblu, atât componenta exocrină, cât şi cea endocrină pancreatică au o structură oarecum comparabilă cu cea înregistrată la lotul martor (Fig. 10).

Comparativ cu juvenilii, ***adulții*** se remarcă printr-o mai mare rezistență la atacul pancreaticotoxic al Cisplatinului, în sensul că modificările histopatologice, care sunt similare cu cele înregistrate la juvenili, devin decelabile microscopo-optic abia la 4 zile de la tratament, ating un maxim la 11 zile, după care aproape că dispar către finele perioadei experimentale. Însă, trebuie subliniat că toate perturbările

structurale (staza sanguină, congestia vasculară, coagularea intravasculară diseminată, edemele, staza pancreatică intraacinară şi intraductală) au fost mult mai discrete şi nu au avut un caracter generalizat ca la juvenili, ci doar unul zonal şi moderat. În plus, nu au fost deloc semnalate fenomene necrotice la nivelul insulelor Langerhans, în timp ce distrofia granulo-vacuolară a fost extrem de discretă şi de foarte scurtă durată.



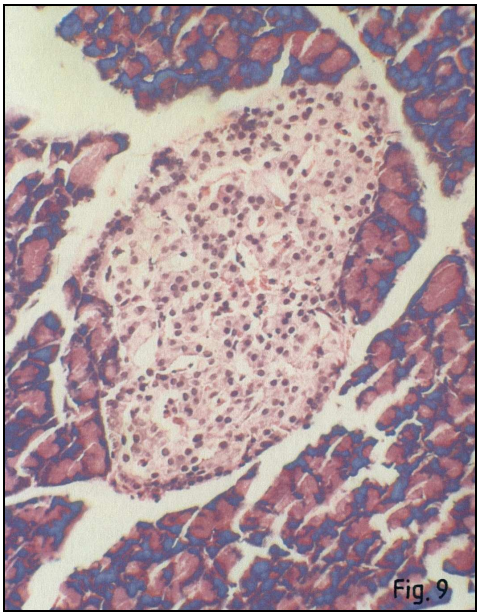
F i g. 7. *Stază sanguină pancreatică generalizată şi edeme interstiţiale (x 1280).*



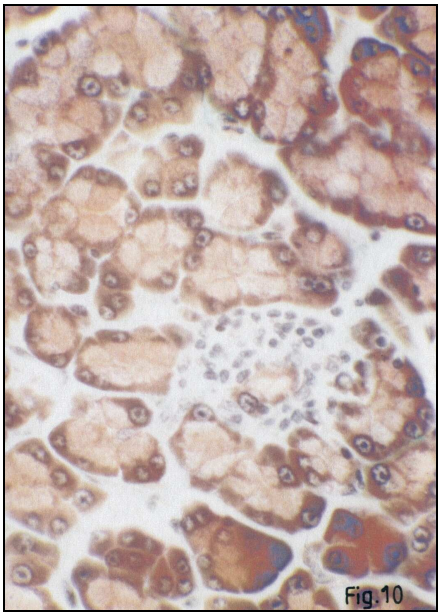
F i g. 8. *Infiltrat limfogranulocitar interstiţial masiv cu preponderenţa eozinofilelor (x 1280).*

Subliniem că, deşi atât la adulţi, cât mai ales la juvenili au fost înregistrate modificări structurale la nivelul insulelor Langerhans, ele nu au fost corelate cu perturbări funcţionale ale acestora, deci cu alterarea secreţiei de insulină şi glucagon, ceea ce justifică menţinerea glicemiei în limite normale pe toată perioada desfăşurării experimentului.

Investigaţiile histologice şi biochimice realizate în cadrul experimentului nostru demonstrează că Cisplatinul administrat chiar şi într-o singură doză terapeutică şi în monochimioterapie poate conduce la instalarea unor modificări structurale semnificative la nivelul pancreasului şobolanului alb Wistar, care prin gravitatea, amploarea şi evoluţia lor în timp pot altera, într-o oarecare măsură, funcţiile acestui organ vital şi, implicit, pot compromite calitatea vieţii acestor organisme. Cercetările noastre au demonstrat că acţiunea pancreaticotoxică a acestui citostatic cu platină pare a fi strâns corelată atât cu vârsta organismului supus tratamentului, cât şi cu intervalul de timp scurs din momentul administrării medicamentului.



F i g. 9. *Stază vasculară uşoară şi edem la nivelul insulelor Langerhans (x 512).*



F i g. 10. *Pancreas cu un aspect histologic foarte apropiat de cel al lotului martor (x 1280).*

Astfel, cele mai pregnante şi extinse modificări histopatologice au fost înregistrate la *juvenili*, la care efectul pancreaticotoxic devine decelabil la doar 24 de ore de la administratea medicamentului, se accentuează progresiv şi semnificativ în prima jumătate a perioadei experimentale, după care începe să scadă atât ca intensitate, cât şi ca extindere. La finele celor 18 zile ale experimentului se ajunge la o refacere structurală avansată a ambelor componente pancreatice, deşi, zonal, se mai păstrează încă amprenta acțiunii toxice exercitate de acest xenobiotic.

Comparativ cu juvenilii, *adulții* se remarcă printr-o mai mare rezistentă la atacul pancreaticotoxic al Cisplatinului. Aşa se explică de ce modificările histopatologice, care sunt similare cu cele înregistrate la juvenili, au fost semnalate microscopo-optic mai târziu (abia după 4 zile de la administrare), ating un maxim la 11 zile, după care scad rapid sub raportul amploarei şi extinderii lor, pentru ca la finele experimentului să dispară aproape complet, structura histologică a pancreasului fiind comparabilă cu cea a lotului de control.

La ambele serii experimentale, dar mai pregnant la juvenili, efectul toxic pancreatic s-a concretizat în apariția unor modificări structurale moderate atât la nivelul componentei vasculare (congestie, stază sanguină, fenomene de coagulare intravasculară diseminată), cât mai ales la nivelul componentei celulare implicate în îndeplinirea funcției exocrine şi endocrină a acestei glande.

Mai afectat pare a fi *pancreasul exocrin*, unde s-a înregistrat instalarea stazei sanguine şi congestiei vasculare, care concură la apariția unui edem difuz la nivelul

acinilor. În plus, efectul iritativ exercitat de acest medicament justifică prezența a numeroase infiltrate limfohistiocitare şi limfogranulocitare interstiţiale, cu dispoziţie preponderent perivasculară.

Tranzitul secreţiei pancreatice este şi el perturbat, fapt materializat prin stagnarea produsului secretor atât intracelular cât şi intraductal. Aşa se justifică hipertrofia acinară generalizată şi dilatarea canaliculelor secretorii. Posibil ca această alterare a fluxului de suc pancreatic să fie corelată cu modificările semnificative înregistrate la nivel intestinal, mai excact cu perturbarea sintezei şi/sau activităţii enzimelor marginii în perie implicate în activarea enzimelor sucului pancreatic (potrivit unor aspecte semnalate de noi, dar încă nepublicate). Deci, acest citostatic cu platină pare să afecteze atât procesul secretor, cât şi tranzitul produsului de secreţie pancreatică.

Componenta endocrină (insulele Langerhans) se dovedeşte a fi mai rezistentă la acţiunea toxică a medicamentului, care, şi în acest caz, interesează atât componenta vasculară (congestie, stază sanguină şi procese tranzitorii de coagulare intravasculară diseminată), cât şi pe cea celulară. Tulburările de hemodinamică de tipul congestiei vasculare şi stazei, în condiţiile alterarării permeabilităţii peretelui vascular, par a fi creat condiţii propice apariţiei unui edem difuz insular. Alterarea aportului adecvat de substanţe nutritive şi O_2, corelată cu stagnarea locală a produşilor de catabolism cu un anumit potenţial toxic, justifică apariţia în unele insule a unei distrofii granulo-vacuolare discrete şi tranzitorii şi a câtorva celule necrozate. Aceste aspecte histo-patologice semnalate în cadrul experimentului nostru sunt în concordanţă cu datele furnizate de diverşi histopatologi, care susţin că diferiţi factori toxici, infecţioşi şi radiaţiile pot cauza tulburări circulatorii de intensităţi diferite, care pot fi generatoare de fenomene distrofice şi chiar necrotice insulare, care uneori interesează un număr semnificativ de celule de tip B, ajungându-se la instalarea unui diabet aloxanic [1].

Aşa cum reiese din cele prezentate mai sus, fenomenele de coagulare intravasculară diseminată interesează atât vasele sanguine tributare componentei exocrine, cât şi endocrine. Conform datelor din literatură, coagularea intravasculară diseminată este un proces patologic destul de complex, care inplică participarea sistemului de coagulare sanguin şi formarea de trombi în multe vase mici, cu instalarea unor alterări ischemice consecutive în diferite organe. Prezenţa cordoanelor de fibrină în vasele sanguine mici cauzează deformarea şi lezarea hematiilor la trecerea lor prin reţeaua de fibrină intravasculară şi, astfel, la organismele cu fenomene de coagulare intravasculară diseminată, apare, implicit, şi o anemie hemolitică corelată cu prezenţa unor eritrocite anormale sub aspect morfologic – mai exact apare ceea ca se numeşte anemie hemolitică micro-angiopatică [14].

De altfel, în prezent este cunoscut că Cisplatinul provoacă destul de frecvent o anemie hemolitică cu caracter progresiv, care deseori impune realizarea unor transfuzii cu masă eritrocitară [9]. Una dintre cauzele majore ale coagulării intravasculare diseminate o reprezintă perturbările hematologice şi, în special, trombocitopenia [4, 14]. Aceasta explică amploarea deosebită a coagulării intravasculare diseminate înregistrată

la şobolanii trataţi cu Cisplatin în cadrul investigaţiilor noastre, acest citostatic fiind cunoscut ca un mielodepresiv foarte puternic, factorul limitant al dozei fiind trombocitopenia, maximă la 3 săptămâni de la tratament, revenirea la normal având loc abia după 4 săptămâni [2, 3, 5, 7, 12, 15]. Putem concluziona că este posibil ca trombocitopenia indusă de acest agent alchilant cu platină să declanşeze fenomene de coagulare intravasculară diseminată, care, graţie cheagurilor de fibrină constituite, creează condiţii propice instalării unor tulburări ischemice în diferite organe şi a unei anemii hemolitice.

Subliniem că, pe baza literaturii de specialitate destul de bogată consultată de către noi, instalarea unor astfel de fenomene de coagulare intravasculară diseminată la nivel pancreatic, după chimioterapie cu Cisplatin, este un aspect histopatologic nou, care nu a mai fost semnalat.

Referitor la intensitatea şi dinamica modificărilor histopatologice induse de Cisplatin la cele două serii experimentale trebuie subliniat că la adulţi staza sanguină, congestia vasculară, coagularea intravasculară diseminată, edemele, staza pancreatică intraacinară şi intraductală au fost mult mai discrete şi nu au avut un caracter generalizat ca la juvenili, ci doar unul zonal şi moderat. În plus, nu au fost deloc semnalate fenomene necrotice la nivelul insulelor Langerhans, în timp ce distrofia granulo-vacuolară a fost discretă şi de foarte scurtă durată.

Subliniem şi faptul că, la nici una dintre cele două serii experimentale, alterările structurale înregistrate la nivelul insulelor Langerhans nu au condus la instalarea unui diabet aloxanic (indus prin distrofii şi necroze ale celulelor B pancreatice), ceea ce explică menţinerea glicemiei în limite normale pe parcursul întregii perioade experimentale.

Cu toate că unele aspecte histopatologice pancreatice înregistrate atât la nivelul componentei exocrine, cât şi endocrine au fost destul de puternice, extinse şi de durată (în principal la juvenili), ele au avut un caracter reversibil, tranzitoriu, făcând posibilă refacerea naturală în timp a tuturor structurilor afectate.

Concluzii. 1. Cisplatinul, administrat într-o doză terapeutică unică de 13 mg/kg corp, în condiţii de monochimioterapie, conduce la instalarea unor modificări structurale semnificative atât la nivelul pancreasului, cât mai ales la nivelul ficatului şobolanului alb Wistar, care prin gravitatea, amploarea şi evoluţia lor în timp pot altera într-o oarecare măsură funcţiile acestor organe vitale şi, implicit, pot compromite calitatea vieţii acestor organisme.

2. Cercetările noastre au demonstrat că acţiunea pancreaticotoxică a Cisplatinului pare a fi strâns corelată atât cu vârsta organismului supus tratamentului, cât şi cu intervalul de timp scurs din momentul administrării medicamentului.

3. Cele mai pregnante şi extinse modificări histopatologice au fost înregistrate la *juvenili,* la care efectul pancreaticotoxic devine decelabil la doar 24 de ore de la administratea medicamentului, se accentuează progresiv şi semnificativ în prima jumătate a perioadei experimentale, după care începe să scadă atât ca intensitate,

cât şi ca extindere. La finele celor 18 zile ale experimentului se ajunge la o refacere structurală avansată a ambelor componente pancreatice, deşi, zonal, se mai păstrează încă amprenta acţiunii toxice exercitate de acest xenobiotic.

4. Comparativ cu juvenilii, *adulţii* se remarcă printr-o mai mare rezistentă la atacul pancreaticotoxic al Cisplatinului. Aşa se explică de ce modificările histopatologice, care sunt similare cu cele înregistrate la juvenili, au fost semnalate microscopo-optic mai târziu (abia după 4 zile de la administrare), ating un maxim la 11 zile, după care scad rapid sub raportul amploarei şi extinderii lor, pentru ca la finele experimentului să dispară aproape complet, structura histologică a pancreasului fiind comparabilă cu cea a lotului de control.

5. La ambele serii experimentale, dar mai pregnant la juvenili, efectul toxic pancreatic s-a concretizat în apariţia unor modificări structurale moderate atât la nivelul componentei vasculare (congestie, stază sanguină, fenomene de coagulare intravasculară diseminată), cât mai ales la nivelul componentei celulare implicate în îndeplinirea funcţiei exocrine şi endocrine a acestei glande.

6. Tulburările hemodinamice pancreatice induse de Cisplatin, de tipul stazei, congestiei şi edemului, prin inducerea unui deficit de substanţe nutritive şi O_2 şi prin blocarea îndepărtării produşilor de catabolism cu potenţial toxic, este posibil să se afle la baza fenomenelor distructive ce interesează celulele acinilor şi insulelor Langerhans.

7. Mai sensibil la acţiunea Cisplatinului pare a fi *pancreasul exocrin* unde s-a înregistrat instalarea stazei sanguine şi congestiei vasculare, care concură la apariţia unui edem difuz la nivelul acinilor şi a numeroase infiltrate limfohistiocitare şi limfogranulocitare interstiţiale, cu dispoziţie preponderent perivasculară.

8. Tranzitul secreţiei pancreatice este şi el perturbat (tranzitoriu), fapt materializat prin instalarea stazei produsului secretor atât intracelular, cât şi intraductal.

9. Componenta endocrină (insulele Langerhans) pare a fi mai rezistentă la acţiunea toxică a medicamentului, constatându-se doar prezenţa unui edem difuz şi a unor procese tranzitorii de coagulare intravasculară diseminată pe fondul unei distrofii granulo-vacuolare discrete şi tranzitorii.

10. La nici una dintre cele două serii experimentale, alterările structurale înregistrate la nivelul insulelor Langerhans nu au fost corelate cu modificări semnificative ale glicemiei pe parcursul perioadei experimentale.

11. La adulţi, staza sanguină, congestia vasculară, coagularea intravasculară diseminată, edemele, staza pancreatică intraacinară şi intraductală au fost mult mai discrete şi nu au avut un caracter generalizat ca la juvenili, ci doar unul zonal şi moderat. În plus, nu au fost deloc semnalate fenomene necrotice la nivelul insulelor Langerhans, în timp ce distrofia granulo-vacuolară a fost discretă şi de foarte scurtă durată.

12. Cu toate că unele modificări histopatologice pancreatice înregistrate la nivelul componentei vasculare şi celulare (exocrine şi endocrine) au fost destul de puternice, extinse şi de durată (în principal la juvenili), toate au avut un caracter tranzitoriu, fiind posibilă refacerea naturală în timp a tuturor structurilor afectate.

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FUNCTIONAL CORRELATIONS BETWEEN THE PERMEABILITY TRANSITION, MEMBRANE POTENTIAL COLLAPSE AND CALCIUM RELEASE BY LIVER MITOCHONDRIA OF ETHANOL-FED RATS

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SUMMARY. – Male white Wistar rats were maintained in our animal facility for 14-18 weeks and the evolution of their body weight was assessed periodically. One group of the rats, kept on a normal diet, served as control (C), while in another group (A) each rat was supplemented individually with 1.5 ml of 48% ethanol/100 g body weight daily. At the end of the period, the rats were sacrificed and their liver used for the preparation of mitochondria. For membrane potential ($\Delta\Psi$), calcium (Ca^{2+}) and swelling measurements, 1 mg of mitochondrial protein/ml was usually incubated directly in the spectrophotometer cuvettes, at room temperature (around $24^{O}C$), in different media, usually containing 5 mM Hepes buffer (pH 7.37), 1 mM KP_i and various concentrations of mannitol, sucrose, KCl and $MgCl_2$. 8 μM rotenone was always added to the mitochondrial suspension, as well as the appropriate probe for either $\Delta\Psi$ (2.5 μM diS-C_2-(5)) or Ca^{2+} (30 μM arsenazo III). The respiration and all the associated phenomena monitored by us ($\Delta\Psi$, Ca^{2+} fluxes and swelling) were usually triggered by the addition of succinate (2.5 mM). Different amounts of $CaCl_2$ or other modulating factors were either added gradually or in one pulse, up to the desired final concentration. Recordings of either $\Delta\Psi$ or calcium fluxes in parallel with the matrix swelling were performed by a diode-array spectrophotometer. In addition, for quantitative measurements of the extension of swelling, special runs were also performed in which mitochondrial protein was used as the triggering factor. From the comparison of the spectrophotometric recordings, one can observe a strong correlation between the concentration of calcium added to the mitochondrial suspension, the moment of the $\Delta\Psi$ collapse, of the swelling and of the calcium release (massive calcium efflux). As expected, these phenomena occur faster (at shorter times and/or lower calcium concentrations) in ionic media, especially in those that lack magnesium. However, important differences were also noticed between the mitochondria of the control (C) rats and those of the animals treated with ethyl alcohol (A) regarding the degree (amplitude) and the kinetics of the absorbance changes associated with the generation/collapse of $\Delta\Psi$, Ca^{2+} movements and swelling. In general, mitochondria from ethanol-treated rats are more sensitive to calcium. Even though the amplitude of membrane potential does not seem to be affected, it collapses at much lower concentrations of added calcium. Even though, in general, the extension of swelling does not differ statistically significant, this sensitivity is also valid for the matrix swelling and the release of calcium. We have quantified this relationship by considering the number of calcium pulses (of 12.5 μM each) added to the cuvette before massive calcium efflux (release) and $\Delta\Psi$ collapse occur. As expected, this number is lower in ionic media (with the lowest in the swelling medium), but for the same medium it is also lower for the ethanol-fed rats and the differences are in general statistically very significant ($p<0.01$) in all media tested.

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Chronic ethanol consumption (in humans) or administration (to laboratory mammals) has been generally associated with specific hepatic structural and functional alterations known under the name of alcoholic liver disease (ALD). Despite many studies, the exact mechanism by which ethanol induces the disease is not entirely known. Whether ALD is determined by the nutritional defects induced by alcohol or by its direct hepatotoxic effect is still a matter of debate [17, 25, 40, 41]. For decades, alcoholic liver disease has been attributed to necrotic events associated, among others, with the production of proinflammatory cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) [11, 44, 68]. However, relatively recent data indicate that under certain circumstances both the acute and chronic ethanol administration is associated with a process of liver apoptosis [1, 12, 26, 36, 46], which, paradoxically, can facilitate latter necrotic events leading to ALD [1, 12, 13, 29, 45, 68]. On the other hand, progresses made during the last decade in the study of apoptosis (physiologic or programmed cell death) have placed mitochondria in a central position in controlling this very complicated process [3, 5, 27, 32-34, 42, 49, 52-54, 56, 57].

Excessive alcohol consumption has been known to be associated with a series of intracellular stresses, particularly detrimental to mitochondria [7, 9, 15, 35, 62, 68]. One of the possible ways of alcohol attack may be through distorting the oxidant/ antioxidant balance of the cell. Oxidative stress, associated with chronic ethanol consumption, leads to the depletion of mitochondrial glutathione, decreased synthesis of the respiratory chain components and acetaldehyde adduct formation [2, 3, 11, 18, 25, 45, 71]. The result is an increase in the concentration of reactive oxygen species (ROS), such as superoxide anion, hydroxyl and peroxyl radicals [15, 19, 20, 29, 35, 36, 39], which are considered to be among the main causes of triggering a drastic change in mitochondrial membrane permeability, known as the mitochondrial permeability transition (MPT), associated with the opening of a permeability transition pore (PTP). This permeability change seems to be a central event in different types of cell death, either apoptosis or necrosis [9, 24, 27, 32, 38, 42, 45, 50, 62, 68]. What exactly determines the type of cell death (apoptosis or necrosis) is not perfectly understood, although it seems to be related finally to the amount of ATP produced by mitochondria, as described elsewhere [37, 47, 62].

Without getting into the details of the structure and function of the pore, we mention that PTP is probably formed at the so-called membrane contact sites by the association of several proteins belonging to the two mitochondrial membranes and the intermembrane space, thus penetrating the whole insulating system of the mitochondrial matrix. The opening of this pore is accompanied by membrane potential ($\Delta\Psi$) collapse, uptake of electrolytes and water, matrix swelling and ruptures of the mitochondrial outer membrane. As a consequence, several factors present in the intermembrane space, among which cytochrome c (cyt c), are liberated into the cytosol. There is, however, an alternative point of view, which holds that cyt c can be released without a permeability transition and outer membrane breaking, due to certain pores created into the outer membrane by several proapoptotic factors (proteins), such as Bax and Bid [14, 31, 43, 69]. Indeed, the implication of Bax and

Bid in apoptosis can not be negated, but many investigators consider that these proteins only interact with the PTP components, activating or regulating the opening of the PT pores, a fact which could explain certain contradictory observations regarding the dependence/independence of cyt c release on $\Delta\Psi$ collapse [22, 70]. The groups of Martinou [14] and Orrenius [21] demonstrated in fact that there are two distinct mechanisms by which cyt c can be liberated: a Ca^{2+}-dependent one, which takes place apparently by matrix swelling and outer membrane rupture, and a Ca^{2+}-independent mechanism, in which the oligomeric form of Bax mediates or regulates the release of cyt c, without permeability transition. On the other hand, the implication of calcium itself in the cell death, in general, is also well documented [8, 21, 23, 24, 48, 51, 55, 58, 67[.

In trying to clarify some aspects of such controversial problems of mitochondrial involvement in apoptosis (specifically in liver apoptosis), especially the relationship between cyt c release and membrane permeability, we selected chronic ethanol feeding as a natural model of producing hepatic apoptosis in combination with the use of suspending media of different ionic content and addition of different concentrations of calcium, while monitoring spectrophotometrically, in parallel, membrane potential, calcium release and matrix swelling. Liver slices and aliquots of both supernantant and mitochondrial sediment, taken at specific moments of incubation, were prepared or saved for later analysis, including electron microscopy. The present article describes and discusses only the spectrophotometric results of our study. A preliminary report has already been presented [65].

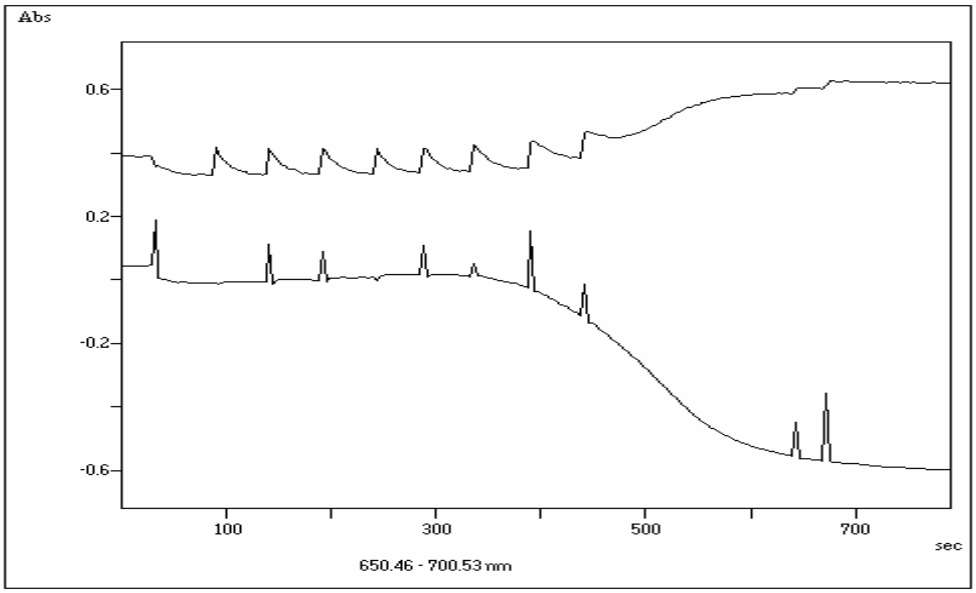
Material and methods. *Animals, treatment protocols and preparation of mitochondria.* Male white Wistar rats were kept under normal conditions in our animal facility for 14-18 weeks, starting from an average weight of 120 g/individual, while the evolution of their weight was assessed periodically. The rats were kept on a normal diet (a premix containing all the ingredients of the Larsen diet), with free access to water. One group served as control (C), while in another group (A) each rat was supplemented daily with 1.5 ml of 48% ethanol/100 g body weight, administered in the morning, on a little piece of bread, before getting access to the food. At the end of the period, the rats were fasted for 24 hrs and sacrificed by decapitation after a slight anaesthesia. Small pieces of liver were taken in some cases and prepared for electron microscopy, while the rest of the liver was used for preparation of mitochondria, essentially according to Johnson and Lardy [30], in a medium containing 200 mM mannitol, 70 mM sucrose, 5 mM Hepes-KOH (pH 7.37) and 0.5 mM Na-EDTA. The washing and preserving medium lacked the chelating agent (EDTA).

Spectrophotometric measurements. A diode-array spectrophotometer (Specord S 100B, Analytik Jena, Germany), which permits concomitant measurements at different wavelengths, was used for measurements of membrane potential and/or calcium in parallel with the matrix swelling, while the extension of swelling was quantitatively measured (at 540 nm) on separate recordings initiated by the addition of mitochondria to a medium containing succinate as a respiratory substrate. In parallel

recordings, membrane potential was estimated as a difference between 670 and 700 nm, by the use of 2.5 μM diS-C_2-(5) as a potential sensitive probe [59, 60], while calcium was measured by the use of 30 μM asenazo-III (650–700 nm) as a calcium specific dye. The change in absorbance at 700 nm (which represents a quasi-isobestic point for both dyes) was used for assessing the extent (amplitude) of swelling. For measurements, 1 mg of mitochondrial protein/ml was usually incubated directly in the spectrophotometer cuvettes, at room temperature (around 24^{O}C), in different media, all containing 5 mM Hepes buffer (pH 7.37), 1 mM KP_i and various concentrations of mannitol, sucrose, KCl and $MgCl_2$, as specified below. The respiration and the associated phenomena monitored by us ($\Delta\Psi$, Ca^{2+} fluxes and swelling) were triggered by the addition of succinate (2.5 mM) in the presence of 8 μM rotenone. Different amounts of $CaCl_2$ were either added gradually, in several pulses, or in one pulse, up to the desired final concentration. The suspending media used are designated as follows: MS (210 mM mannitol, 70 mM sucrose, 1.5 mM $MgCl_2$); MSK (110 mM mannitol, 40 mM sucrose, 65 mM KCl, 1.5 mM $MgCl_2$); MSK–Mg (the same as MSK, but without Mg). In addition, a special swelling medium (KSW) was also used. It contained 100 mM KCl, 50 mM sucrose, 10 mM Hepes and 5 mM KP_i. Although we made some attempts at obtaining precise values of membrane potential amplitude and calcium concentrations (as decribed elsewhere [65]), the most important quantitative result was obtained by counting the number of calcium pulses (of 12.5 μM each) necessary to be added to the mitochondrial suspension for producing the calcium release, which is always more-or-less associated with the permeability transition (as monitored by matrix swelling) and $\Delta\Psi$ collapse. The statistical significance of the differences between different groups or subgroups was computed by the Student *t* test.

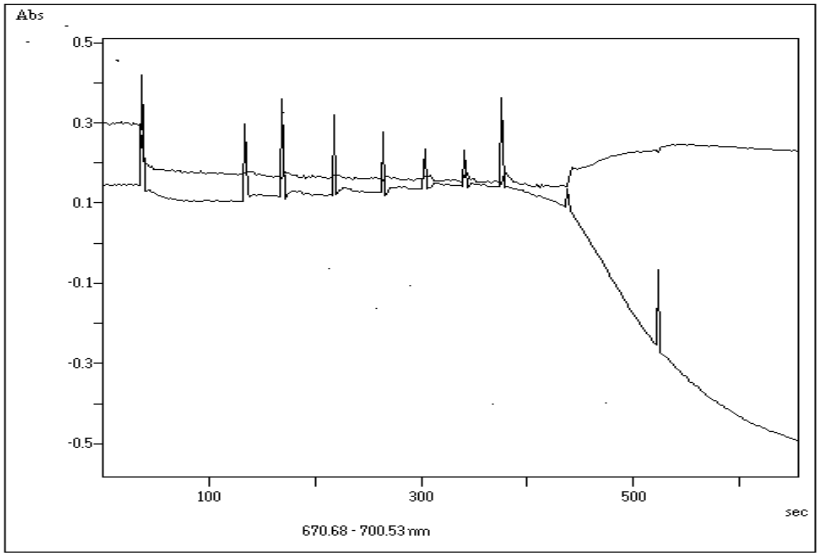
Results and discussion. *Mitochondria of the control rats.* The behaviour of the control mitochondria (C) in the 4 media used for suspension (incubation), regarding the kinetics of calcium fluxes, membrane potential and swelling, under stress conditions induced by calcium, are presented in Figs. 1-8. One of the curves in these figures (usually, the upper trace or the one noted *A*), represents the variation of absorbance difference between 650 nm (in the case of calcium fluxes) or 670 nm (in the case of $\Delta\Psi$) and 700 nm, whereas the second trace represents the absorbance change at 700 nm (the quasi-isobestic point of the two dyes), associated with the matrix swelling. Whereas the first is a continuous curve, lacking any electronic accidents (due to the differential way in which it was recorded), the "spikes" on the second trace reflect the electronic noise associated with the additions made into cuvettes. Although this noise is in general very visible (hence, the moments of addition are well marked), it must be mentioned that in some of the additions (see, for example, Figs. 1 and 6) the noise is barely perceivable. The 8 figures in this group should be considered in pairs (1-2, 3-4, 5-6 and 7-8), each pair reflecting the behaviour of mitochondria in one of the 4 media: MS, MKS, MKS–Mg and KSW, respectively.

From the analysis of the illustrations presented in these figures, one can notice the "saw tooth" behaviour in the upper traces of the odd number figures (1, 3, 5, 7), recorded by sequential additions of 12.5-μM calcium pulses. The number of teeth varies from 7-9 in the MS medium to 2-3 in the KSW medium, after which the absorbance increases gradually in a hyperbolic fashion, indicating the massive calcium efflux (release) associated with the permeability transition, as indicated by the strong absorbance decrease in the lower curve (or the one noted B) of the same figure. The behaviour of membrane potential is illustrated in the upper traces of the even number figures (2, 4, 6, 8), also in parallel with the absorbance decrease (lower trace) associated with the extensive swelling due to the permeability transition. As expected, the permeability transition, $\Delta\Psi$ collapse and calcium release occur at a lower and lower calcium loads as we pass from a medium almost devoid of ions (MS) to an almost completely ionic medium (KSW), which demonstrates the involvement in these events of ion transport phenomena at the level of the inner mitochondrial membrane. In addition, the lack of Mg enhances even more this behaviour, a fact which is in accord with what is known from the literature with regard to the regulating role of Mg^{2+} in the calcium fluxes [4, 64, 72].

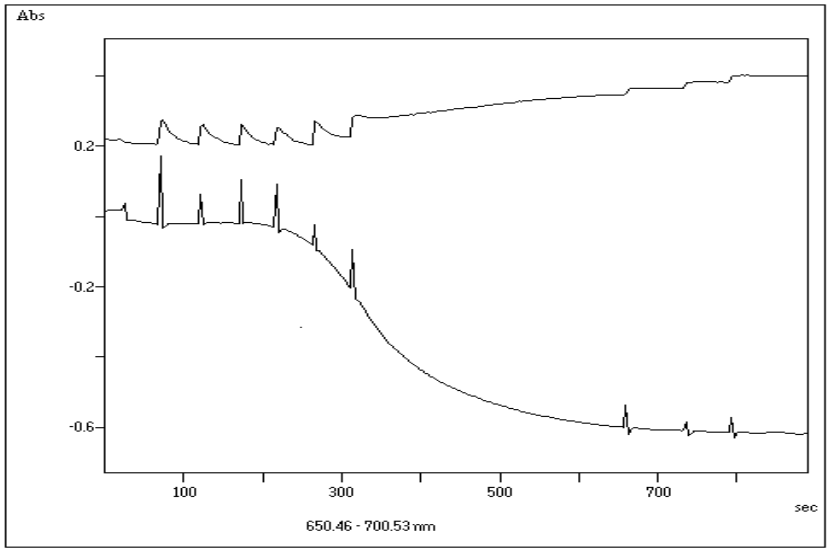


F i g . 1 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MS medium.*

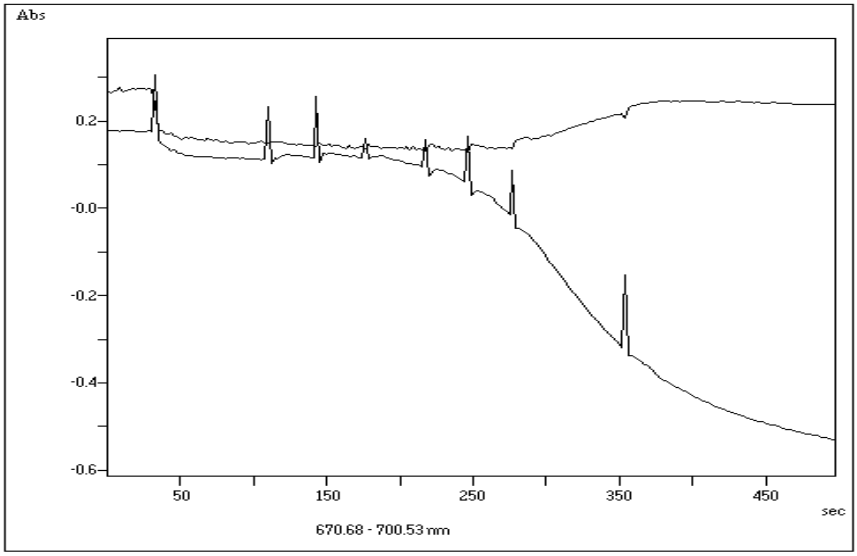
In all figures, the first spike is associated with the injection of succinate (2.5 mM), the rest being associated with $CaCl_2$ pulses of 12.5 μM each, except those added after Ca^{2+} release (or $\Delta\Psi$ collapse).



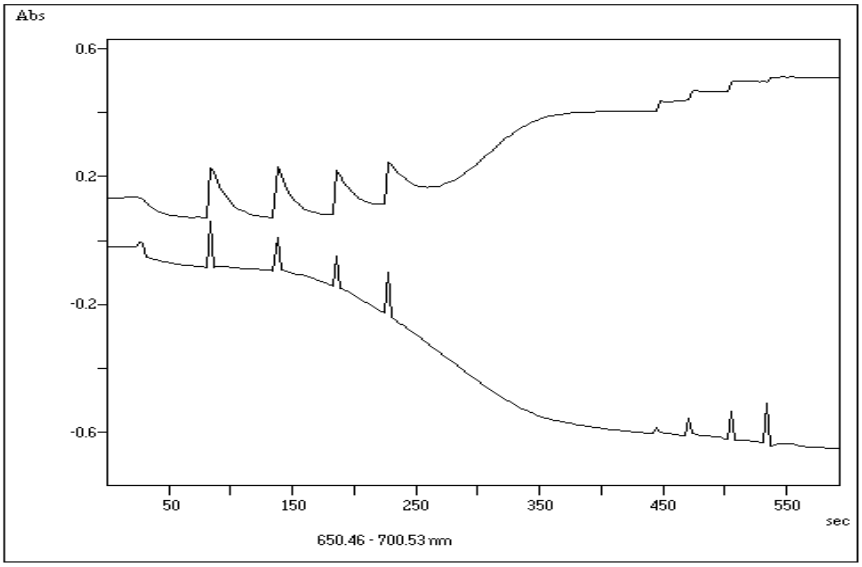
F i g . 2 . *Membrane potential* (upper curve) *and mitochondrial swelling* (lower curve) *in the MS medium*. Note the relatively close association between the initiation of $\Delta\Psi$ collapse (absorbance increase on the upper curve) and of mitochondrial swelling (absorbance decrease on the lower curve).



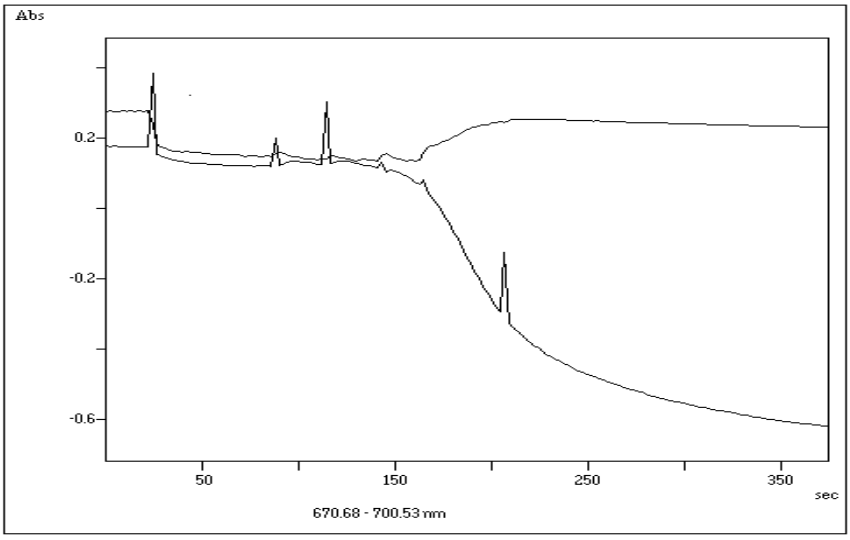
F i g . 3 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK medium*. Note the fewer pulses of Ca^{2+} (than in the MS medium) to which the mitochondria resist.



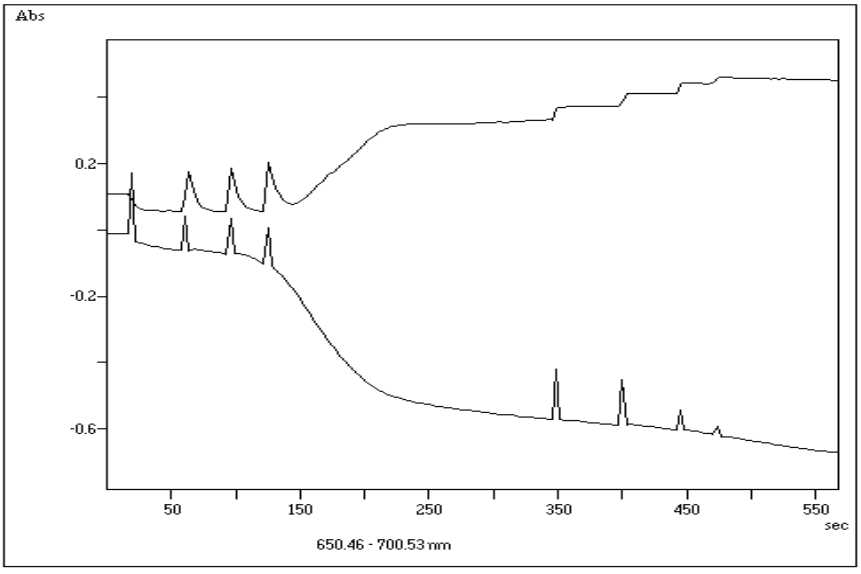
F i g . 4 . *Membrane potential* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK medium*. Note that the swelling begins sooner than the initiation of $\Delta\Psi$ collapse.



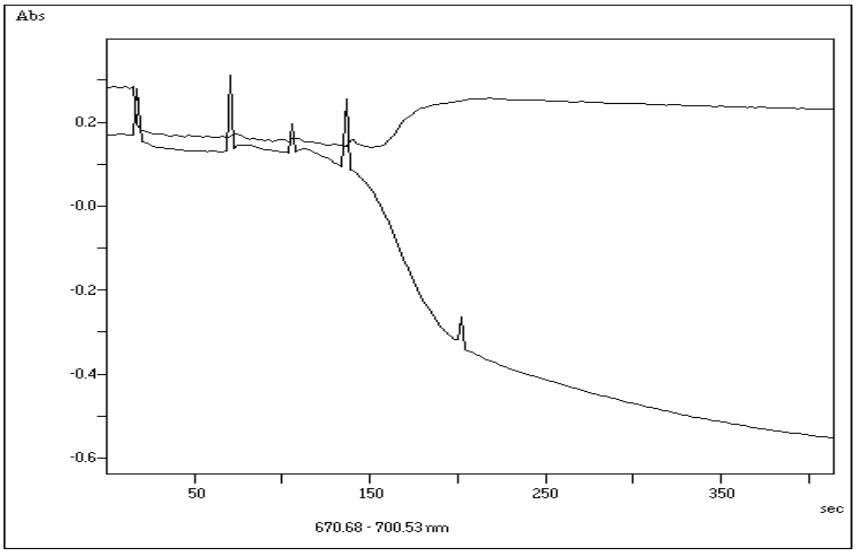
F i g . 5 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK–Mg medium*. The massive calcium efflux (release) occurs easier than in the presence of Mg^{2+}.



F i g . 6 . *Membrane potential* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK–Mg medium*. Calcium pulses 3 (140 s) and 4 (160 s) are accompanied by very small electronic noises.



F i g . 7 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the KSW medium*. Note that the swelling begins sooner than the release of Ca^{2+}.

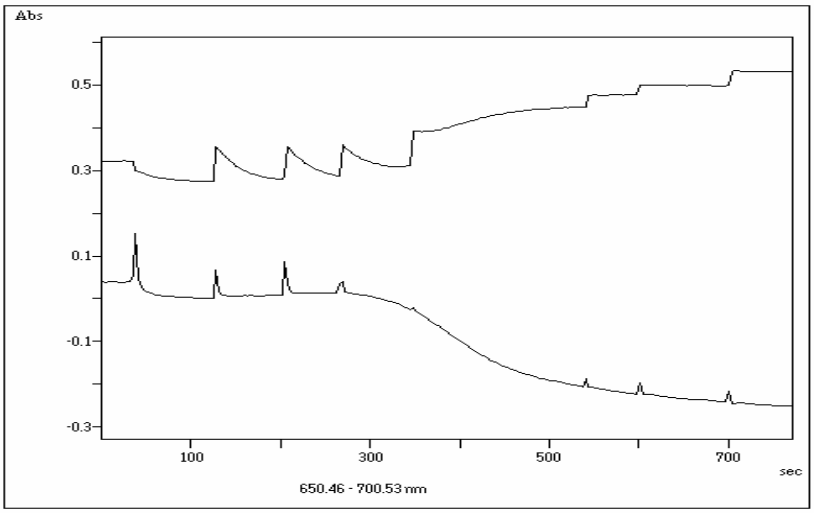


F i g . 8 . *Membrane potential* (upper curve) *and mitochondrial swelling* (lower curve) *in the KSW medium*. Note the difference between the beginning of swelling and of the ΔΨ collapse.

Despite a rather evident parallelism, at a closer analysis, one can observe that the swelling slightly precedes the other two phenomena. Thus, in Fig. 1, one can see that the swelling begins after the 6th calcium pulse while calcium release occurs after the 8th pulse. In Fig. 2, the swelling occurs after the 7th calcium pulse whereas ΔΨ collapse begins only after the 8th pulse. From Figs. 5-6 one can observe that the swelling begins after the second pulse while ΔΨ collapse and calcium release only after the 4th pulse. There are many discussions in the literature with regard to the succession of these events. Some researchers claim that the permeability transition (PT) may occur after ΔΨ collapse while others that ΔΨ can be preserved or restored after PT and cyt c release, leaving space for different scenarios regarding the exact relationship (conditioning) among these events (see [6, 14, 21, 31, 69, 70]). Our work proves that under relatively physiological conditions the first phenomenon to be triggered is the swelling, followed shortly by ΔΨ collapse and calcium release, which proceed apparently in parallel with the moment of maximum rate of swelling (hence, PT), and there is no recovery. One should not forget, however, that the phenomena recorded by us represent a statistical result of a relatively variable behaviour of a mitochondrial population *in vitro*. Therefore, we can not exclude that under *in situ* physiological conditions some of the mitochondria, that have suffered less, may recover. This possibility probably depends also on the intensity and duration of the stress factor. As regards the relationship between ΔΨ and calcium fluxes, this is more complicated and the relatively slow response of our instrument does not allow a clear-cut conclusion. In fact, we think that the problem has

already been solved more than two decades ago by Bernardi and Azzone [2], which showed that the massive calcium efflux begins when $\Delta\Psi$ decreases below 130 mV.

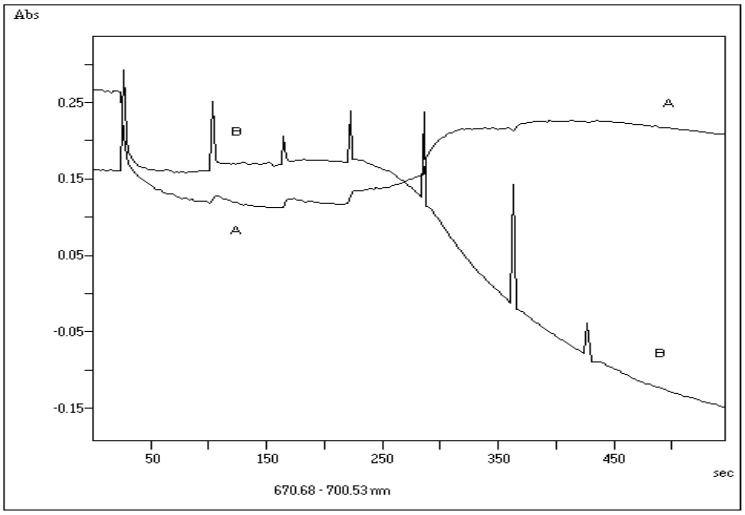
Mitochondria of ethanol-fed rats. Before presenting the spectrophotometric recordings, we have to make the observation that of the 8 treated animals only 6 behaved in a consistent manner, characteristic for alcohol-fed rats, while two of them had a totally different behaviour. The quantitative measurements that we are going to present later in this article will prove that the two (sub)groups differ significantly from a statistic point of view. While the group of 6 (designated $A_{1\text{-}6}$) showed a higher sensitivity to the calcium stress, the other group (designated $A_{7,8}$) demonstrated a behaviour somewhat similar to that of the control group (C), one of the rats even displaying a higher resistance to the calcium stress. The behaviour of mitochondria obtained from both subgroups of alcohol-fed rats is illustrated in Figs. 9-16.



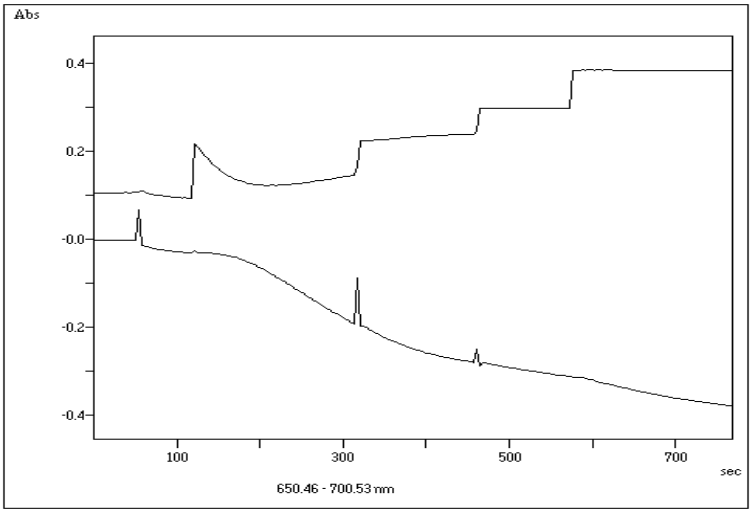
F i g . 9 . *Calcium fluxes* (upper curve) *and matrix swelling* (lower curve), *in mitochondria of an alcoholic rat with typical behaviour ($A_{1\text{-}6}$), recorded in the MS medium*.

Figs. 9-12 present illustrations taken from the $A_{1\text{-}6}$ subgroup. The most striking observation is the increased sensitivity of mitochondria to calcium. For example, in the MS medium, where the resistance is the highest, PT occurs after the third calcium pulse (Figs. 9-10) while in the C group this happens after 6-7 pulses. The same is true for calcium release (Fig. 9) and $\Delta\Psi$ collapse (Fig. 10). The situation is worse in the ionic media. Thus, in the KSW medium, the phenomena discussed occur after a single pulse of calcium (Fig. 11). Moreover, as can be seen from Fig. 12, if the addition of calcium is a little delayed, PT and $\Delta\Psi$ collapse occur spontaneously. This demonstrates that the chronic ethanol feeding had a rather grave effect on the

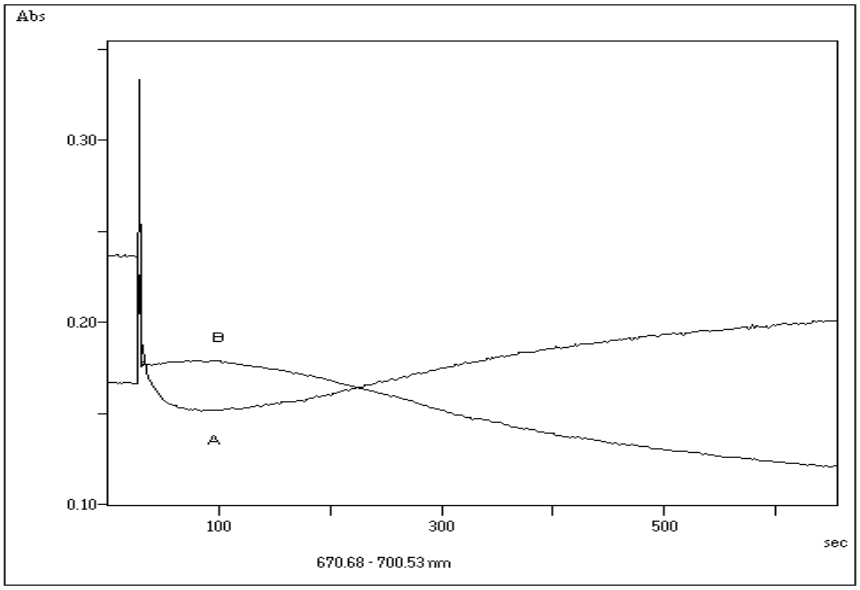
liver of the animals, despite the fact that their average body weight was slightly higher than in the control animals, although not statistically significant. These results point to the fact that the mitochondrial membranes of the alcoholic rats are extremely fragile, with a high degree of ion permeability, ready to give up to slight metabolic stresses and release the components of the intermembrane space and probably even of the matrix, despite the apparent well-being of the animals.



F i g . 1 0 . *Membrane potential* (curve A) *and matrix swelling* (curve B), *in mitochondria of an alcoholic rat with typical behaviour (A_{1-6}), recorded in the MS medium.*



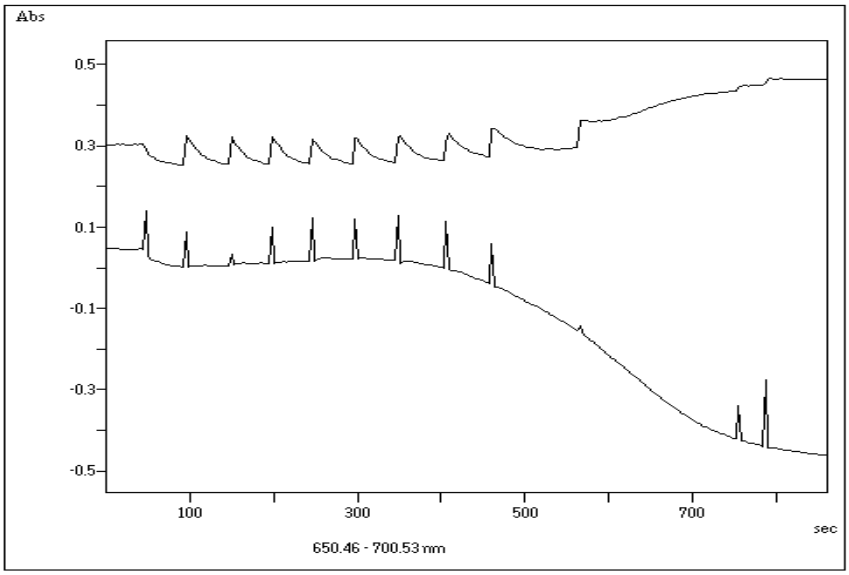
F i g . 1 1 . *Calcium fluxes* (upper curve) *and matrix swelling* (lower curve), *in the KSW medium, in mitochondria of an alcoholic rat with typical behaviour (A_{1-6}).*



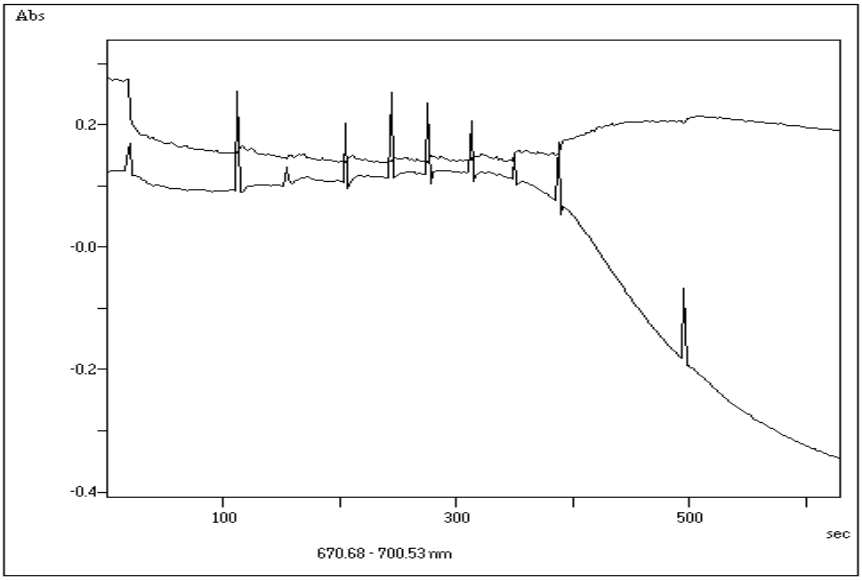
F i g . 1 2 . *Spontaneous dissipation of membrane potential* (curve A) *and matrix swelling* (curve B), *in the KSW medium, in mitochondria of an alcoholic rat with typical behaviour (A_{1-6}).*

As regards the behaviour of mitochondria from atypical alcoholic rats ($A_{7,8}$), it is illustrated in Figs. 13-16. One can observe that in the basically nonionic medium (MS), the release of calcium and $\Delta\Psi$ collapse occur after 8 pulses of calcium and the swelling begins after 6 pulses (Figs. 13-14), while in ionic media devoid of Mg^{2+} these phenomena occur after 3-4 pulses of calcium (Figs. 15-16), similar to what we have seen in the control group. Taking into consideration the inter-individual variability in regard to the enzymes (and enzyme induction capacity) involved in the degradation of ethanol and acetaldehyde (the metabolite basically responsible for alcohol intolerance), such a behaviour is not totally unexpected. In fact, the phenomenon is known under the name of alcohol tolerance (see, for example, [16]).

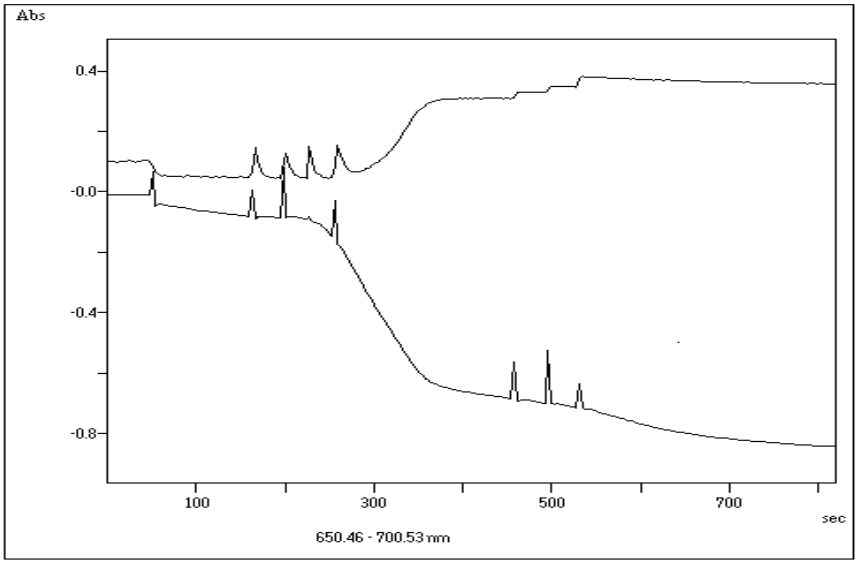
Quantitative comparison regarding the sensitivity of mitochondria to calcium. As mentioned before, if we take into consideration the number of calcium pulses (12.5 μM each or 12.5 nmols/mg protein) necessary to induce calcium release, the sensitivity of mitochondria can readily be quantified using relatively small integers or fractions equal to 0.5 and computing means that can be compared by the use of the Student *t* test or other more sophisticated tests. The first comparison of this kind that is presented in Table 1 proves that the alcoholic group (A) did not respond in a homogeneous manner to ethanol treatment and that there are statistically significant differences between the A_{1-6} and $A_{7,8}$ subgroups.



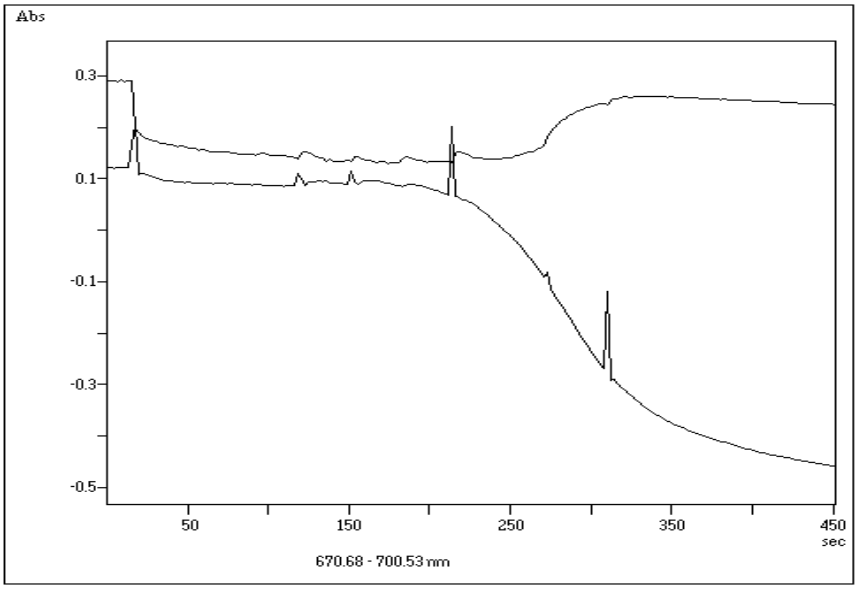
F i g . 1 3 . *Calcium fluxes* (upper curve) *and matrix swelling* (lower curve), *in mitochondria of an alcoholic rat with atypical behaviour ($A_{7,8}$), recorded in MS medium.*



F i g . 1 4 . *Membrane potential* (upper curve) *and matrix swelling* (lower curve), *in mitochondria of an alcoholic rat with atypical behaviour ($A_{7,8}$), recorded in MS medium.*



F i g . 1 5 . *Calcium fluxes* (upper curve) *and matrix swelling* (lower curve), *in mitochondria of an alcoholic rat with atypical behaviour ($A_{7,8}$), recorded in KSW medium.*



F i g . 1 6 . *Membrane potential* (upper curve) *and matrix swelling* (lower curve), *in mitochondria of an alcoholic rat with atypical behaviour ($A_{7,8}$), recorded in MSK–Mg medium.*

Table 1

Comparison of mitochondrial sensitivity in different groups of alcohol-fed rats

Statistical parameter	$\bar{x}$ ± SEM		t	n' = $n_1 + n_2 - 2$	p
Suspending medium \ Group	A_{1-6}	$A_{7,8}$			
MS	4.750 ± 0.750	10.000 ± 1.000	4.200	2	≥0.05
MSK	2.333 ± 0.105	5.500 ± 0.881	10.408	6	< 0.001
MSK–Mg	2.000 ± 0.129	5.000 ± 1.000	5.692	6	< 0.002
KSW	1.000 ± 0.000	3.500 ± 0.500	6.708	3	< 0.01

The fact that for the MS medium the differences, although very large, are only at the limit of significance can be explained by the small number of measurements performed in this medium. For the rest of the media, however, the differences between means are from very to highly significant. Under these circumstances it is recommended to consider the two subgroups as distinct and to compare them to the control. By doing this, we established that there is no statistical difference between $A_{7,8}$ and C. In turn, between A_{1-6} and C, with one exception, all the differences are statistically highly significant ($p < 0.001$), as can be seen from Table 2. The exception is again due to the small number of measurements performed in the MS medium.

Table 2

Mitochondrial sensitivity in typical alcohol-fed rats in comparison to the control

Statistical parameter	$\bar{x}$ ± SEM		t	n' = $n_1 + n_2 - 2$	p
Suspending medium \ Group	C	A_{1-6}			
MS	7.750 ± 0.250	4.750 ± 0.750	3.795	2	≥0.05
MSK	5.214 ± 0.343	2.333 ± 0.105	7.487	11	< 0.001
MSK–Mg	3.286 ± 0.184	2.000 ± 0.129	5.520	11	< 0.002
KSW	3.143 ± 0.143	1.000 ± 0.000	9.488	8	< 0.01

These quantitative data confirm the conclusions drawn from comparing the spectrophotometric kinetic recordings, *i.e.*, the alcoholic rats are characterised by a high sensitivity to metabolic stress, specifically to that exerted by an increased calcium concentration. Even if the comparison is made with the entire alcoholic group (A), there is a very significant difference ($p < 0.01$) in the case of the most relevant medium (MSK), where the number of recordings is the largest.

We have also tried to see whether the extension of swelling at different calcium loads is also different for different groups and subgroups, because in a previous work [63] we observed a higher tendency to swelling for mitochondria of alcoholic rats, especially at high calcium loads (50-250 μM) and long incubation times (20-30 min). The conclusion is that although there are certain differences

they are in most cases not significant and not always in the expected direction. On the contrary, the amplitude of swelling is usually smaller in alcoholic rats, even though the differences are statistically significant only in a few cases, a fact that can be seen from Table 3, where our data are presented as percent of absorbance decrease with reference to the maximum absorbance (A_0), usually attained immediately after the addition of mitochondria to the complete incubation medium. We shall try an explanation of this apparent discrepancy with our previous observations in an accompanying paper [66].

Table 3

Amplitude of mitochondrial swelling (mean values) **at different calcium loads and times** (SEM = standard error of mean; n = number of animals tested; * $p < 0.05$; ** $p < 0.01$)

Time (min)		1	5	10	20	30
Group	$[Ca^{2+}]$ (mM)	$-\Delta A/A_0$ (%) ±SEM (n)	$-\Delta A/A_0$ (%) ±SEM (n)	$-\Delta A/A_0$ (%) ±SEM (n)	$-\Delta A/A_0$ (%) ±SEM (n)	$-\Delta A/A_0$ (%) ±SEM (n)
C	0	2.57±0.59 (8)	4.83±0.46 (8)	6.55±1.12 (8)	21.62±3.77 (8)	45.15±2.82 (6)
	25	2.91±0.45 (8)	19.43±4.51 (8)	43.98±2.42 (8)	56.34±1.78 (8)	60.39±0.90 (5)
	200	27.39±2.62 (7)	46.61±7.03 (7)	52.73±2.31 (7)	57.22±2.04 (7)	61.89±0.30 (2)
A_{1-6}	0	1.80±0.39 (6)	2.97±0.58 (6)*	3.66±0.87 (6)	17.35±4.23 (6)	45.75±4.16 (4)
	25	4.65±1.90 (6)	22.84±6.65 (6)	40.33±4.01 (6)	49.27±3.34 (6)*	–
	200	26.36±1.87 (6)	41.57±2.16 (6)	46.30±2.34 (6)	51.07±2.53 (6)	52.54±4.07 (4)
A	0	1.80±0.29 (8)	3.25±0.46 (8)*	3.92±0.66 (8)	17.76±2.91 (8)	41.53±4.46 (6)
	25	3.99±1.46 (8)	18.26±5.71 (8)	32.84±5.80 (8)	48.54±1.07(8)**	55.36±0.95(2)*
	200	26.49±1.67 (8)	42.19±1.63 (8)	47.15±1.84 (8)	52.11±2.00 (8)	54.84±2.98 (6)

As regards the aim of the present study, *i.e.*, trying to clarify the succession of the phenomena responsible for (or at least associated with) the release of the apoptotic factors from mitochondria, in the first place that of cyt c, necessary for establishing a correct causal relationship in the interpretation of the apoptotic process, we have succeeded in identifying indubitably the matrix swelling as the event that precedes both $\Delta\Psi$ collapse and calcium release and established that the latter events are triggered as the swelling approaches the maximum rate, *i.e.*, they are caused by the permeability transition and not vice-versa. Of course, one should not forget that our recordings are an expression of the statistical behaviour of a mitochondrial population, these phenomena stimulating each other, as more and more mitochondria suffer the permeability transition. The sigmoid aspect of the swelling curve is in fact suggestive of co-operative phenomena. Also, along with the loss of membrane potential there is a loss of mitochondrial ability to perform oxidative phosphorylation (see [10, 28, 61] and the references therein). Consequently, the lack of energy (under the form of ATP) enhances the metabolic stress and accentuates the permeability transition and all the associated phenomena, even being able to change the type of cell death, from apoptosis to necrosis (see, for example, [37, 47]). The mutual conditioning of these phenomena is proved also by the fact that when the metabolic stress is very intense, the association between the 3 phenomena studied by us is so tight that

the limited time resolution of our spectrophotometer does not allow their temporal dissociation. We observed this situation with very high concentrations of calcium, added in one pulse, or in the presence of other strong apoptotic inducers [65].

In **conclusion**, our present results are a promising preliminary step, which, hopefully, in corroboration with the electron microscopy and biochemical assays that we are going to perform on the material taken at different points in our experiments, will allow us to clarify other controversial problems of apoptosis, or at least of the cell death induced by alcohol, *i.e.*, of the general relationship between apoptosis and necrosis in the alcoholic liver disease.

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EFFECT OF ACUTE ENDOTOXIN ADMINISTRATION TO RATS ON MEMBRANE PERMEABILITY AND RELATED FUNCTIONAL PARAMETERS OF LIVER MITOCHONDRIA

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SUMMARY. − Male white Wistar rats were maintained in our animal facility for 14-18 weeks and the evolution of their body weight was assessed periodically. 24 hrs before sacrificing, part of the control animals were injected with either 0.5 or 2 mg bacterial endotoxin/kg body weight. Another group of rats was chronically fed ethanol (1.5 ml of 48% alcohol/100 g body weight, daily) and some of these alcoholic rats also received 0.5 or 2 mg endotoxin/kg body weight 24 hrs prior to sacrifice. The liver of the animals was used for mitochondrial preparations and in some cases also for samples of electron microscopy. Mitochondrial membrane permeability was assessed indirectly from the matrix swelling in the presence of different concentrations of calcium. In addition, membrane potential ($\Delta\Psi$) and calcium fluxes were also monitored spectrophotometrically, as described in our previous paper (Tarba and Suărăşan [46]). The capacity of mitochondria to resist to different calcium loads was estimated qualitatively from such spectrophotometric recordings and also quantified by counting the number of calcium pulses (of 12.5 μM each) needed for the triggering of the permeability transition, $\Delta\Psi$ collapse and calcium release. Predictably, all the phenomena tested occur faster (at shorter times and/or lower calcium concentrations) in ionic media, especially in those that lack magnesium. However, statistically, no significant differences were observed between the results obtained with the two doses of endotoxin. Also, a certain degree of similarity could be observed between the ethanol-fed and the endotoxin-treated rats. On the other hand, very significant differences were observed between the mitochondria of the endotoxin-treated rats (as a single group) and of the control rats. Thus, mitochondria from endotoxin-treated animals are more sensitive to calcium loads than those of the (non-alcoholic) control, they approaching the behaviour of the mitochondria from alcohol-fed rats, although the sensitivity to calcium is somewhat lower. This lower sensitivity can be seen in ionic media, especially in the swelling medium (KSW), where the differences are highly significant ($p<0.001$). The mitochondria of double-treated animals (endotoxin-injected alcohol-fed rats) show a more complex behaviour and less homogeneity. Their sensitivity to metabolic stress is in general higher than that of the simply endotoxin-treated animals, but comparable to that of the simply ethanol-fed rats, although in ionic suspending media membrane permeability actually tends to be lower, a fact that must be attributed to the endotoxin treatment. A certain degree of dissociation of the $\Delta\Psi$ collapse from the matrix swelling and calcium release was also observed. The fact that $\Delta\Psi$ collapse occurs apparently at a later time than the matrix swelling and calcium release is interpreted by us as an indication of a differential sensitivity of different types of hepatic mitochondria to the metabolic stress factors, such as chronic ethanol feeding and acute LPS administration.

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As discussed in a more extensive way in previous articles [44, 46], chronic ethanol administration has been generally associated with specific hepatic structural and functional alterations known as the alcoholic liver disease (ALD). However, the exact mechanism of this disease is not entirely known. For decades, ALD has been attributed to necrotic events associated, among other things, with the production of proinflammatory cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) [6, 10, 25, 47]. However, relatively recent data indicate that under certain circumstances both the acute and chronic ethanol administration is associated with a process of liver apoptosis [1, 7, 14, 20, 30, 34] which, paradoxically, can facilitate latter necrotic events leading to ALD [1, 7, 8, 16, 26, 33, 47]. What exactly determines the type of cell death (apoptosis or necrosis) is not entirely understood, although it seems to be related finally to the amount of ATP produced by mitochondria, as described elsewhere [21, 22, 31]. Different properties, such as the rate and the intensity of the triggering agents, as well as the presence/absence of certain factors having sensitising, modulating or even protective effects determine the final outcome. Among other factors, for example, the excessive alcohol consumption increases the absorption from the gut of bacterial endotoxin or lipopolysaccharide (LPS) [3, 27, 32, 37], a toxin produced by Gram-negative bacteria, which is associated with the stimulation of the cytokine secreting cells (the hepatic macrophages or Kupffer cells) [5, 8, 16, 25, 26]. LPS seems to exert its effects mainly through TNF-related events [6, 8, 13, 16, 25, 47], although contradictory results are sometimes reported by different authors (see [23, 28, 33]). On the other hand, as anticipated above, progresses made during the last decade in the study of apoptosis have placed mitochondria in a central position in controlling this very complicated process [1, 2, 10, 16, 18, 19, 39]. It seems that the main event in the control of apoptosis by mitochondria is the formation of the so-called permeability transition pore (PTP), which represents the structural basis for a drastic change in membrane permeability associated with the release of the apoptogenic factors, including cytochrome c [4, 14, 18, 19, 24, 35, 36, 39]. Many studies have tried lately to link alcohol consumption and/or endotoxicosis to changes in mitochondrial membrane permeability, suggesting an increased permeability [14, 20, 26, 33, 34], but, in our opinion, the results are controversial [38, 43-46].

The present article is part of a larger study aimed at establishing the order of events leading to the release of the apoptogenic factors from the mitochondrial intermembrane space and uses alcohol and endotoxin as metabolic stress factors for the induction of apoptosis and/or necrosis in animals kept under more natural conditions (see *Material and Methods*) than usually reported in the literature. In a previous article [46] we dealt mainly with the effect of alcohol, whereas the present study is dedicated to the effect of acute endotoxin administration, both in control (*i.e.*, non-alcoholic) and alcoholic rats. Two preliminary reports on our results have also appeared [38, 45].

Material and methods. *Animal treatments and preparation of mitochondria*. Male white Wistar rats were kept under normal conditions in our animal facility for 14-18 weeks, starting from an average weight of 120 g/individual, while the evolution of their weight was assessed periodically. The rats were kept on a normal diet (a premix containing all the ingredients of the Larsen diet), with free access to water. One group served as control (C), while in another group (A) each rat was supplemented daily with 1.5 ml of 48% ethanol/100 g body weight. In both groups, 24 hrs before the sacrifice, part of rats were injected with either 0.5 or 2 mg LPS/kg body weight (b.w.), administered in the caudal vein. All the rats were fasted for the last 24 hrs and sacrificed by decapitation after a slight anaesthesia. Small pieces of liver were taken in some cases and prepared for electron microscopy, while the rest of the liver was used for preparation of mitochondria, essentially according to Johnson and Lardy [17], in a medium containing 200 mM mannitol, 70 mM sucrose, 5 mM Hepes-KOH (pH 7.37) and 0.5 mM Na-EDTA. The washing medium (which also served as a preserving medium) lacked the chelating agent (EDTA).

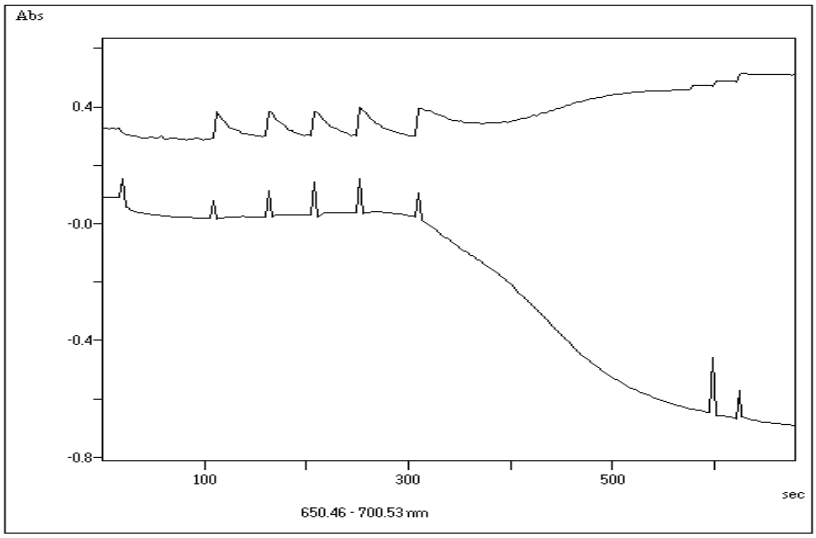
Spectrophotometric measurements. Using the principles and methodology described in our previous paper [46], a diode-array spectrophotometer (Specord S 100B, Analytik Jena, Germany) was employed for measurements of membrane potential and/or calcium fluxes in parallel with the matrix swelling, while the extension (amplitude) of swelling was quantitatively assessed on separate recordings, where the reaction was triggered by the addition of mitochondrial protein itself. The suspending media used for incubations are designated as follows: MS (210 mM mannitol, 70 mM sucrose, 1.5 mM $MgCl_2$); MSK (110 mM mannitol, 40 mM sucrose, 65 mM KCl, 1.5 mM $MgCl_2$); MSK–Mg (the same as MSK, but without Mg). All these media also contained 5 mM Hepes (pH 7.37) and 1 mM KP_i. Mg (as $MgCl_2$) and phosphate were usually added directly to the cuvettes. In addition, a special swelling medium (KSW) was also used. It contained 100 mM KCl, 50 mM sucrose, 10 mM Hepes and 5 mM KP_i. Different amounts of $CaCl_2$ were either added gradually to the spectrophotometer cuvettes, where the incubation took place, or in one pulse, up to the desired final concentration. Since succinate (2.5 mM) was used as a respiratory substrate, 8 μM rotenone was also present in the cuvettes. Although we made some attempts at obtaining precise values of membrane potential amplitude and calcium concentrations (as described elsewhere [44]), the most reliable quantitative result was reached by counting the number of calcium pulses (of 12.5 μM each) necessary to be added to the mitochondrial suspension in order to induce the calcium release, a phenomenon which is always more-or-less associated with the permeability transition (as monitored by matrix swelling) and $\Delta\Psi$ collapse. The statistical significance of the differences between groups was computed by the Student *t* test.

Results and discussion. *Mitochondria of non-alcoholic LPS-injected rats.* Based on previous experiments in which we observed a dose-dependent response to acute LPS administration [43], we selected two different concentrations of endotoxin to be tested: 0.5 mg LPS/kg body weight (b.w.) and 2 mg LPS/kg b.w. Although there are clear differences in comparison with the non-alcoholic group (control, C), the

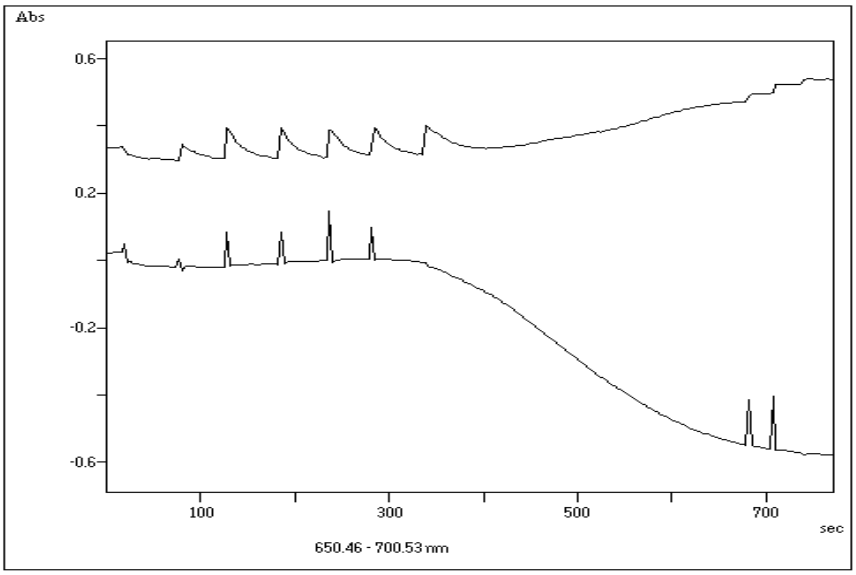
differences between the two LPS-injected subgroups are in general small. The spectrophotometric results obtained in several media with the two subgroups (designated as 0.5-LPS and 2-LPS, respectively) are compared in Figs. 1-10.

From Figs. 1 and 2 we can compare the behaviour of mitochondria belonging to the two subgroups, as regards the calcium fluxes, whereas from Figs. 3 and 4 the corresponding aspects of the membrane potential can be compared. It can be seen that not only the number of calcium pulses taken up by mitochondria before the beginning of calcium release and $\Delta\Psi$ collapse are very close (if not identical), but the general aspects of the recordings are similar, including the lower curves which represent the matrix swelling. At the same time, if we compare these recordings with those of the control rats (see our previous paper [46]), a higher sensitivity to calcium can be observed in the present experiment.

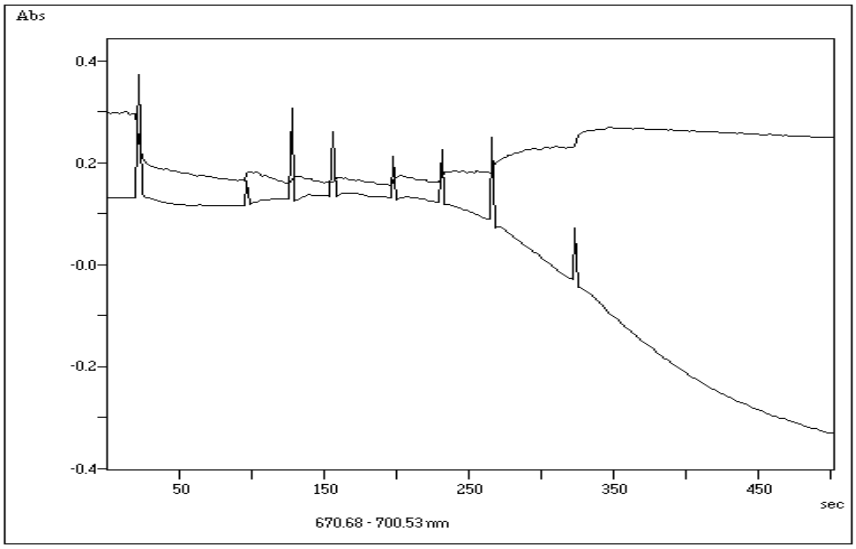
In general, the similarity of behaviour of the two LPS subgroups is also preserved in media with a higher ionic content (Figs. 5-10), where the number of calcium pulses taken up by mitochondria is smaller, but certain subtle differences also become apparent, as can be best seen from comparing Figs. 7 and 8. An obvious dissociation between the initiation of the permeability transition (absorbance decrease on the lower curve) and the beginning of $\Delta\Psi$ collapse (absorbance increase on the upper curve) can be observed in Fig. 8. This type of behaviour is in general more evident for mitochondria of the 2-LPS subgroup, especially in media lacking Mg^{2+} (*i.e.*, MSK–Mg and KSW). Figs. 9 and 10 show the similarity of behaviour of the two subgroups with respect to calcium fluxes.



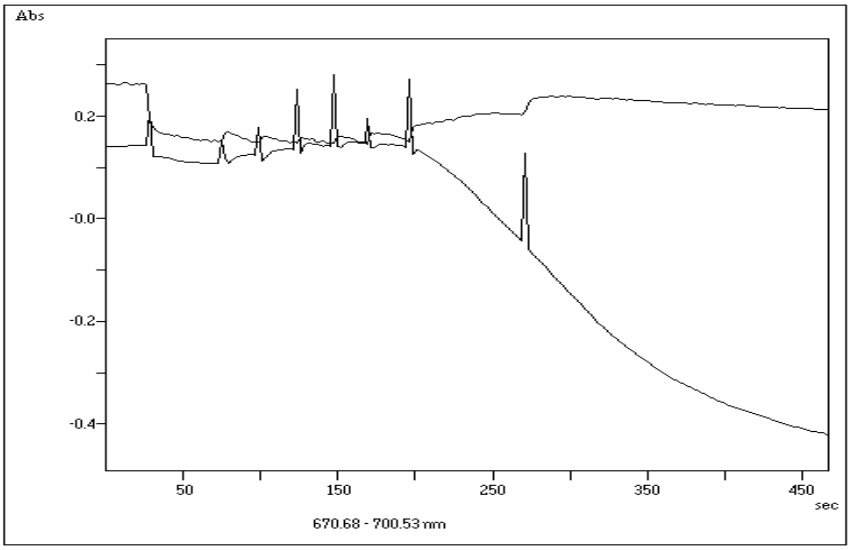
F i g . 1 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MS medium, in a preparation belonging to a rat from the 0.5-LPS subgroup.* In all figures, the first spike is associated with the injection of succinate (2.5 mM), the rest being associated with $CaCl_2$ pulses of 12.5 μM each, except those added after Ca^{2+} release (or $\Delta\Psi$ collapse).



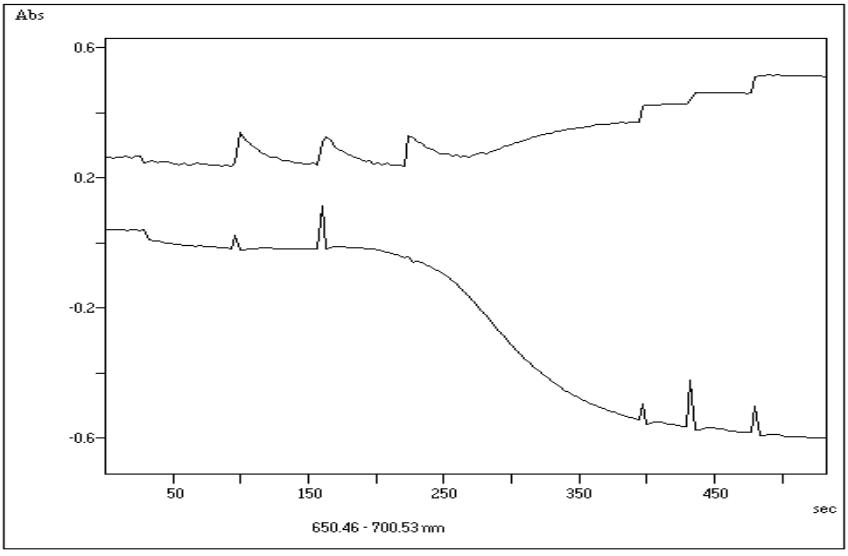
F i g . 2 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MS medium, in a preparation belonging to a rat from the 2-LPS subgroup.*



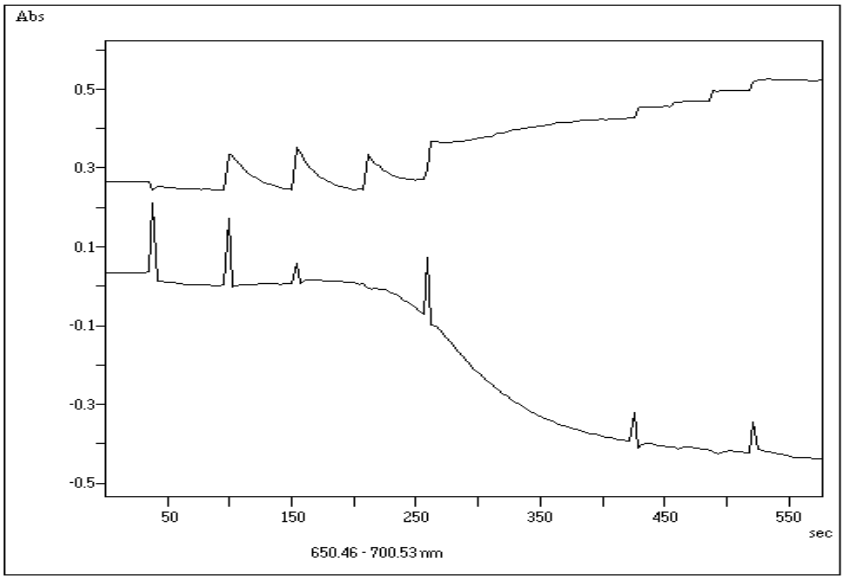
F i g . 3 . *Membrane potential* (upper curve) *and mitochondrial swelling* (lower curve) *in the MS medium, in a preparation belonging to a rat from the 0.5-LPS subgroup.* Note the relatively close association between the initiation of $\Delta\Psi$ collapse (absorbance increase on the upper curve) and of the mitochondrial swelling (absorbance decrease on the lower curve).



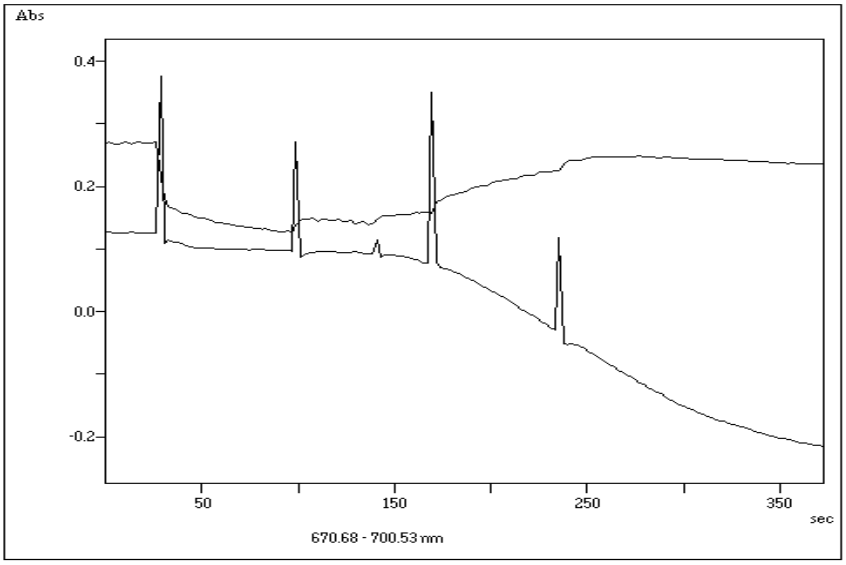
F i g . 4 . *Membrane potential* (upper curve) *and mitochondrial swelling* (lower curve) *in the MS medium, in a preparation belonging to a rat from the 2-LPS subgroup.*



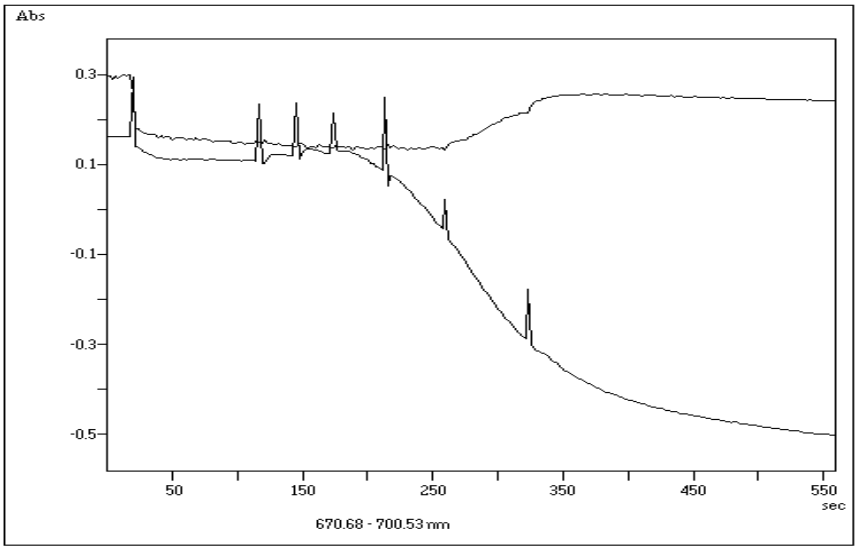
F i g . 5 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK medium, in a preparation belonging to a rat from the 0.5-LPS subgroup.* Note the smaller number of calcium pulses (than in the MS medium) to which mitochondria can resist (cf. Fig. 1).



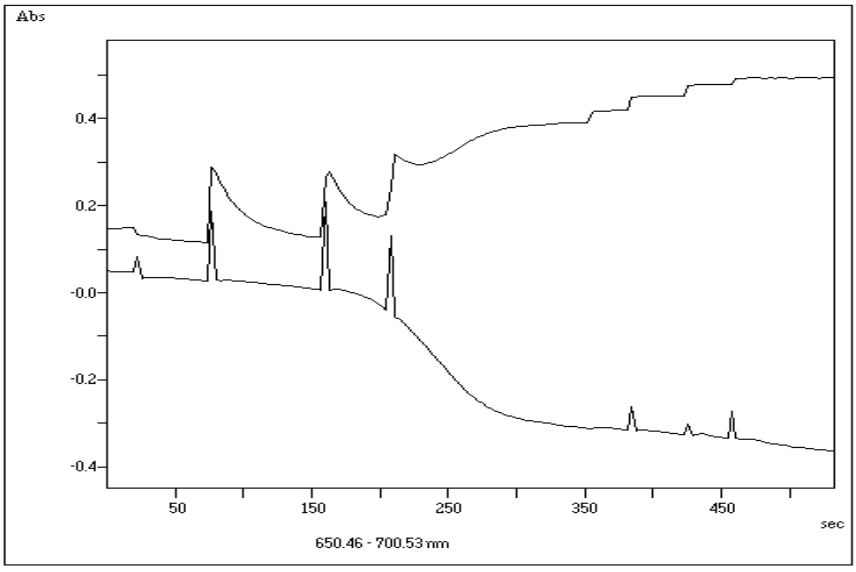
F i g . 6 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK medium, in a preparation belonging to a rat from the 2-LPS subgroup.*



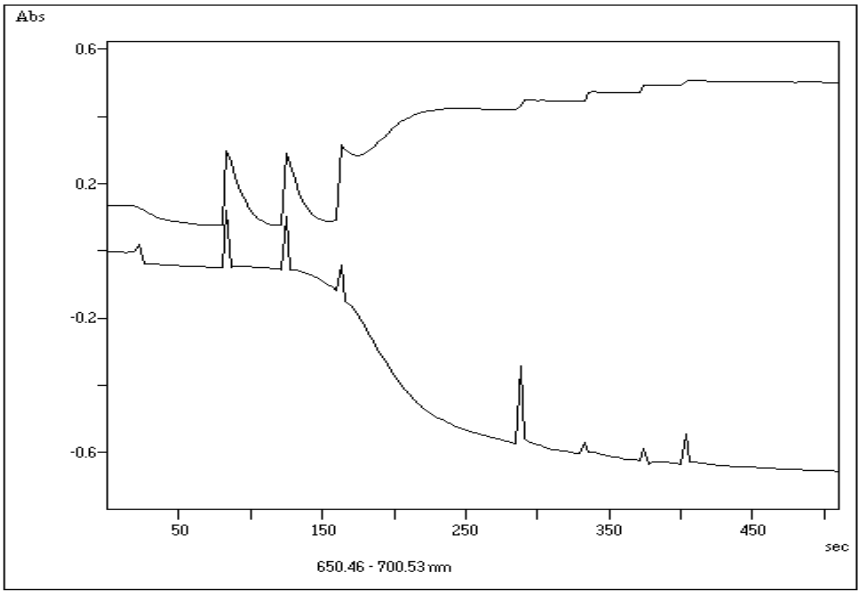
F i g . 7 . *Membrane potential* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK medium, in a preparation belonging to a rat from the 0.5-LPS subgroup.*



F i g . 8 . *Membrane potential* (upper curve) *and mitochondrial swelling* (lower curve) *in the MS medium, in a preparation belonging to a rat from the 2-LPS subgroup.* Note the dissociation between the initiation of swelling and the beginning of $\Delta\Psi$ collapse.



F i g . 9 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK-Mg medium, in a preparation belonging to a rat from the 0.5-LPS subgroup.*



F i g . 1 0 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK-Mg medium, in a preparation belonging to a rat from the 2-LPS subgroup.*

If a strictly quantitative comparison is made, in terms of the number of calcium pulses to which mitochondria resist before starting to swell, the differences between the two LPS subgroups are not statistically significant. Therefore, for comparisons with other groups, we pooled together the two LPS subgroups. Statistically, very significant differences are obtained in this way if the comparison is made with the control group (C), as can be observed from Table 1, whereas if the comparison is made with the alcoholic group (A) the differences are smaller and significant only in MSK and KSW media, as can be seen from Table 2. It must be stressed, however, that the differences are negative in comparison with the control and positive in comparison to the alcohol-fed rats, meaning that the effect of the acute LPS administration, even though obvious, is less strong than that of the chronic ethanol feeding.

Table 1

Mitochondrial sensitivity of the LPS-treated group as compared to the control

Statistical parameter	$\bar{X}$ ± SEM		T	n' = $n_1 + n_2 - 2$	p
Suspending medium \ Group	C	LPS			
MS	7.750 ± 0.250	5.250 ± 0.339	3.981	6	< 0.01
MSK	5.214 ± 0.343	3.125 ± 0.190	7.567	19	< 0.001
MSK–Mg	3.286 ± 0.184	2.300 ± 0.200	3.567	10	< 0.01
KSW	3.143 ± 0.143	2.083 ± 0.091	6.122	11	< 0.001

Table 2

Mitochondrial sensitivity of the LPS-treated group as compared to the alcohol-fed group

Statistical parameter	$\bar{x}$ ± SEM		T	n' = $n_1 + n_2 - 2$	p
Suspending medium \ Group	A_{1-6}	LPS			
MS	4.750 ± 0.750	5.250 ± 0.339	0.750	6	>> 0.1
MSK	2.333 ± 0.105	3.125 ± 0.190	4.683	18	< 0.001
MSK–Mg	2.000 ± 0.129	2.300 ± 0.200	1.303	9	> 0.1
KSW	1.000 ± 0.000	2.083 ± 0.091	8.878	7	< 0.001

Approximately the same conclusions can be drawn if the amplitude of swelling (calculated as percent of absorbance decrease relative to the maximum absorbance) is compared at different times, especially for higher calcium loads (25 and 200 μM). From Table 3, one can see significant differences in comparison to the control (C), whereas the differences are in general not significant if comparison is made with the ethanol-fed group (not presented).

Table 3

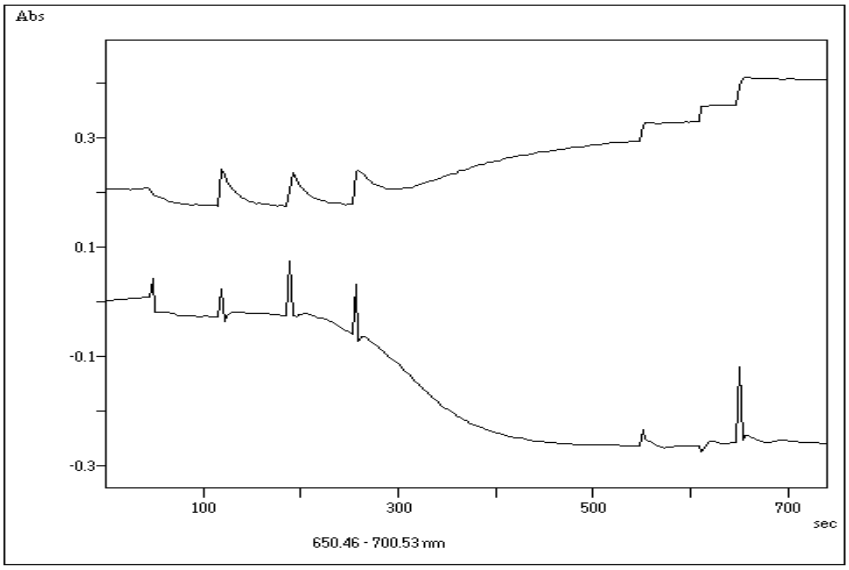
Amplitude of mitochondrial swelling (mean values) **at different calcium loads and times** (SEM = standard error of mean; n = number of animals tested; * $p < 0.05$; ** $p < 0.01$)

Time (min)		1	5	10	20	30
Group	$[Ca^{2+}]$ (mM)	$-\Delta A/A_0$ (%) ±SEM (n)	$-\Delta A/A_0$ (%) ±SEM (n)	$-\Delta A/A_0$ (%) ±SEM (n)	$-\Delta A/A_0$ (%) ±SEM (n)	$-\Delta A/A_0$ (%) ±SEM (n)
C	0	2.57±0.59 (8)	4.83±0.46 (8)	6.55±1.12 (8)	21.62±3.77 (8)	45.15±2.82 (6)
	25	2.91±0.45 (8)	19.43±4.51 (8)	43.98±2.42 (8)	56.34±1.78 (8)	60.39±0.90 (5)
	200	27.39±2.62 (7)	46.61±7.03 (7)	52.73±2.31 (7)	57.22±2.04 (7)	61.89±0.30 (2)
LPS	0	2.23±0.22 (14)	3.44±0.50 (14)	5.81±1.41 (14)	26.25±2.46 (14)	41.83±2.99 (6)
	25	2.83±0.47 (13)	20.63±4.20(13)	34.53±3.69(13)	45.10±2.79(11)**	56.60±1.29(4)*
	200	22.28±2.07(14)	39.02±2.10(14)	43.64±2.38(14)*	48.77±2.27(14)*	54.84±2.98 (6)

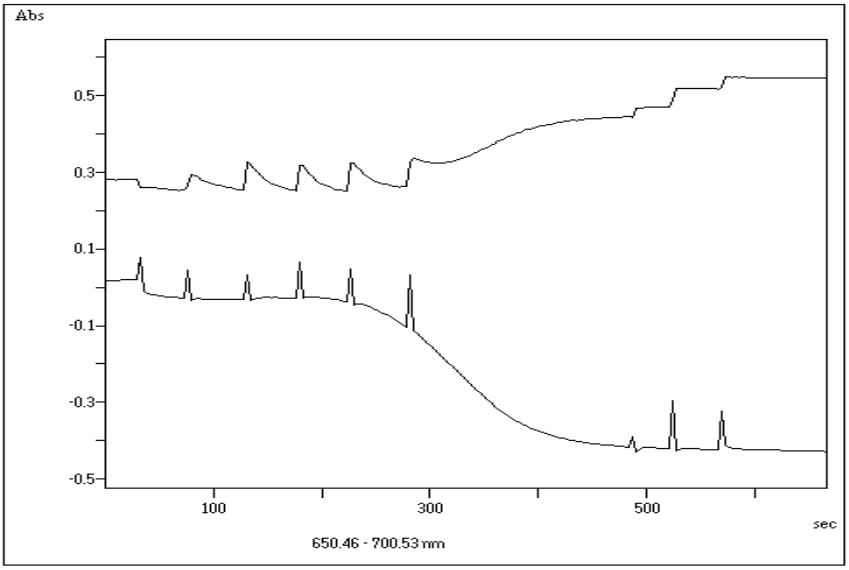
Mitochondria of alcohol-fed LPS-injected rats. The spectrophotometric results with this type of mitochondria are presented in Figs. 11-22. The alcoholic rats injected with 0.5 mg LPS/kg b.w. and 2 mg LPS/kg b.w. are designated as A+0.5LPS and A+2LPS, respectively. Although there are certain small differences between the two subgroups, they turned out to be statistically not significant. However, as in the case of simply alcohol-fed rats (see [46]), we observed (in both subgroups injected with LPS) the presence of rats with atypical responses. Therefore, this time, the figures are so arranged as to make obvious the differences between the rats with typical $(A+LPS)_t$ and atypical $(A+LPS)_a$ responses, for each subgroup. Differences can be observed in all the media and for all the parameters monitored or measured.

Illustrations from the typical and atypical (A+0.5LPS) subgroups are presented in Figs. 11-16. Figs. 11-12 and 13-14 present aspects of the calcium fluxes and membrane potential, respectively, each associated with the matrix swelling, in the MSK medium, while Figs. 15-16 present aspects of the calcium fluxes and matrix swelling of the same subgroups in KSW medium. It can be seen that the calcium load tolerated by the mitochondria of the atypical subgroup is higher than that tolerated by the typical subgroup. In addition, a certain degree of dissociation can be observed between the moments of initiation of matrix swelling and $\Delta\Psi$ collapse, especially in ionic media, for mitochondria of the atypical subgroup (see Fig. 14). There is also a difference in the extent of matrix swelling at relatively low calcium loads, as can be seen from Figs. 15-16, the amplitude of swelling being larger in mitochondria of the atypical subgroup.

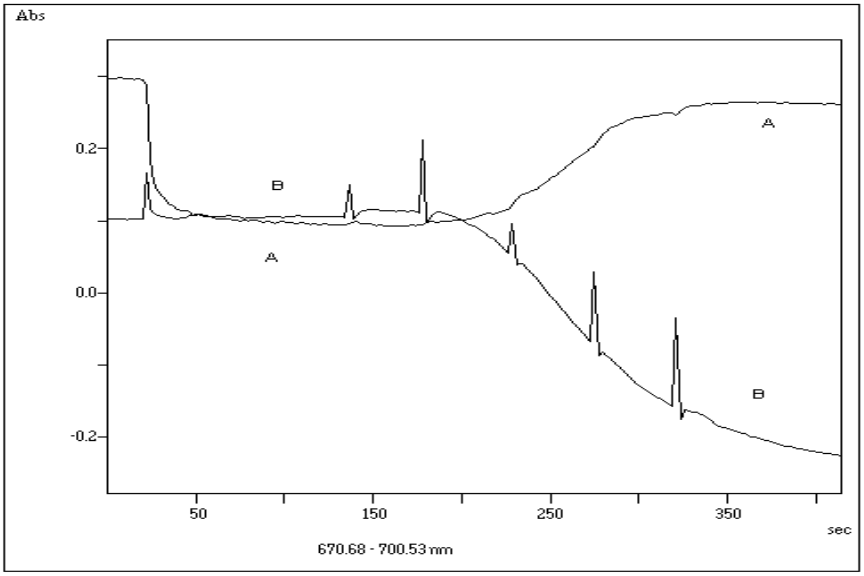
Figs. 17-22 present illustrations taken from the two (A+2LPS) subgroups. From Figs. 17-20, one can see the really large differences, recorded in the MS medium, existing between the typical and atypical subgroups, regarding the number of calcium pulses that mitochondria are able to take up before the triggering of the matrix swelling, $\Delta\Psi$ collapse and calcium release. Figs. 21-22 demonstrate that such differences are also evident in ionic media devoid of Mg^{2+}, such as MSK-Mg, although they are not so impressive as in a basically non-ionic medium (MS).



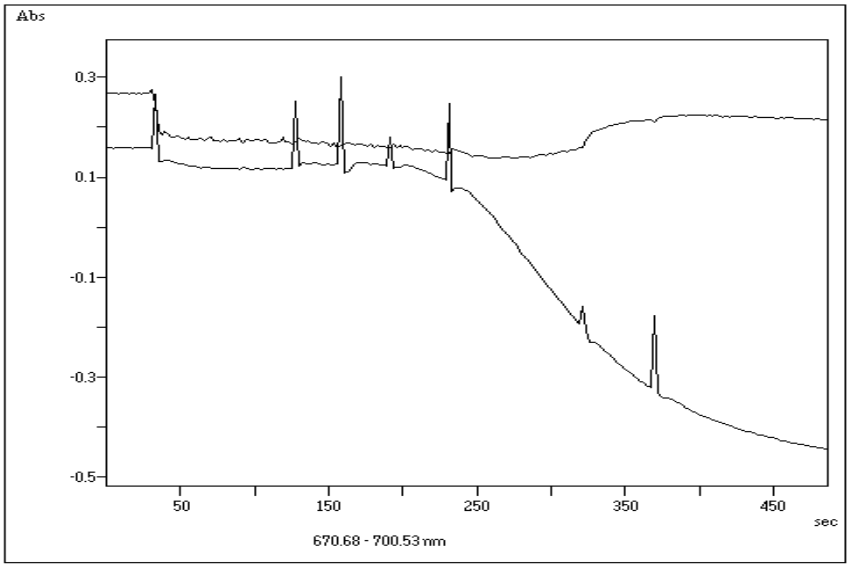
F i g . 1 1 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK medium, in a preparation belonging to a rat from the* $(A+0.5LPS)_t$ *subgroup.*



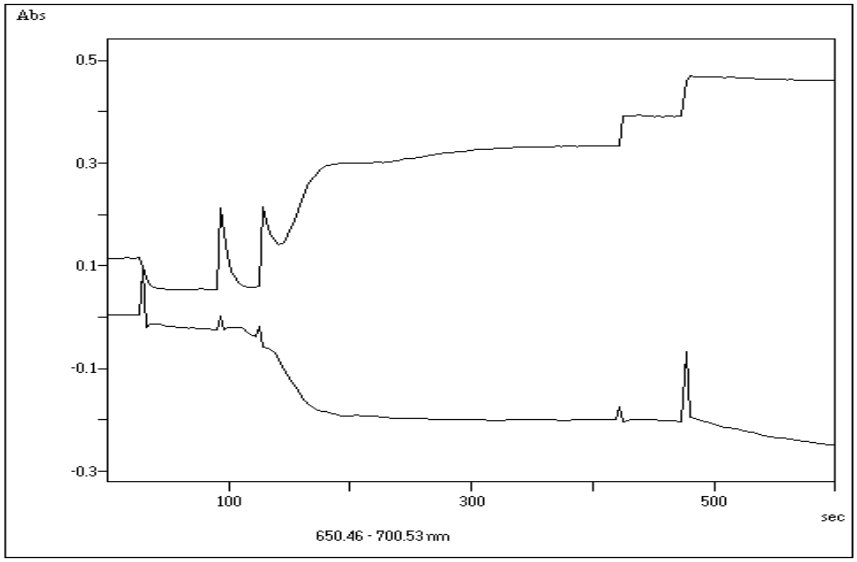
F i g . 1 2 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK medium, in a preparation belonging to a rat from the* $(A+0.5LPS)_a$ *subgroup.*



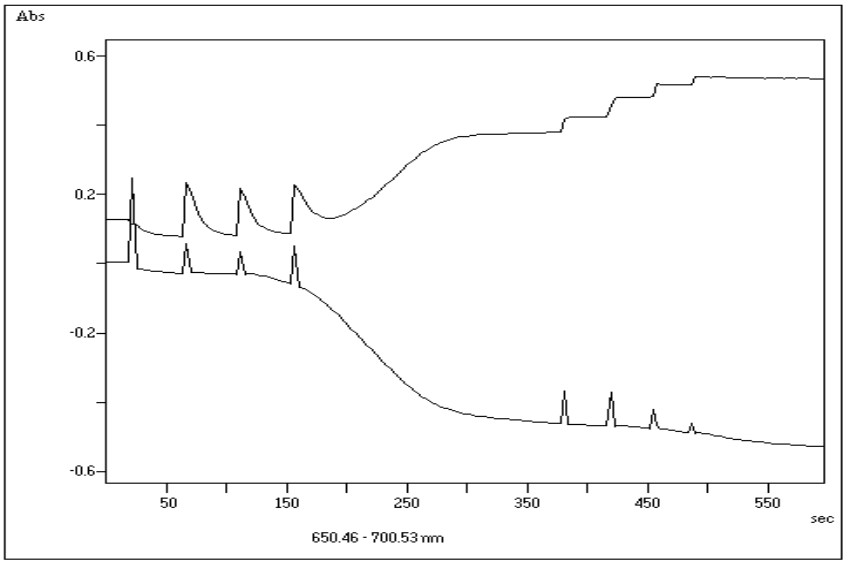
F i g . 1 3 . *Membrane potential* (curve A) *and mitochondrial swelling* (curve B) *in the MSK medium, in a preparation belonging to a rat from the* $(A+0.5LPS)_t$ *subgroup.*



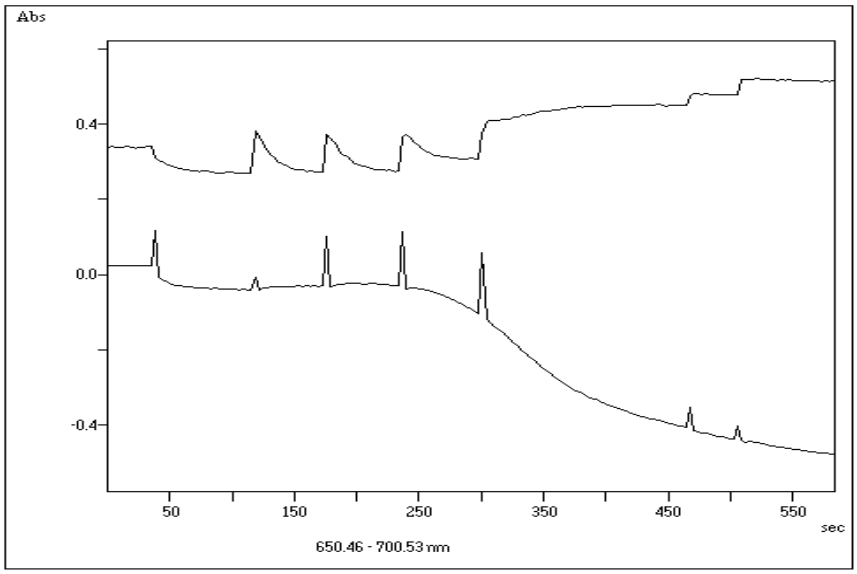
F i g . 1 4 . *Membrane potential* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK medium, in a preparation belonging to a rat from the* $(A+0.5LPS)_a$ *subgroup.* Note the dissociation between the initiation of swelling and the beginning of $\Delta\Psi$ collapse.



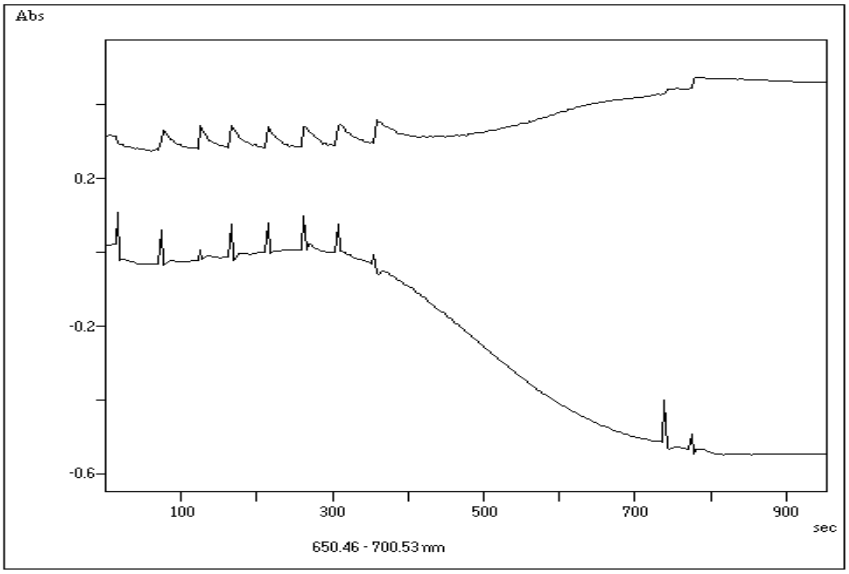
F i g . 1 5 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in KSW medium, in a preparation belonging to a rat from the* $(A+0.5LPS)_t$ *subgroup.* Note the small number of Ca^{2+} pulses to which the mitochondria resist and a rapid but shallow swelling, until further Ca^{2+} is added.



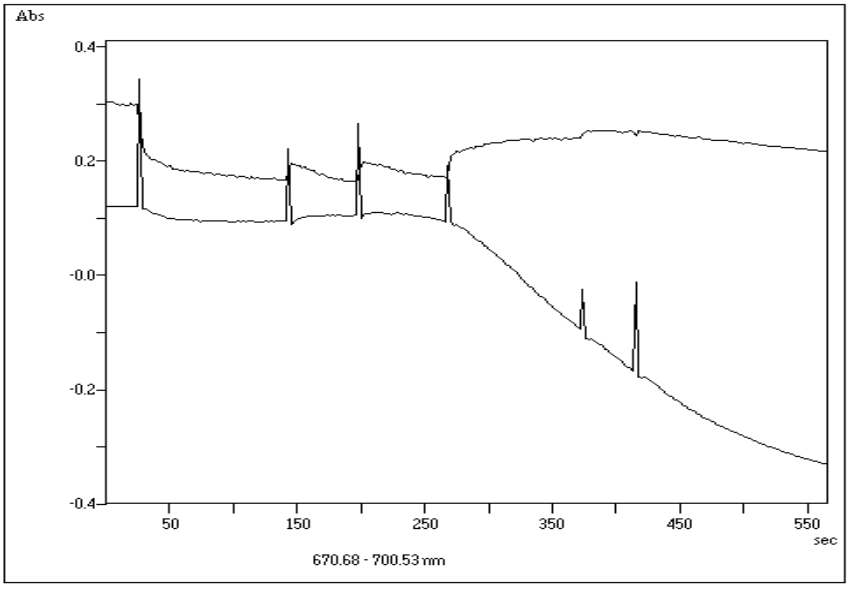
F i g . 1 6 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in KSW medium, in a preparation belonging to a rat from the* $(A+0.5LPS)_a$ *subgroup.*



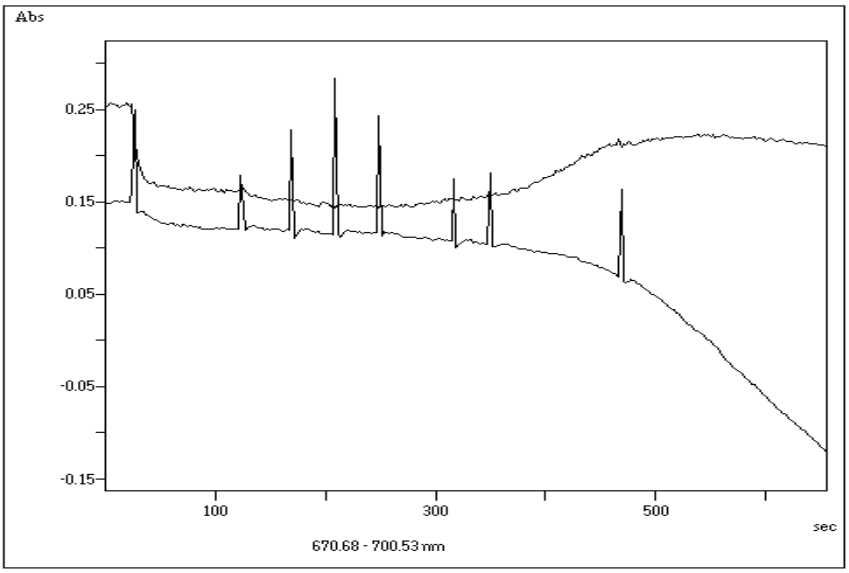
F i g . 1 7 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MS medium, in a preparation belonging to a rat from the* $(A+2LPS)_t$ *subgroup.*



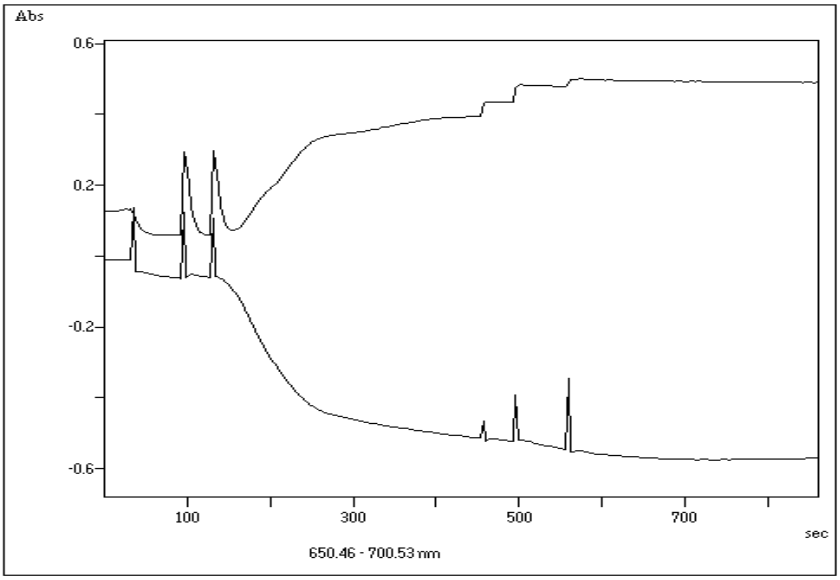
F i g . 1 8 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MS medium, in a preparation belonging to a rat from the* $(A+2LPS)_a$ *subgroup.* Note the high resistance of mitochondria to calcium loading.



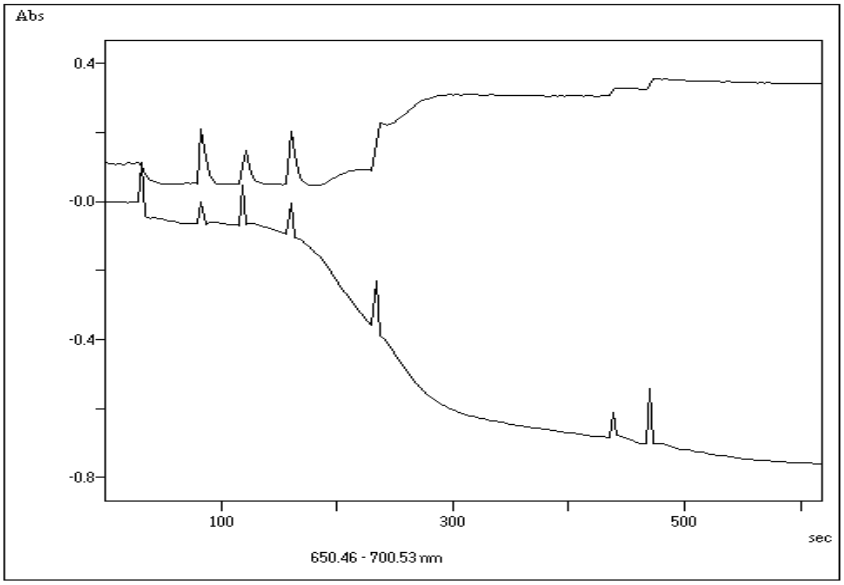
F i g . 1 9 . *Membrane potential* (upper curve) *and mitochondrial swelling* (lower curve) *in the MS medium, in a preparation belonging to a rat from the* $(A+2LPS)_t$ *subgroup.*



F i g . 2 0 . *Membrane potential* (upper curve) *and mitochondrial swelling* (lower curve) *in the MS medium, in a preparation belonging to a rat from the* $(A+2LPS)_a$ *subgroup.*



F i g . 2 1 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK–Mg medium, in a preparation belonging to a rat from the* $(A+2LPS)_t$ *subgroup.*



F i g . 2 2 . *Calcium fluxes* (upper curve) *and mitochondrial swelling* (lower curve) *in the MSK–Mg medium, in a preparation belonging to a rat from the* $(A+2LPS)_a$ *subgroup.*

Quantitative comparisons regarding the number of calcium pulses (of 12.5 μM each), taken up by mitochondria before the initiation of calcium release, are presented for the (A+0.5LPS) and (A+2LPS) subgroups in Tables 4 and 5, respectively. As can be seen, the differences between the typical and atypical subgroups are statistically significant or even very significant in all the media tested. On the other hand, despite apparent small differences, no significant statistical differences could be established between the typical subgroups themselves (or the atypical subgroups). Therefore, in further comparisons, the typical subgroups were pooled together in one group.

Table 4

Comparison between the typical and atypical A+0.5LPS groups

Statistical parameter	$\bar{X}$ ± SEM		t	$n' = n_1 + n_2 - 2$	p
Suspending medium \ Group	**$(A+0.5LPS)_t$**	**$(A+0.5LPS)_a$**			
MS	3.750 ± 0.250	5.500 ± 0.500	3.615	4	< 0.05
MSK	2.313 ± 0.237	4.250 ± 0.250	5.012	4	< 0.01
MSK–Mg	2.000 ± 0.000	3.000 ± 0.000	$\rightarrow \infty$	3	$\rightarrow 0$
KSW	1.750 ± 0.250	3.000 ± 0.000	3.333	4	< 0.05

Table 5

Comparison between the typical and atypical A+2LPS groups

Statistical parameter	$\bar{x}$ ± SEM		t	n' = $n_1 + n_2 - 2$	p
Suspending medium \ Group	**$(A+2LPS)_t$**	**$(A+2LPS)_a$**			
MS	3.833 ± 0.183	7.500 ± 0.500	5.389	3	< 0.02
MSK	2.833 ± 0.167	5.000 ± 0.000	1.073	3	< 0.01
MSK–Mg	2.000 ± 0.000	3.250 ± 0.354	4.743	3	< 0.02
KSW	2.000 ± 0.000	3.000 ± 0.000	→ ∞	3	→ 0

In order to establish in a more precise way the contribution of each of the two stress factors used in this experiment (chronic ethanol feeding and acute LPS injection), we compared the typical A+LPS group in turn with the typical alcoholic group (A_{1-6}) and with the (non-alcoholic) LPS-injected group. The results are presented in Tables 6 and 7, respectively. As can be seen from Table 6, with one exception (the KSW medium), there are no significant statistical differences between the typical A+LPS group and the typical alcoholic group. On the other hand, also with one exception (the MSK–Mg medium), there are very significant differences between the typical A+LPS group and the one treated with LPS only, as shown in Table 7.

Table 6

Comparison of the typical A+LPS group with the alcohol-fed group

Statistical parameter	$\bar{x}$ ± SEM		t	n' = $n_1 + n_2 - 2$	p
Suspending medium \ Group	A_{1-6}	$(A+LPS)_t$			
MS	4.750 ± 0.750	3.786 ± 0.214	1.820	7	> 0.1
MSK	2.333 ± 0.105	2.536 ± 0.176	0.946	11	> 0.1
MSK–Mg	2.000 ± 0.129	2.000 ± 0.000	→ 0	10	>>> 0.1
KSW	1.000 ± 0.000	1.857 ± 0.091	3.794	8	< 0.01

Table 7

Comparison of the typical A+LPS group with the LPS-treated group

Statistical parameter	$\bar{x}$ ± SEM		t	n' = $n_1 + n_2 - 2$	p
Suspending medium \ Group	LPS	$(A+LPS)_t$			
MS	5.250 ± 0.339	3.786 ± 0.214	4.965	11	< 0.001
MSK	3.125 ± 0.190	2.536 ± 0.176	3.134	19	< 0.01
MSK–Mg	2.300 ± 0.200	2.000 ± 0.000	1.662	9	> 0.1
KSW	2.083 ± 0.091	1.857 ± 0.091	2.274	11	< 0.02

This observation tells us that of the two stress factors used in our experiments, the chronic ethanol feeding is by far the most important one. This does not mean that the effect of LPS is not visible. In fact, the swelling amplitude (in KSW medium) of the mitochondria obtained from double-treated rats is significantly smaller at almost all times and for all calcium loads, whereas in the case of either (simply) alcoholic or LPS-injected animals this is true only for a few selected combinations of incubation time-calcium load (see Table 3 in [46] and the present work).

General discussion. In accord with previous results obtained with rats kept under tightly controlled conditions and fed a liquid diet [12, 43], we find that the acute LPS administration in small doses also affects the membrane permeability and related parameters of liver mitochondria from animals raised under the present conditions and that it modulates the response to chronic ethanol feeding, by decreasing the membrane permeability, even though the effect is not so obvious as before. In fact, it is very likely that the chronic ethanol feeding for such a long time (4 months) is associated with a constant penetration of LPS through the intestinal wall of the rats, as shown in the literature for similar circumstances [3, 32, 37]. Under these conditions, it is expected that the effect of alcohol should be modulated by the endogenous LPS and that the rats have already gained a certain tolerance to endotoxin. This might explain also why the LPS dose-dependent effects reported previously [43] are not so obvious in the present experiments. It must be stressed, however, that in our previous experiment we monitored only the matrix swelling in KSW medium, while the present work deals with more parameters and several different incubating media, for which we do not have a comparing possibility. Nevertheless, there is one study that can be partly corroborated with ours, showing an increased mitochondrial respiratory capacity after endotoxin pretreatment *in vivo* [12] and several other reports demonstrating protection against oxygen toxicity and an increased synthesis of superoxide dismutase by tracheal insuflation of endotoxin or other similar treatments [9, 11, 29, 40]. These observations and others lend support to the idea that LPS partly counteracts the effects of ethanol by controlling the production of the so-called reactive oxygen species (ROS), which have been demonstrated to be involved in ALD [1, 15, 16, 20, 47].

The problem with our alcohol-fed rats, however, seems to be complicated by the presence of animals with (what we call) atypical response, *i.e.*, rats with very low sensitivity to alcohol, that can be observed even after the LPS treatment of ethanol-fed animals. We found that about 30% of the rats can be included in this category. Of course, considering our model of ethanol administration, one could suspect that the rats insensitive to alcohol (designated as atypical) were in fact rats that somehow managed to avoid taking their share of ethanol. This can be ruled out, however, by the fact that atypical animals were also discovered among the double-treated (alcohol-fed, LPS-injected) animals, whereas no such animals were identified among the non-alcoholic (control) rats injected with LPS. If the effect is due to alcohol avoidance, the mitochondria of such rats should have a similar behaviour to

that of the LPS-injected control. However, this is generally not the case, since the mitochondria of atypical double-treated rats show less sensitivity to metabolic stress than those of the LPS-injected rats.

Another problem that should be addressed is the origin and explanation of the partial dissociation between the initiation of the matrix swelling and the beginning of $\Delta\Psi$ collapse in mitochondria of either alcohol-fed or LPS-injected rats, but especially in mitochondria of double-treated rats. Is this real or is it an artefact? And, if it is real, what is its significance? Since this dissociation phenomenon is not so obvious for the calcium release, which normally should accompany $\Delta\Psi$ collapse, an artefact seems a likely candidate. However, if one understands the response mechanism of the potential-sensitive probe, alternative explanations can be put forward. The mechanism of response of the cyanine dye used in the present experiments was unravelled many years ago [41, 42]. The most important feature of the dye is its moderate hydrophobicity, which makes it membrane permeant, but at the same time membrane bound. When a membrane potential difference is built up, the cationic dye, which is initially symmetrically distributed, simply concentrates on the negative face of the membrane. This surface concentration change leads to a concentration-dependent dimerisation associated with a change in the absorption spectrum. We assume that our preparation is a heterogeneous population of mitochondria, in which some organelles are more sensitive than others to different treatments (ethanol and/or LPS), either by their different origin and/or by their different position in the liver. If this is true, then some of the mitochondria will start depolarising and releasing calcium sooner than others. Since the probe is in a limited amount (2.5 μM), the dye of the inert mitochondria (those that have suffered the permeability transition) can now redistribute among the still active mitochondria, which can concentrate and dimerise or polymerise it, thus keeping the absorbance relatively unchanged until the majority of mitochondria suffer depolarisation. The calcium probe, on the other hand, is in much higher concentration (30 μM) and, being much more polar, it also has a different mechanism of response. Therefore, the release of calcium becomes apparent sooner after the triggering of the matrix swelling. In our opinion, the apparent dissociation of the membrane potential collapse from the other two phenomena studied, is a strong indication that mitochondria from different liver cells and/or different places have a differential sensitivity to apoptotic/necrotic stress factors (in our case ethanol and LPS). We hope that our electron microscopy samples, at least to some extent, will confirm our supposition. However, in order to have a clear-cut response, it is necessary to prepare mitochondria from different liver cells (parenchymal, Kupffer, stellate cells etc.) and find more subtle ways to monitor the activity of these cells *in situ*.

Conclusions. From the results obtained in the present work and partly from those reported in our accompanying paper [46], we can draw four groups of conclusions.

1. For all the experimental groups of rats (hence, for all the treatments), from the three membrane-associated phenomena studied, the one that is triggered first is the matrix swelling. Calcium release and $\Delta\Psi$ collapse occur more or less in parallel, as a consequence of the permeability transition and not vice-versa.

2. In general, in all media used for incubation, the chronic alcohol-feeding increases the sensitivity of mitochondria to acute stress factors (such as LPS and calcium), although the reactivity of the animals vary, some of them displaying alcohol tolerance.

3. The acute administration of bacterial endotoxin (LPS) to control rats, in sublethal doses, has an effect similar to, but less grave than, that of the chronic ethanol-feeding. A certain degree of dissociation of $\Delta\Psi$ collapse from the other two phenomena studied can be observed.

4. LPS administration to alcoholic rats leads to an intensification of the dissociation phenomenon mentioned above, a fact which may point to a differential sensitivity of different types of hepatic mitochondria. At the same time, a decrease in mitochondrial membrane permeability can be observed in the swelling medium, when moderate concentrations of calcium are used, suggesting that LPS may contribute to the phenomenon of alcohol tolerance.

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UTILIZAREA INDICATORULUI ENZIMATIC AL CALITĂŢII NĂMOLULUI ÎN SCOPUL VALORIFICĂRII ŞI PROTECŢIEI LACURILOR SALINE

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SUMMARY. – Utilisation of the Enzymatic Indicator of Mud Quality for Exploitation and Protection of Salt Lakes. We have determined six enzymatic activities (phosphatase, urease, protease, catalase, actual dehydrogenase, potential dehydrogenase) and three nonenzymatic catalytic activities (H_2O_2 splitting in autoclaved samples, TTC-[2,3,5-triphenyltetrazolium chloride] reduction in autoclaved samples, without or with glucose addition) in the muds of the salt lakes Techirghiol, Nuntaşi and Costineşti, during four seasons (summer and autumn 2002, winter and spring 2003, respectively). The enzymatic and the nonenzymatic catalytic activities varied depending on the season, lake and the collection zone. The highest values were obtained in the mud of the Techirghiol lake, being followed by the Costineşti and the Nuntaşi lakes. Generally, no evident seasonal variations were registered.. Based on the enzymatic indicator of mud quality (EIMQ), we could determine the quality of the mud in the three analysed lakes. The mud of the Techirghiol lake is a very active one, the EIQM values being between 0.5 and 0.59. The mud of the Costineşti lake had an EIQM between 0.4 and 0.6, while the mud of the Nuntaşi lake had the EIQM of 0.3-0.45. Taking into consideration the biological stability of the mud through the variability coefficients, we were able to establish that the muds of the Nuntaşi and Costineşti lakes indicate an evolution towards high quality muds. By means of the EIQM we could estimate the evolution of the mud in the three lakes, in comparison to the situation registered 20 years ago. In the case of Techirghiol lake the value of EIQM increased with 0.4 units, in the case of Costineşti lake it increased with 0.2 units and in the Nuntaşi lake with 0.1 units. The EIQM may be used for determining the quality of the muds, in order to use them in balneotherapy or in decting of their some negative modifications – having this way the possibility to protect the lakes.

Este bine cunoscut că, în sedimentele acvatice, procesele microbiene stau la baza mineralizării materiei organice, precum şi la baza degradării poluanţilor organici.

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Cataliza enzimatică, sub controlul direct sau indirect al microorganismelor, mediază acest proces complex ce asigură desfăşurarea ciclurilor biologice ale elementelor C, N, P şi S.

Determinarea activităţilor enzimatice în sedimentele acvatice, de altfel ca şi în sol, constituie un instrument de cercetare pentru a evalua diversitatea funcţională a microbiotei, cu alte cuvinte a proceselor biochimice din aceste medii naturale şi pentru găsirea unor indicatori ai calităţii solului [11], respectiv ai calităţii sedimentelor acvatice [10].

Nămolul din lacurile saline este produsul proceselor microbiologice ce au loc într-un ecosistem acvatic în strânsă conexiune cu condiţiile de mediu [15]. Potenţialul enzimatic al nămolului reflectă în mod direct sau indirect activitatea microbiotei, influenţa diferiţilor factori fizici, chimici, antropogeni asupra microbiotei, precum şi asupra procesului de acumulare a enzimelor şi chiar a intensităţii activităţilor enzimatice. Funcţionarea unui ecosistem nu poate fi înţeleasă fără participarea activă a proceselor enzimatice [11].

Primele cercetări care au abordat determinarea activităţii enzimatice a unui sediment acvatic au fost cele realizate de L e n h a r d [8, 9] la sedimentele unor râuri din Africa de Sud, ca mai târziu cercetările să se extindă şi la sedimentele unor lacuri saline, apreciindu-se potenţialul enzimatic al nămolului printr-un indicator enzimatic al calităţii obţinut pe baza determinării mai multor activităţi enzimatice şi catalitice neenzimatice [1-3, 5-7, 10, 12-14]. Prima clasificare a lacurilor saline din ţara noastră pe baza activităţilor enzimatice şi catalitice neenzimatice ale nămolului terapeutic a fost publicată în 1986 de către K i s s şi colab. [6], fiind comparate valorile indicatorului enzimatic al calităţii nămolului din 37 lacuri saline, iar apoi s-a efectuat o a doua clasificare a 56 lacuri saline de către M u n t e a n şi colab. în 1996 [10], datele obţinute ulterior fiind comparate cu acest din urmă sistem de clasificare a lacurilor saline.

In lucrarea de faţă ne-am propus să urmărim valorile indicatorului enzimatic al calităţii nămolului la trei lacuri saline din zona litoralului românesc, determinate în anii 2002 şi 2003, în vederea aprecierii calităţii nămolului, ca factor de bază în valorificarea acestuia în balneoterapie, respectiv în vederea stabilirii unor măsuri de protecţie a lacurilor în cazul când apar influenţe antropogene nedorite.

Materiale şi metode. Au fost studiate sedimentele a 3 lacuri saline din zona litoralului românesc şi anume Techirghiol, Nuntaşi şi Costineşti. Prelevarea probelor de nămol s-a realizat în vara şi toamna anului 2002, respectiv în iarna şi primăvara anului 2003. In cazul lacului Techirghiol, probele de nămol s-au recoltat din 3 puncte (Debarcader, Coada lacului, Băile reci), din lacul Nuntaşi colectarea s-a realizat din 4 puncte (Camping, Istria 1, Istria 2, E-lacului), iar din lacul Costineşti din 3 puncte (Debarcader, Zona Obelisc, Camping).

Au fost determinate următoarele 9 activităţi enzimatice şi catalitice neenzimatice: fosfatazică, ureazică, proteazică, catalazică, catalitică neenzimatică, reducerea TTC în probe de nămol neautoclavate, fără glucoză (activitate dehidrogenazică

actuală), respectiv cu glucoză (activitate dehidrogenazică potențială), reducerea TTC în probe de nămol autoclavate, fără glucoză, respectiv cu adaos de glucoză. In vederea determinării activităților catalitice neenzimatice, părți din probele de nămol s-au autoclavat la 121^0C, timp de o oră, în trei zile consecutive.

Activitatea fosfatazică s-a determinat prin metoda lui K r á m e r şi E r d e i [4], în amestecuri de reacție ce au constat din 2,5 g sediment + 2 ml toluen (ca agent antiseptic) + 10 ml substrat enzimatic (fenilfosfat disodic, soluție 0,5 %) sau 10 ml apă distilată. Activitatea ureazică s-a determinat prin metoda S u m n e r [4], în amestecuri de reacție constând din 2,5 g sediment + 2 ml toluen + 5 ml soluție tampon fosfat (pH 6,7) + 5 ml soluție de uree 3%. Activitatea protează s-a determinat prin metoda B e c k [4], în amestecuri de reacție conținând 2,5 g sediment + 2 ml toluen + 20 ml soluție de gelatină 2%. O tehnică bazată pe metoda lui K a p p e n [4] a fost aplicată pentru determinarea activității catalazice, în amestecuri de reacție cu 1,5 g sediment + 2 ml H_2O_2 3% + 10 ml apă distilată. Aceeaşi tehnică a fost utilizată şi pentru determinarea activității catalitice neenzimatice, dar probele de nămol au fost inactivate termic prin autoclavare. Reducerea TTC în probe de nămol neautoclavate (activitatea dehidrogenazică) şi în probe de nămol autoclavate s-a determinat conform metodei lui C a s i d a şi colab. [4]. Compoziția amestecurilor de reacție a fost următoarea: 0,5 g nămol + 1 ml apă distilată sau 1 ml soluție de glucoză 3% + 0,5 ml soluție de clorură de 2,3,5-trifeniltetrazoliu (TTC) sau 0,5 ml apă distilată.

Activitățile au fost exprimate astfel: activitatea fosfatazică în mg fenol/ 2,5 g sediment uscat/ 24 ore, la 37^0C; activitatea ureazică în mg NH_4^+/ 2,5 g sediment uscat/24 ore, la 37^0C; activitatea protează în mg NH_2-N/2,5 g sediment uscat/24 ore, la 37^0C; activitatea catalazică şi catalitică neenzimatică în mg H_2O_2/1,5 g sediment uscat/1 oră, la 20^0C; activitățile de reducere a TTC în mg trifenilformazan/0,5 g sediment uscat/24 ore, la 37^0C. Pentru fiecare activitate enzimatică şi catalitică neenzimatică a fost calculat coeficientul de variație [4].

Rezultate şi discuții. Rezultatele obținute sunt prezentate în Tabelele 1-5, respectiv în Fig. 1-5.

Tabelul 1 prezintă datele privind activitățile enzimatice şi catalitice neenzimatice ale probelor de nămol colectate în vara anului 2002 din cele 3 lacuri litorale studiate (Techirghiol, Nuntaşi şi Costineşti). Se poate constata, din analiza rezultatelor, că au fost evidențiate toate cele 6 activități enzimatice (fosfatazică, ureazică, protează, catalazică, dehidrogenazică actuală, dehidrogenazică potențială) şi cele 3 activități catalitice neenzimatice (scindarea catalitică a H_2O_2, reducerea neenzimatică a TTC în probe fără glucoză, reducerea neenzimatică a TTC în probe cu glucoză).

Valorile activităților determinate au oscilat în funcție de lacul studiat, respectiv în funcție de locul de colectare. Lacul Techirghiol, unul din cei mai importanți producători de nămol sapropelic din Europa, se situează pe primul loc în privința potențialului enzimatic al nămolului, valorile activităților enzimatice, în special, fiind cele mai ridicate, comparativ cu cele determinate în celelalte două lacuri (Nuntaşi şi Costineşti). In privința locului de colectare, cele mai ridicate valori s-au înregistrat

în zona Băile reci, la toate cele 6 activități enzimatice, urmate de zonele Debarcader şi Coada lacului. In ceea ce priveşte activitățile catalitice neenzimatice, nu se constată deosebiri marcante între cele 3 zone de colectare, valori uşor mai ridicate fiind obținute în zona Debarcader, respectiv în cea de la Coada lacului. Subliniem că lacul Techirghiol are un nămol negru onctuos, cu un grad ridicat de peloidizare, care posedă un potențial enzimatic ridicat.

Din analiza Tabelului 1 reiese, de asemenea, că după lacul Techirghiol, valorile activităților enzimatice sunt destul de însemnate şi în nămolul din lacul Costineşti, dar mai reduse comparativ cu cele întâlnite la toate zonele lacului Techirghiol. Lacul Costineşti, se cunoaşte, are o salinitate mai scăzută, iar biocenozele sunt mai sărace, fapt ce se reflectă şi în potențialul enzimatic. Nămolul este tot unul negru, dar cu un grad mai scăzut de onctuozitate. In cazul acestui lac, nămolul din zona Obelisc este cel mai activ pe plan enzimatic, prezentând valorile cele mai ridicate atât la activitățile enzimatice cât şi la cele neenzimatice.

Nămolul din lacul Nuntaşi prezintă cele mai scăzute valori ale activităților enzimatice, chiar şi ale activităților catalitice neenzimatice, cu excepția scindării H_2O_2, unde la toate punctele de prelevare nămolul a prezentat valori mai ridicate decât probele colectate din celelalte două lacuri. Intre cele 4 zone de colectare, există deosebiri în funcție de activitatea enzimatică determinată, dar nămolul din punctele Istria 1 şi Istria 2 s-a dovedit a fi cel mai activ pe plan enzimatic. Subliniem că lacul Nuntaşi are un nămol cenuşiu, uşor onctuos sau lipsit de onctuozitate, cu un grad mai mic de peloidizare, care s-a demonstrat a avea şi un potențiual enzimatic mai scăzut. Lacul Nuntaşi prezintă o salinitate mai mică, probabil şi biocenozele sunt de alt tip, ceea ce se răsfrânge şi asupra procesului de peloidogeneză, respectiv asupra potențialului biologic (enzimatic) al nămolului.

In Fig.1 se prezintă evoluția potențialului enzimatic al nămolului din cele 3 lacuri litorale, în perioada de vară, din toate punctele de colectare, pe baza indicatorului enzimatic al calității nămolului (IECN). Se poate constata mai pregnant diferențierea între cele 3 lacuri litorale, precum şi între punctele de colectare de la fiecare lac în parte. Se confirmă faptul că pe baza acestui indicator global al activității enzimatice, lacurile Techirghiol şi Costineşti se situează în categoria lacurilor cu sedimente active din punct de vedere enzimatic, având valori ale IECN cuprinse între 0,5 şi 1, pe când lacul Nuntaşi are nămol de calitate mai redusă din punct de vedere enzimatic, valorile IECN fiind cuprinse între 0,3 şi 0,5. De asemenea, este evidentă diferențierea şi între punctele de colectare, fiecare lac prezentând o zonă cu nămol mai activ din punctul de vedere al potențialului enzimatic.

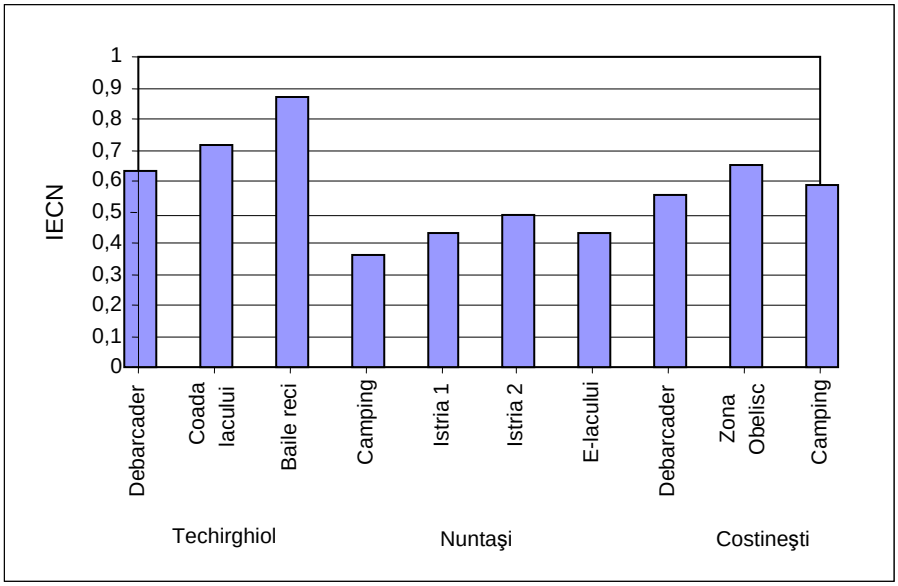
După cum se poate vedea, în cazul lacului Techirghiol, diferențierile între cele 3 zone sunt mai pregnante, în sensul că zona de la Coada lacului se distanțează la o diferență de 0,2 unități, de zona Băile reci, pe când la celelalte două lacuri nămolul este mai omogen, diferența între punctele de colectare fiind doar de circa 0,1 unități.

Tabel 1

Valorile activităţilor enzimatice şi catalitice neenzimatice ale probelor de nămol prelevate vara din lacurile litorale studiate

Lacul	Locul de prelevare	Activitatea fosfatazică	Activitatea ureazică	Activitatea proteazică	Activitatea catalitică		Reducerea TTC				IECN*
					Enzi-matică	Neenzi-matică	Nămol neautoclavat		Nămol autoclavat		
							Fără glucoză	Cu glucoză	Fără glucoză	Cu glucoză	
Techirghiol	Debarcader	5,925	1,450	1,925	41,560	24,680	4,500	5,825	0,920	0,985	0,635
	Coada lacului	4,116	1,160	2,524	48,440	22,450	4,025	4,940	1,100	1,200	0,715
	Băile reci	7,220	2,350	3,286	46,200	21,600	5,116	6,200	1,090	1,195	0,868
Nuntaşi	Camping	0,900	0,490	0,526	27,222	23,670	2,160	2,905	0,526	0,520	0,364
	Istria 1	1,270	0,600	0,724	28,324	28,953	2,612	3,100	0,620	0,700	0,435
	Istria 2	1,925	0,702	0,803	29,966	29,440	2,928	3,625	0,725	0,800	0,488
	E-lacului	0,830	0,395	0,640	31,445	30,166	1,900	2,400	0,832	0,790	0,431
Costineşti	Debarcader	3,250	0,936	0,983	32,160	23,100	3,100	4,100	0,940	1,030	0,555
	Zona Obelisc	4,500	1,025	1,124	39,260	24,525	3,900	4,600	1,095	1,200	0,652
	Camping	3,800	0,986	1,090	35,166	23,900	3,125	4,250	0,990	1,050	0,586

*IECN - Indicatorul enzimatic al calităţii nămolului.



F i g . 1. *Indicatorul enzimatic al calităţii nămolului – vara.*

In Tabelul 2 sunt trecute rezultatele obţinute la determinările activităţilor enzimatice şi catalitice neenzimatice la probele de nămol colectate în toamna anului 2002. Se poate observa că există deosebiri, dar nu de ordin global, ci mai mult oscilaţii ale valorilor obţinute la diferite activităţi enzimatice. Se menţine poziţia lacurilor din punctul de vedere al potenţialului enzimatic, cu specificarea că probele din lacul Techirghiol prezintă chiar activităţi uşor mai ridicate, pe când nămolurile din lacurile Nuntaşi şi Costineşti au furnizat valori cu ceva mai scăzute decât în perioada de vară, ceea ce se poate remarca şi pe baza analizei Fig. 2. Se constată că se menţine şi poziţia locurilor de colectare, cu o singură excepţie, în cazul lacului Techirghiol, unde s-au înregistrat valori mai crescute la nămolul din zona Coada lacului, acestea fiind foarte apropiate de valorile obţinute la cel din zona Debarcader. Se poate afirma că în mediul acvatic nu se semnalează variaţii sezoniere decât de mică intensitate, la toate cele 3 lacuri litorale luate în studiu.

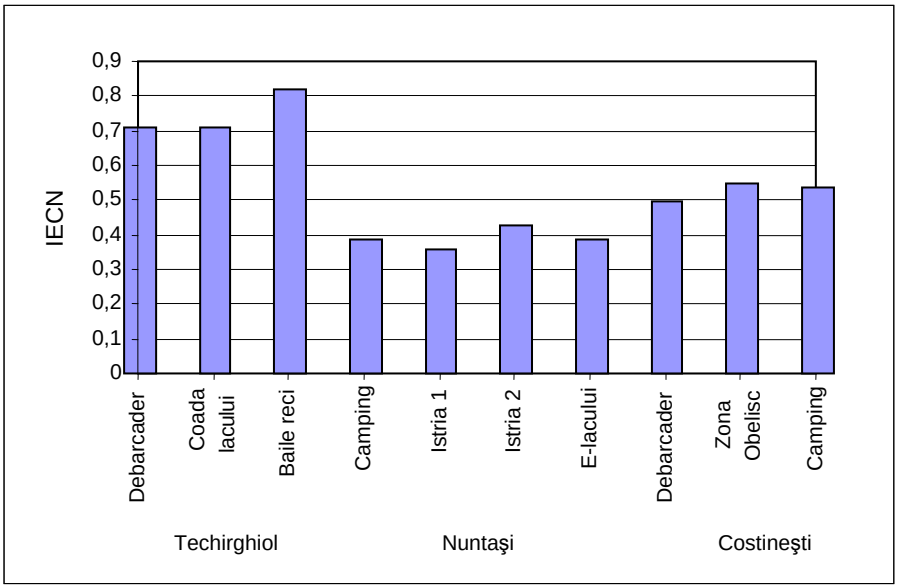
Tabel 2

Valorile activităţilor enzimatice şi catalitice neenzimatice ale probelor de nămol prelevate toamna din lacurile litorale studiate

Lacul	Locul de prelevare	Activitatea fosfatazică	Activitatea ureazică	Activitatea protează	Activitatea catalitică		Reducerea TTC				IECN*
					Enzi-matică	Neenzi-matică	Nămol neautoclavat		Nămol autoclavat		
							Fără glucoză	Cu glucoză	Fără glucoză	Cu glucoză	
Techirghiol	Debarcader	6,240	1,829	2,146	46,606	21,096	6,379	9,200	1,725	1,650	0,707
	Coada lacului	4,600	1,700	2,788	45,305	20,366	5,925	8,311	1,402	1,495	0,708
	Băile reci	8,200	2,688	3,929	43,200	23,725	6,800	9,025	1,200	1,290	0,822
Nuntaşi	Camping	1,320	0,700	0,680	29,708	26,600	2,400	3,100	0,400	0,525	0,388
	Istria 1	1,425	0,925	0,932	28,609	24,425	2,800	3,600	0,325	0,613	0,359
	Istria 2	1,640	0,990	0,998	31,405	28,306	3,100	4,000	0,788	0,780	0,428
	E-lacului	1,304	0,650	0,720	29,983	23,405	2,900	3,300	0,902	0,844	0,387
Costineşti	Debarcader	3,996	1,126	1,090	34,507	24,705	3,820	4,222	0,980	1,010	0,494
	Zona Obelisc	5,200	1,300	1,485	25,900	25,900	4,129	4,900	0,991	1,115	0,546
	Camping	4,326	1,250	1,325	26,100	26,100	4,016	4,725	0,940	0,975	0,537

*IECN - Indicatorul enzimatic al calităţii nămolului.

Rezultatele obţinute la determinările efectuate asupra probelor de nămol colectate în iarna anului 2003 sunt cuprinse în Tabelul 3, respectiv în Fig. 3. Se poate observa că, practic, oscilaţiile valorilor activităţilor enzimatice şi ale activităţilor catalitice neenzimatice sunt foarte restrânse, menţinându-se constatarea că influenţa sezonieră este mică în cazul lacului Techirghiol şi foarte mică în cazul lacurilor Nuntaşi şi Costineşti (lacuri cu adâncimi mai mici, comparativ cu lacul Techirghiol). Este de semnalat că la nămolurile din lacurile Nuntaşi şi Costineşti chiar s-au obţinut valori ale IECN mai ridicate în perioada de iarnă decât în perioada de toamnă, la toate punctele de colectare, ceea ce întăreşte afirmaţia că în sedimentele acvatice ale lacurilor saline se face mai puţin simţită influenţa sezonieră, datorată în primul rând factorilor climaterici.



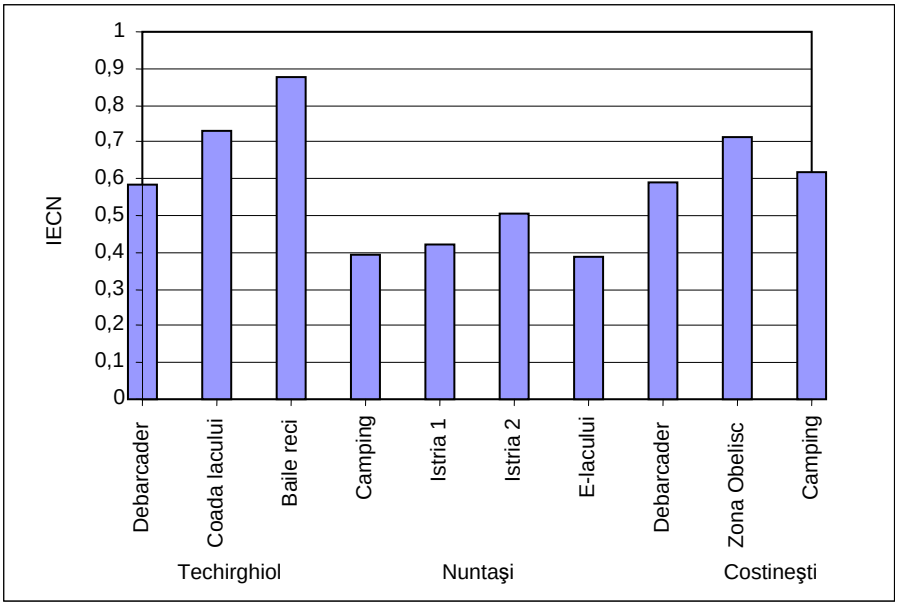
F i g . 2. *Indicatorul enzimatic al calităţii nămolului – toamna.*

Tabel 3

Valorile activităţilor enzimatice şi catalitice neenzimatice ale probelor de nămol prelevate iarna din lacurile litorale studiate

Lacul	Locul de prelevare	Activitatea fosfatazică	Activitatea ureazică	Activitatea protează	Activitatea catalitică		Reducerea TTC				IECN*
					Enzi-matică	Neenzi-matică	Nămol neautoclavat		Nămol autoclavat		
							Fără glucoză	Cu glucoză	Fără glucoză	Cu glucoză	
Techirghiol	Debarcader	3,200	0,995	1,100	38,006	25,506	2,125	2,800	0,560	0,610	0,583
	Coada lacului	2,100	0,800	1,500	36,305	24,425	3,000	3,926	0,912	0,980	0,728
	Băile reci	3,800	1,326	2,100	40,126	26,100	3,600	3,728	0,990	1,012	0,878
Nuntaşi	Camping	0,840	0,330	0,310	22,448	26,516	1,740	2,305	0,415	0,418	0,393
	Istria 1	0,990	0,460	0,450	25,556	27,412	1,925	2,604	0,306	0,350	0,420
	Istria 2	1,100	0,520	0,560	28,312	29,500	2,010	2,925	0,525	0,600	0,503
	E-lacului	0,930	0,280	0,380	26,405	31,040	1,500	2,040	0,280	0,300	0,389
Costineşti	Debarcader	2,115	0,720	0,700	30,802	24,940	2,117	3,116	0,726	0,800	0,590
	Zona Obelisc	3,090	0,940	0,910	32,516	25,518	2,924	3,041	0,980	0,990	0,714
	Camping	2,240	0,880	0,830	33,400	22,416	2,200	2,900	0,800	0,860	0,620

*IECN - Indicatorul enzimatic al calităţii nămolului.



F i g . 3. *Indicatorul enzimatic al calității nămolului – iarna.*

Determinările efectuate în perioada de primăvară 2003, la nămolurile din cele 3 lacuri studiate, sunt trecute în Tabelul 4, respectiv în Fig. 4. Acestea au confirmat concluzia noastră că schimbările sezoniere în sedimentele celor 3 lacuri analizate sunt de mică importanță, comparativ cu sedimentele acvatice din apele curgătoare şi în contrast cu cele semnalate de mulți cercetători în cazul solurilor din zonele temperate. Mai mult, la lacurile Nuntaşi şi Costineşti, chiar se înregistrează valori mai mici ale IECN în perioada de primăvară, comparativ cu cele calculate în celelalte 3 anotimpuri.

Tabel 4

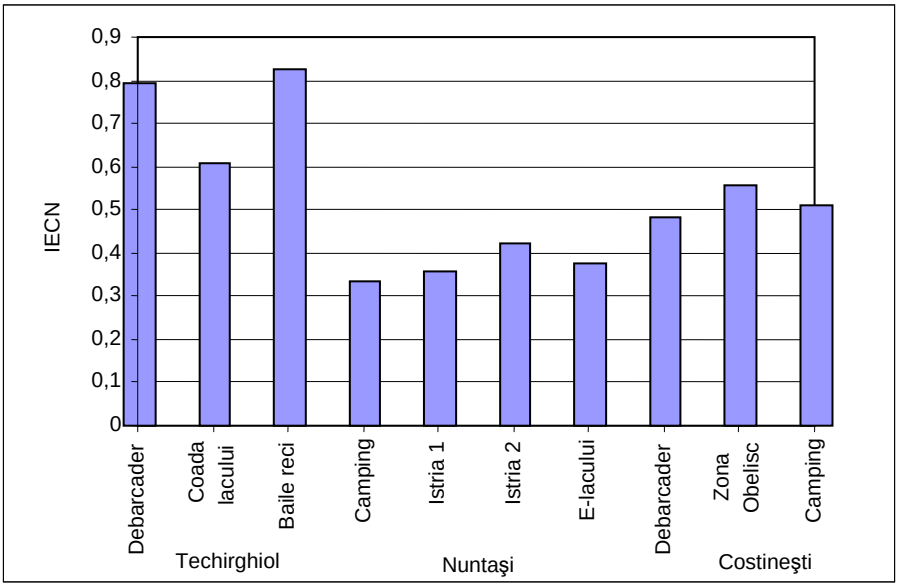
Valorile activităților enzimatice şi catalitice neenzimatice ale probelor de nămol prelevate primăvara din lacurile litorale studiate

Lacul	Locul de prelevare	Activitatea fosfatazică	Activitatea ureazică	Activitatea protează	Activitatea catalitică		Reducerea TTC				IECN*
					Enzi-matică	Neenzi-matică	Nămol neautoclavat		Nămol autoclavat		
							Fără glucoză	Cu glucoză	Fără glucoză	Cu glucoză	
Techirghiol	Debarcader	8,659	1,955	2,800	48,365	20,345	6,328	9,306	2,900	2,980	0,795
	Coada lacului	5,275	1,624	3,112	40,200	22,284	5,210	6,800	1,480	1,600	0,609
	Băile reci	7,935	2,985	3,560	46,121	24,386	7,251	10,245	2,100	2,300	0,824
Nuntaşi	Camping	2,112	0,825	0,805	30,900	25,300	2,700	3,400	0,500	0,504	0,335
	Istria 1	2,614	0,991	1,220	29,300	26,525	3,112	3,700	0,380	0,400	0,359
	Istria 2	2,912	1,200	1,405	31,225	27,627	3,345	4,114	0,804	0,860	0,420
	E-lacului	2,557	0,850	0,980	30,011	24,428	3,091	3,900	0,900	0,990	0,377

Tabel 4 (continued)

Lacul	Locul de prelevare	Activitatea fosfatazică	Activitatea ureazică	Activitatea protează	Activitatea catalitică		Reducerea TTC				IECN*
					Enzi-matică	Neenzi-matică	Nămol neautoclavat		Nămol autoclavat		
							Fără glucoză	Cu glucoză	Fără glucoză	Cu glucoză	
Costineşti	Debarcader	4,925	1,400	1,800	37,500	25,624	3,990	4,466	0,900	0,906	0,484
	Zona Obelisc	6,129	1,890	1,925	42,829	26,120	4,500	5,290	1,050	1,012	0,555
	Camping	5,300	1,750	1,550	41,925	24,928	4,346	5,016	0,968	0,900	0,512

*IECN - Indicatorul enzimatic al calităţii nămolului.



F i g . 4. *Indicatorul enzimatic al calităţii nămolului – primăvara.*

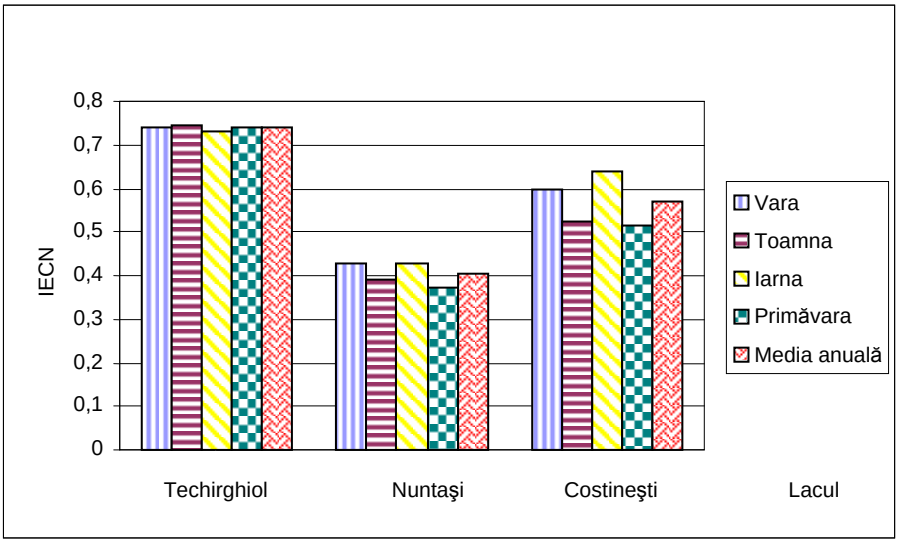
Trebuie să subliniem că în determinările noastre, 4 dintre activităţile enzimatice se datoresc enzimelor acumulate (fosfatază, urează, protează, catalază) care sunt în mai mică măsură influenţate de factorii climaterici în mediul acvatic, comparativ cu activităţile enzimatice datorate microbiotei proliferante (dehidrogenaza actuală şi cea potenţială), care pot suferi oscilaţii în funcţie de dezvoltarea microbiotei, dar fără a modifica semnificativ valoarea IECN. De asemenea, nici activităţile catalitice neenzimatice nu sunt influenţate în mod vizibil de influenţele sezoniere, ceea ce a dus la obţinerea unor valori ale IECN destul de apropiate, indiferent de anotimpul în care s-au colectat probele de nămol pentru analiză (Fig. 5).

Datele din Tabelul 5 ne furnizează informaţii despre stabilitatea biologică a nămolurilor din cele 3 lacuri, pe baza coeficienţilor de variabilitate calculaţi. Se înregistrează diferenţe în privinţa valorilor coeficienţilor, în funcţie de activitatea

Tabel 5

Coeficienţii de variabilitate sezonieră şi anuală ai activităţilor enzimatice şi catalitice neenzimatice în nămolul lacurilor litorale studiate (%)

Lacul	Sezonul	Activitatea fosfatazică	Activitatea urează	Activitatea protează	Activitatea catalitică		Reducerea TTC			
					Enzi-matică	Neenzi-matică	Nămol neautoclavat		Nămol autoclavat	
							Fără glucoză	Cu glucoză	Fără glucoză	Cu glucoză
Techirghiol	Primăvara	24,400	32,400	11,700	7,600	9,000	16,300	20,000	32,900	30,000
	Vara	27,100	37,500	26,400	7,700	6,900	12,000	11,400	9,700	9,900
	Toamna	28,400	25,900	30,500	3,800	8,100	6,800	5,100	18,300	12,200
	Iarna	28,400	25,500	32,100	5,000	3,300	25,500	16,900	25,700	14,600
	Anual	24,075	30,320	18,580	6,020	6,500	15,150	13,350	21,650	16,450
Nuntaşi	Primăvara	12,900	17,700	30,200	2,700	2,300	8,700	6,100	38,000	40,400
	Vara	31,100	24,300	17,100	6,400	6,000	19,100	16,800	19,500	18,400
	Toamna	10,800	11,800	39,300	4,700	8,500	10,500	10,100	46,100	22,300
	Iarna	11,300	28,000	23,500	9,500	7,100	12,600	15,400	31,400	29,200
	Anual	16,520	20,450	27,520	5.820	5,970	12,720	12,100	33.750	27,570
Costineşti	Primăvara	10,600	15,000	10,800	6,900	2,300	6,100	9,200	7,700	6,700
	Vara	16,200	4,500	6,900	7,100	2,900	13,400	5,900	7,700	7,500
	Toamna	13,800	7,300	15,200	9,500	2,900	3,900	7,700	2,700	4,200
	Iarna	19,000	13,400	13,600	4,000	6,700	18,300	3,600	10,900	15,000
	Anual	14,900	10,050	11,620	6,870	3,700	10,420	6,600	7,250	8,350



F i g . 5. *Indicatorul enzimatic global.*

enzimatică determinată, de data colectării probelor şi de proveniența lor în funcție de lacul studiat. Valori mai ridicate ale acestora denotă stabilitate biologică mai mică şi invers. Comparând lacurile între ele, din acest punct de vedere, putem constata că nămolul din lacul Costineşti prezintă cea mai bună stabilitate biologică, urmat de nămolul din lacul Nuntaşi şi apoi de cel din lacul Techirghiol. Aceste rezultate ne permit să afirmăm că nămolul din cele 3 lacuri prezintă o bună stabilitate biologică, ceea ce denotă evoluția acestora spre nămoluri cu potențial microbiologic (enzimatic) ridicat, intrând în categoria nămolurilor valoroase pentru balneoterapie. Determinarea activităților descrise mai sus, la perioade mai scurte de timp, vor putea sesiza modificările apărute în potențialul biologic al nămolului, impunându-se măsuri de protecție.

Concluzii. 1. In nămolurile colectate din lacurile litorale Techirghiol, Nuntaşi şi Costineşti au fost evidențiate 6 activități enzimatice (fosfatazică, ureazică, proteazică, catalazică, dehidrogenazică actuală, dehidrogenazică potențială) şi 3 activități catalitice neenzimatice (scindarea catalitică a H_2O_2, reducerea neenzimatică a TTC în probe fără glucoză, respectiv reducerea neenzimatică a TTC în probe cu glucoză), în toate cele 4 anotimpuri (vara şi toamna anului 2002, respectiv iarna şi primăvara anului 2003).

2. Activitățile enzimatice şi cele catalitice neenzimatice au oscilat în funcție de anotimp, de lac şi de zona de colectare. Cele mai ridicate valori ale activităților enzimatice determinate s-au înregistrat în nămolul lacului Techirghiol, urmat de cel din lacul Costineşti şi apoi de cel din lacul Nuntaşi. La toate lacurile nu s-au semnalat variații sezoniere, decât de mică intensitate, ceea ce ne-a condus la constatarea că în mediul acvatic, în special în lacurile saline, influența factorilor climaterici asupra activității microbiologice a sedimentului este de mai mică importanță.

3. Pe baza indicatorului enzimatic al calității nămolului (IECN) s-a putut aprecia valoarea nămolului din cele 3 lacuri, pe primul loc situându-se cel din lacul Techirghiol, urmat de cel din lacul Costineşti şi apoi de cel din lacul Nuntaşi. Lacul Techirghiol dispune de un nămol foarte activ din punct de vedere enzimatic, cu un potențial microbiologic ridicat (IECN cuprins între 0,5 şi 0,9), încadrat pe baza datelor obținute între cele mai active nămoluri terapeutice de la noi din țară. Cel din lacul Costineşti se încadrează în categoria nămolurilor active (IECN cuprins între 0,4 şi 0,6), iar cel din lacul Nuntaşi în categoria nămolurilor mai puțin active (IECN cuprins între 0,3 şi 0,45).

4. Pe baza coeficienților de variabilitate calculați s-a putut aprecia stabilitatea biologică a sedimentului, care s-a dovedit a fi mai bună la nămolurile din lacurile Costineşti şi Nuntaşi, permițând evoluția acestora spre un nămol de calitate superioară.

5. Cu ajutorul IECN s-a putut aprecia evoluția nămolului din cele 3 lacuri, cel din lacul Techirghiol crescând cu 0,4 unități, față de perioada 1988, iar nămolul din celelalte lacuri, cu o rată mai mică. Pe baza valorilor IECN se poate monitoriza evoluția unui nămol terapeutic, luându-se măsuri de protecție, în anumite situații, respectiv se poate aprecia valoarea acestuia pentru utilizarea lui în balneoterapie.

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CONTRIBUŢII LA STUDIEREA DROJDIILOR INDUSTRIALE

LETIŢIA OPREAN*

SUMMARY. - Contributions to the Study of Industrial Yeasts. Twenty yeast strains have recently been isolated in pure cultures from industrial sources and identified based mainly on the morphological properties of their cells and cultures on solid and liquid nutrient media. Majority of the strains (15) are alcohologenic belonging to the genus *Saccharomyces* and comprising two brewer's (beer) yeast strains (*S. carlsbergensis* = *S. uvarum* A and B), two baker's yeast strains (*S. cerevisiae* CA and CP), one spirit yeast strain (*S. cerevisiae* CF) and ten wine yeast strains (*S. cerevisiae* var. *ellipsoideus* 1, 3, 4, 6, 8 and 9; *S. bayanus* var. o*viformis* 2, 5 and 7; and *S. uvarum* 10). The other, 5 yeast strains belong to different species: *Kloeckera apiculata, Candida mycoderma (Mycoderma vini), Pichia membranaefaciens, Rhodotorula glutinis* and *Torulopsis holmii*, respectively.

Examinarea caracterelor morfologice celulare şi culturale ale tulpinilor de drojdii izolate în culturi pure prezintă o importanţă deosebită în caracterizarea şi identificarea acestora [1, 2, 4].

În lucrarea de faţă au fost examinate caracterele morfologice ale tulpinilor de drojdii izolate în culturi pure prin examen microscopic, caracterul culturilor pe mediu solid şi lichid, capacitatea de formare a sporilor şi aspectul coloniilor gigant.

Materiale şi metode. Am întreprins un studiu asupra caracterelor morfologice ale unui număr de 20 tulpini de drojdii, majoritatea cu aplicaţii în industria fermentativă, dintre care 15 tulpini de drojdii alcooligene aparţinătoare genului *Saccharomyces:* două de bere (*S. carlsbergensis* A si B), două de panificaţie (*S. cerevisiae* CA şi CP), una de spirt (*S. cerevisiae* CF),10 de vin (*S. cerevisiae* var. *ellipsoideus* 1, 3, 4, 6, 8 şi 9, *S. bayanus* var. *oviformis* 2, 5 şi 7 şi *S. uvarum* 10) şi 3 tulpini de drojdii de contaminare (*Candida mycoderma, Pichia membranaefaciens* şi *Rhodotorula glutinis*) au fost izolate sub formă de culturi pure, identificate şi selecţionate în cadrul Laboratorului de microbiologie al Catedrei de Biotehnologii alimentare, Universitatea "Lucian Blaga" Sibiu. Am supus tulpinile de drojdii, izolate în culturi pure, unei examinări biologice complexe, determinând caracterele lor morfologice celulare şi culturale prin examenul microscopic al celulei vegetative de drojdie şi a sporilor (în cazul drojdiilor ascosporogene), prin examenul caracterelor culturilor pe mediu solid şi lichid şi a aspectului coloniilor gigant [1, 3].

Pe preparate umede şi frotiuri colorate Gram am examinat la microscop (cu grosisment 900X şi, respectiv, 450X) forma şi mărimea celulelor, formarea mugurilor, locul apariţiei lor pe celula mamă, formarea de pseudomiceliu (abundente ramificaţii). Dimensiunile celulelor au fost măsurate cu un ocular sau cu o lamă micrometrică [3].

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Sporularea a fost urmărită prin cultivarea tulpinilor de drojdii pe mediul Gorodkova sau acetat-agar. Examinarea caracterelor culturale (macroscopice) ale tulpinilor de drojdii izolate în culturi pure şi dezvoltate în/pe medii lichide şi solide specifice a permis caracterizarea şi diferenţierea lor. Observaţiile s-au efectuat după 3 şi 7 zile de incubare în termostat la 25^0C şi după 30 zile de menţinere la 17^0C.

Dezvoltarea tulpinilor de drojdii pe medii solide a permis caracterizarea coloniilor obţinute după formă, dimensiuni, profil, aspect, consistenţă şi culoare. Sunt evidenţiate şi caracterele coloniei gigant pe mediul adecvat. Am utilizat următorul procedeu care a dat rezultate foarte bune. Cu ajutorul unei pipete Pasteur am lăsat să cadă o picătură din cultura de drojdie în centrul unei plăci Petri cu mediul adecvat. Incubarea s-a efectuat în termostat 7 zile la 25 ^{0}C şi 30 de zile la 17 ^{0}C. Am obţinut şi culturi în strii pe mediul înclinat adecvat [3].

În cazul drojdiilor de vin s-a studiat şi aspectul coloniilor pe must de struguri gelificat, precum şi puterea de lichefiere a gelatinei (proteoliză). Ultimul caracter reprezintă un criteriu de diferenţiere a celor două tulpini de drojdii predominante în mustul de fermentaţie (*S. cerevisiae* var. *ellipsoideus*) şi vin tânăr (*S. bayanus* var. *oviformis*) [3].

Rezultate. Caracterele morfologice (macroscopice şi microscopice) ale tulpinilor de drojdii izolate şi obţinute sub formă de culturi pure în laborator sunt prezentate în continuare.

Tulpinile de drojdii ***Saccharomyces carlsbergensis*** **(**sin.: ***S. uvarum*****) A, B** (bere) şi **10** (vin) formează pe mediul must de bere-geloză, după 3 zile de incubare la 25^{o}C, colonii de tip S (smooth), cu diametrul de 2 - 3,5 mm, cu aspect neted şi mat, de culoare alb-crem, cu perimetrul circular, uneori lobat triangular, cu profil bombat sau lenticular şi consistenţă cremoasă. După 7 zile, coloniile ajung la diametrul de 3,5 – 8 mm şi îşi menţin caracterele morfologice. După 30 zile, coloniile gigant se colorează de la alb-crem la crem închis sau maroniu şi prezintă un perimetru lobat cu suprafaţa netedă sau pliată, verucoasă, restrânsă sau împrăştiată, profil plat sau bombat şi margini ondulate, festonate sau lobiforme. Pe must cu gelatină, coloniile nu lichefiază gelatina.

Culturile în strii la *S. uvarum* 10 (vin), pe must de bere sau extract de malţ-geloză, sunt mate cu suprafaţa netedă, de consistenţă cremoasă, de culoare bej, uniforme şi fără relief; la *S. carlsbergensis* A şi B (bere), culturile în strii sunt albe lucioase, mai puţin strălucitoare în centru.

Culturile de drojdii în mediul lichid (must de bere), după 24 ore de incubare la 25^{o}C, produc o tulburează opacă, densă, la suprafaţă formează o spumă slab persistentă, iar după 3 zile un sediment compact aderent (depozit flocunos). După 7 zile, aceste drojdii nu formează peliculă la suprafaţa mediului. *S. uvarum* 10 (vin), în mustul de bere, după 7 zile, formează la suprafaţă, ocazional, un inel incomplet, care se păstrează şi după 30 zile.

Microscopic, după 3 zile de termostatare la 25^{o}C, în must de bere, celulele au formă ovală sau sferică, globoasă, cu dimensiuni: (3-7) x (4-10) μm şi dispuse singular sau în perechi, uneori în lanţuri scurte sau ciorchini mici. S-au observat şi

celule înmugurite cu muguri dispuşi polar. În mustul de struguri, celulele sunt ovale, puțin alungite, cu dimensiuni mari: (4-8) x (5-12) μm. Pe mediul Gorodkova sau acetat-agar, se formează 1 - 4 ascospori într-un asc (de obicei 2 sau 4), prin partenogeneză sau copulare.

Tulpinile de drojdii ***Saccharomyces cerevisiae*** **CA** şi **CP** (panificație) şi **CF** (spirt) formează pe must de bere-geloză, după 3 zile de incubare la 25^{o}C, colonii de tip S, cu diametrul de 1 - 2 mm, cu perimetrul circular, uneori lobat triangular, profil convex, consistență cremoasă, aspect neted, lucios şi de culoare alb-crem (*S. cerevisiae* CF) sau alb-cenuşiu (*S. cerevisiae* CA şi CP). După 7 zile, coloniile ajung la diametrul de 2,5 - 5 mm şi îşi mențin caracterele morfologice. După 30 zile, coloniile gigant devin plate, uşor bombate, de culoare crem şi maroniu închis, cu suprafața netedă sau pliată, zbârcită.

Culturile în strii pe must de bere-geloză sau extract de malț-agar sunt lucioase de culoare albă-bej (*S. cerevisiae* CF) sau alb-cenuşiu (*S. cerevisiae* CA şi CP), uniforme, fără relief.

Culturile de drojdii în mediul lichid (must de bere), după 24 ore de incubare la 25°C, produc tulbureală, iar la suprafață formează o spumă fină, cu aspect mat. După 3 zile, se formează un sediment compact, aderent (depozit flocunos), ceea ce demonstrează că drojdiile de panificație şi de spirt, ca şi cele de bere, sunt drojdii floculante. Ocazional, unele tulpini (*S. cerevisiae* CF) formează şi un inel incomplet la suprafața mediului. Nu se formează peliculă nici în culturile bătrâne (peste 30 zile).

Microscopic, după 3 zile, celulele crescute pe must de struguri au formă, în general, eliptică, cu dimensiuni: (5-9) x (4-8) μm, unele fiind mai alungite, altele mai rotunjite. Ele sunt dispuse izolat, în perechi sau lanțuri. S-au observat şi celule cu muguri dispuşi multipolar. Uneori, după incubare prelungită pe extract de malț-agar, precum şi pe mediul Gorodkova, s-au format ascospori. Ascii provin din celule vegetative, când sporii s-au format prin partenogeneză. Un asc conține, de cele mai multe ori, 3 - 4 ascospori sferici sau ovali cu suprafața netedă .

Tulpinile de drojdii ***Saccharomyces cerevisiae*** **var. *ellipsoideus* 1, 3, 4, 6, 8** şi **9** formează pe must de struguri-geloză, după 3 zile, colonii de tip S, cu diametrul de 2-4 mm cu perimetru circular, slab lobat, uneori triangular, cu profil convex, de consistență umedă, cremoasă, mai puțin netede sau granuloase, lucioase de culoare albă sau alb-cenuşiu. După 7 zile, coloniile ajung la diametrul de 4-6 mm, unele colonii devin proeminente, țuguiate, iar restul caracterelor morfologice se mențin constante. După 30 zile, coloniile gigant prezintă în funcție de tulpini aspecte multiple. Sunt bine dezvoltate, mai plate sau uşor bombate, de culoare crem-maroniu, cu suprafața netedă sau pliată, cu margini bine dezvoltate ondulate, larg festonate.

Culturile în strii pe must de malț-geloză sau must de struguri-geloză sunt lucioase, de culoare albă, uneori alb-gălbui, uniforme, fără relief. Pe must cu gelatină, coloniile sunt mate sau strălucitoare, de dimensiuni mari (3-5 mm diametru), în general destul de groase, deseori proeminente, țuguiate. Prezintă proprietatea de a lichefia gelatina.

Culturile în mediul lichid (mustul de struguri), după 24 ore de incubare la 25°C, produc tulbureală, o spumă fină, cu aspect mat, iar după 72 ore, formează un sediment granular, aderent (depozit flocunos), ceea ce demonstrează că şi aceste tulpini sunt drojdii floculante. Separarea rapidă la sfârşitul fermentaţiei prin floculare reprezintă o proprietate tehnologică importantă, după care sunt selecţionate în laborator drojdiile de vin. Caracterele de sedimentare sunt variabile de la o tulpină la alta. Pe culturile mai în vârstă (după 30 zile), nu se remarcă o peliculă la suprafaţă, dar poate rămâne pe eprubetă un inel format de drojdiile ridicate de spumă în timpul fermentaţiei active.

Microscopic, după 3 zile de cultivare la 25^{o}C, în must de struguri, celulele au o formă elipsoidală, globoasă sau elipsoidal alungită, cu dimensiuni de (5-8) x (4-8) μm, dispuse singular, în perechi sau lanţuri. S-au observat şi celule înmugurite, cu muguri dispuşi, de obicei, polar şi care au rămas alipiţi de celula mamă. Pe mediul Gorodkova sau acetat-agar, *S. cerevisiae* var. *ellipsoideus* sporulează uşor. După o lună s-au observat asce ce conţin 1-4 ascospori rotunzi sau uşor ovali. Formarea sporilor este precedată de copulare.

Tulpinile de drojdii ***Saccharomyces bayanus* var. *oviformis* 2, 5** şi **7** formează pe mediul must de bere-geloză sau must de struguri-geloză, după 3 zile de incubare la 25^{o}C, colonii de tip S, cu diametrul de 2-3,5 mm, cu contur circular sau lobat triangular, cu profil bombat lenticular, de consistenţă cremoasă, cu suprafaţa netedă, lucioasă şi de culoare albă sau alb-cenuşie (se aseamănă cu coloniile de *S. cerevisiae* var. *ellipsoideus*). După 7 zile, colonia ajunge la diametrul de 2,5-5 mm; unele colonii devin ţuguiate, iar restul caracterelor morfologice se menţin la fel. După 30 zile, coloniile gigant prezintă aspecte variate: se aseamănă şi se confundă cu cele ale lui *S. cerevisiae* var. *ellipsoideus*. Unele colonii devin mai plate, altele cu suprafaţa zbârcită, cutată, cu margini ondulate şi festonate de culoare mai închisă (maroniu).

Culturile în strii pe must de malţ-geloză şi must de struguri-geloză sunt destul de abundente şi compacte uneori netede şi lucioase, de culoare albă. Cultivate pe must cu gelatină, aceste drojdii nu lichefiază gelatina (nu produc proteoliză).

Culturile de drojdii în mediul lichid (must de struguri), după 24 ore de incubare la 25°C, produc o slabă opalescenţă şi rareori o spumă fină cu aspect mat, iar după 3 zile, se formează un sediment pulverulent, granular, neaderent, uşor de îndepărtat. După 7 zile, se poate forma o peliculă la suprafaţa vinurilor, uneori se formează un inel superficial.

Microscopic, celulele de *S. bayanus* var. *oviformis* crescute în must de struguri, după 3 zile, au formă ovală, eliptică, sunt uneori aproape rotunde, cu dimensiuni de (4-7) x (5-9) μm, dispuse singular, în perechi sau lanţuri. Pe must de struguri-geloză, celulele sunt mai alungite, uneori foarte alungite. Pe unele celule se observă muguri dispuşi polar, care în majoritate rămân alipiţi de celula mamă. Pe must-geloză, celulele sunt mai puţin înguste şi mai alungite în mediu solid: (3-6) x (5-9) μm, cu câteva celule foarte alungite. Pe mediul Gorodkova sau acetat-agar, aceste drojdii formează celule mai alungite şi sporulează mai greu decât *S. cerevisiae* var. *ellipsoideus*; formează 2 - 3 spori rotunzi într-un asc.

Kloeckera apiculata formează pe must de struguri-geloză sau pe must de malț-geloză, după 3 zile la 25°C, colonii de tip S cu diametrul de 1,5-2,5 mm, aproape semisferice, slab lobate, cu margini circulare, aspect neted şi lucios, de culoare alb-crem. După 7 zile, coloniile ajung la diametrul de 3-6 mm, unele prezentând un aspect rugos, puțin încrețit; restul caracterelor morfologice se mențin. După 30 zile, majoritatea coloniilor gigant devin gri sau de culoare gălbui spre crem. Coloniile sunt de obicei catifelate, lucioase, uneori încrețite cu marginea întreagă până la neregulată. Majoritatea coloniilor au o regiune centrală joasă şi fine striații radiale, ocazional observându-se şi cratere mici.

Culturile în strii pe must de malț-geloză sunt lucioase, subțiri, de culoare crem, uniforme, fără relief. Pe must-gelatină, coloniile lichefiază gelatina.

Cultura de drojdie în mediul lichid (must de struguri), după 3 zile de incubare, nu produce tulbureală. Lichidul este limpede, cu urme de inele superficiale. Nu se formează peliculă la suprafața mediului.

Microscopic, celulele de *K. apiculata*, crescute pe must de struguri, după 3 zile la 25°C, au formă fie elipsoidală, subțiri, mici: (3-1,5) μm sau groase, mai mari: (5-6) x (3-4) μm, fie apiculată cu dimensiuni de (3-6) x (7-10) μm, datorită mugurilor terminali bipolari. Pe malț-geloză, celulele sunt foarte mici şi de aceeaşi formă. Pe mediul Gorodkova, nu formează spori .

Candida mycoderma **(*Mycoderma vini*)** crescută pe must de struguri-geloză formează, după 3 zile, colonii de tip S/R cu un perimetru slab lobat, neregulat cu o proeminență în partea centrală, margini ondulate şi cu diametrul de 2,5-5,8 mm. Suprafața coloniei are un aspect mat, neted lucios sau cutat de culoare alb-cenuşiu, uneori alb-crem. După 7 zile, coloniile ajung la diametrul de 4,5-7 mm şi îşi mențin caracterele morfologice. După 30 zile, coloniile gigant devin mai plate şi prezintă o suprafață zbârcită sau încrețită cu perimetru lobat şi neregulat. În mediu cu gelatină, nu lichefiază gelatina.

Culturile în strii pe must de malț-geloză sunt mate, uniforme, fără relief, de culoare alb-crem.

Cultura în mediu lichid (Rieder) formează tulbureală, iar în scurt timp o peliculă albă sau galbenă la suprafața mediilor slab-alcoolice şi subțire ce devine uscată şi încrețită după 3-4 zile. Cu timpul se îngroaşă şi o parte din ea se destramă şi se depune pe fundul vasului sub formă de sediment pulverulent. Formarea peliculei reprezintă un criteriu taxonomic important în identificarea drojdiilor oxidative (peliculare).

Microscopic, celulele de *C. mycoderma* crescute pe must de struguri-geloză, timp de 3 zile, sunt de formă elipsoidală, alungită, cilindrică, cu extremitățile rotunjite sau slab ascuțite şi cu dimensiuni cuprinse între (2-4) x (6-13) μm. Prezintă una sau două granulații refringente. S-au observat şi celule cu muguri dispuşi multipolar. În culturile mai vechi (14 zile), această drojdie formează pseudomiceliu cu blastospori (muguri grupați sub formă de ciorchine ce rămân alipite de celula mamă). Formarea de pseudomiceliu reprezintă un criteriu taxonomic de bază în identificarea acestei drojdii. În must de struguri, celulele de la suprafață sunt mai alungite, decât cele care cad la fund. Pe mediul Gorodkova, nu se formează spori.

Pichia membranaefaciens formează pe mediu must de bere-geloză, după 3 zile, colonii de tip S/R cu diametrul de 2,5-4 mm, cu perimetrul slab lobat sau chiar neregulat, margini ondulate sau lobiforme, aspect mat, neted, catifelat sau zbârcit, rugos, de culoare albă sau alb-cenuşie. După 7 zile, coloniile ajung la diametrul de 4-5,5 mm şi îşi menţin caracterele morfologice. După 30 zile, colonia gigant este albă-gălbuie spre maro-gălbui, moale, catifelată, parţial sau total zbârcită. Însămânţată pe mediul cu făină de porumb şi cartof, celulele formează pseudomiceliu bine dezvoltat cu aspect de capac sau poate fi absent .

Culturile în strii pe must de bere-geloză sunt mate, prăfoase, de culoare albă, uniforme, fără relief. În anumite cazuri, coloniile pe must gelificat au lichefiat gelatina.

În mustul de struguri după 3 zile, se formează tulbureală, iar la suprafaţa mediului apare o peliculă groasă, plisată, de culoare alb-gălbuie, mată, relativ friabilă, care se depune producând un sediment pulverulent. Formarea peliculei reprezintă un criteriu taxonomic important care stă la baza clasificării acestei drojdii, deoarece prezintă un metabolism predominant oxidativ.

Microscopic, celulele de *P. membranaefaciens*, crescute în must de struguri, sunt ovale alungite, cu capetele uşor ascuţite sau rotunjite, cu dimensiuni de (3-10) x (2-4) μm şi dispuse în lanţuri mai mult sau mai puţin lungi sau ramificate. S-au observat şi celule cu muguri dispuşi pe toată suprafaţa celulei. Formează în culturile mai vechi (30 zile) pseudomicelii cu blastospori rezultaţi prin înmugurire multilaterală sau hife adevărate (cu pori în septum). Pe mediul solid (must de malţ-geloză), celulele sunt mai rotunjite, izolate sau câte două. Pe mediul Gorodkova, se formează spori de dimensiuni variabile cu forme diferite: sferici sau hemisferici, saturn sau de pălărie, cu suprafaţa netedă sau rugoasă care se eliberează uşor din asce. Într-un asc se găsesc 1 - 4 spori, uneori 8. Prezenţa unui număr mare de spori dă culturii culoare maro.

Rhodotorula glutinis formează pe mediul extract de malţ sau must de bere-geloză, după 3 zile, colonii de tip S/R, cu diametrul de 1,5 - 3 mm, cu perimetrul circular, margini drepte, profil convex, cu aspect neted lucios sau consistenţă mucoasă, de culoare roşu pastelat. Prezenţa pigmenţilor carotenoizi de culoare roz şi roşu este foarte caracteristică genului *Rhodotorula* şi reprezintă un criteriu taxonomic delimitativ al acestui gen de drojdii. După 7 zile, coloniile ajung la diametrul de 3,5-7 mm şi îşi menţin caracterele morfologice. După 30 zile, coloniile gigant devin roz spre portocaliu. Suprafaţa coloniei variază de la catifelat, adesea cu striaţii fine transversale, până la zbârcită şi cu aspectul foarte lucios sau semilucios. Consistenţa variază de la mucoasă până la uşor dură. Profilul este plat spre larg convex cu marginea neregulată spre întreagă. Uneori în culturile vechi (30 zile), se diferenţiază şi un pseudomiceliu. În culturile crescute pe mediul cu făină de porumb, pseudomiceliul, în general, lipseşte sau este rudimentar. În ocazii rare, se pot forma pseudohife bine dezvoltate sau hife adevărate. Uneori se produc clamidospori mari pigmentaţi puternic.

Culturile în strii pe extract de malţ sau must de bere-geloză sunt lucioase, de culoare roz-roşie, uniforme, fără relief.

În mustul de struguri, drojdia nu fermentează. După 30 zile, mediul rămâne limpede şi se depune un sediment fin, iar la suprafaţă se formează o peliculă subţire (drojdie strict oxidativă).

Microscopic, celulele de *R. glutinis*, crescute pe mediul extract de malţ, timp de 3 zile, sunt globulare, puternic vacuolate, dispuse izolat sau în perechi cu dimensiuni medii: (4-5,5) x (3-3,5) μm. S-au observat şi muguri pe întreaga suprafaţă a celulei. Unele celule au formă alungită. Se pot dezvolta pe mediul Czapek, formând celule globulare cu dimensiuni de (2,3-5) x (12-16) μm şi uneori pseudomiceliu; deci pot folosi azotaţii ca sursă de azot, dar nu se dezvoltă pe extract de malţ-agar + 0,5 % acid acetic. Pe mediul Gorodkova, nu se formează spori.

Torulopsis holmii formează pe must de malţ-geloză, după 3 zile de cultivare la 25^{o}C, colonii de tip S cu diametrul de 3-5 mm, de culoare albă, perimetru circular, profil slab convex, aplatizate cu margini drepte, cu suprafaţa lucioasă. După 7 zile, coloniile ajung la diametrul de 4-7,5 mm şi îşi menţin caracterele morfologice. După 30 zile, coloniile gigant sunt colorate crem-cenuşiu, au formă circulară, uneori neregulată, margini lobate, aplatizate, lucioase .

Culturile în strii pe must de malţ-geloză sunt lucioase, netede, de culoare albă, uniforme, fără relief.

În mustul de struguri, după 3 zile de cultivare, se observă o tulbureală şi un sediment fin.

Microscopic, celulele crescute pe must de malţ-geloză, timp de 30 zile, sunt mici, elipsoidale, cu dimensiuni de (2-4) x (3-5) μm, izolate sau în lanţuri sau grămăjoare. Pe unele celule se observă muguri dispuşi lateral. Pe mediul Gorodkova, nu se formează spori .

Concluzii. 1. Examinarea caracterelor morfologice celulare ale tulpinilor de drojdii izolate a permis diferenţierea lor după mărimea şi forma celulelor (*Saccharomyces, Pichia, Candida, Rhodotorula, Kloeckera* şi *Torulopsis*).

2. După capacitatea de formare a sporilor s-au diferenţiat tulpinile de drojdii sporogene aparţinătoare genurilor *Saccharomyces* şi *Pichia.*

3. Examinarea caracterelor culturale (macroscopice) ale tulpinilor de drojdii a evidenţiat deosebiri în aspectul coloniilor gigant dezvoltate pe medii adecvate solide, funcţie de care pot fi diferenţiate aceste tulpini de drojdii.

4. Prezenţa pigmenţilor carotenoizi de culoare roz sau roşu la drojdiile aparţinătoare genului *Rhodotorula* a permis identificarea drojdiei de contaminare izolate, *Rhodotorula glutinis.*

5. Proprietatea drojdiilor peliculare (oxidative) de a forma pelicula la suprafaţa mediilor lichide alcooligene este utilizată drept criteriu de identificare a drojdiilor de contaminare izolate (*Candida mycoderma* şi *Pichia membranaefaciens*).

6. Proprietatea drojdiilor de vin izolate de a lichefia gelatina (proteoliză) este utilizată drept criteriu de diferențiere a celor două tulpini de drojdii de vin predominante în mustul de fermentație: *Saccharomyces cerevisiae* var. *ellipsoideus* şi *Saccharomyces bayanus* var. *oviformis*. *S. bayanus* var. *oviformis* nu lichefiază gelatina, iar *S. cerevisiae* var. *ellipsoideus* prezintă acest caracter.

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ADDITIONAL BIBLIOGRAPHY ON ENZYMOLOGY OF DISTURBED SOILS

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This additional bibliography comprises the papers not referred to in any of the three Parts of the book *"Enzymology of Disturbed Soils"* (XIV + 336 pages), which was elaborated by S. Kiss, D. Paşca and M. Drăgan-Bularda and published by Elsevier Science, Amsterdam, in 1998. In Part I. Enzymology of Oil-Contaminated Soils, Part II. Enzymology of Soils Affected by Industrial Emissions and Part III. Enzymology of Technogenic Soils 74, 149 and 237 papers, respectively, were referred to.

The additional bibliography consists of three separate lists, *i.e.* a separate bibliographical list will be added to each of the three Parts of the book. The papers in each bibliographical list are presented based on the system applied in *Enzymology of Disturbed Soils.* If a paper covers topics belonging to two Parts of the book, it is cited only once, namely in the additional bibliographical list of the first of the two Parts. Similarly, the additional bibliographical list of Part I comprises the papers dealing with topics of all Parts.

The soil enzymological investigations described in the 64, 74 and 61 papers cited in the three additional bibliographical lists were carried out in the following countries: Australia, Austria, Azerbaijan, Byelorussia, Canada, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Kuwait, The Netherlands, New Zealand, Poland, Romania, Russian Federation (Russia, Bashkiria, Komi Republic, Tataria), Spain, Switzerland, Turkey, Ukraine, United Kingdom, United States of America, Uzbekistan, Venezuela.

It should be mentioned that of the 64, 74 and 61 papers forming the three additional bibliographical lists, 22, 38 and 19 papers, respectively, were cited in a review article, too [S. Kiss (2001) Advances in soil enzymology (Parts I-III). *Stud. Univ. Babeş-Bolyai, Biol.,* **46** (1), 3-48].

PART I. *ENZYMOLOGY OF OIL-CONTAMINATED SOILS*

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PART II. *ENZYMOLOGY OF SOILS AFFECTED BY INDUSTRIAL EMISSIONS*

ADDENDA. **Nuclear fuel waste disposal. Urban soils. Roadside soils**

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PART III. *ENZYMOLOGY OF TECHNOGENIC SOILS*

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I N M E M O R I A M

Prof. Univ. dr. *TRAIAN CEUCA*
(18 ianuarie 1921 – 12 octombrie 2003)

Profesorul şi diplopodologul Traian Ceuca s-a născut la 18 ianuarie 1921, în localitatea Salva, judeţul Bistriţa-Năsăud, România. A urmat una dintre cele mai prestigioase şcoli din ţară, Liceul ,,George Coşbuc" din Năsăud. In perioada 1940-1942 a urmat cursurile Facultăţii de Ştiinţe la Timişoara, unde s-a refugiat Universitatea clujeană în urma Dictatului de la Viena. După o întrerupere datorată războiului (sfârşitul acestuia găsindu-l pe frontul din Cehoslovacia), şi-a definitivat studiile la Cluj. Profilul ştiinţific, moral şi civic al profesorului Ceuca a fost puternic marcat de personalitatea unor prestigioşi dascăli, precum Vasile Gh. Radu, Emil Pop şi Victor Pop. Din 1946, după revenirea Universităţii la Cluj, şi-a continuat studiile întrerupte cu 4 ani, fiind angajat în acelaşi timp ca desenator la Catedra de Zoologie, condusă de prof. V. Gh. Radu. Calităţile de fin observator şi remarcabil desenator l-au făcut, ca imediat după terminarea studiilor universitare, să fie reţinut la catedră ca preparator, iar în decursul celor 40 de ani de carieră, a urcat toate gradele didactice până în 1974, când a devenit profesor universitar.

Studiul diplopodelor a fost început încă din anul 1949, sub îndrumarea directă a prof. dr. V.Gh. Radu, studiu care s-a transformat într-o veritabilă pasiune de o viaţă şi a cuprins mai multe etape. Studiul faunei de diplopode din Europa – ca cercetare de bază, a inclus în primul rând inventarierea colecţiei ,,Biospeologica'', aflată la Institutul de Speologie ,,Emil Racoviţă'' din Cluj-Napoca. Începând din 1950, prin deplasările făcute pe teren, a colectat peste 10.000 de indivizi, din aproape 500 de localităţi din cele mai diferite regiuni şi biotopuri din ţară. Prima lucrare ştiinţifică a apărut în 1951, referitoare la câteva specii de *Trachisphera (Gervaisia*). Aprofundând studiul acestui grup faunistic, a publicat lucrări şi despre reprezentanţii altor grupe de diplopode din fauna ţării noastre, atât forme epigee, cât şi cavernicole, completând în acelaşi timp aceste lucrări, cu excepţionale desene. De asemenea, a avut colaborări cu specialişti străini, precum şi studii şi comunicări făcute pe material colectat din Italia, Spania, Germania, Franţa, Austria, Polonia, fosta Iugoslavie, Rusia, S.U.A., Anglia, Brazilia, Tunisia, Turcia, Grecia, Bulgaria, Somalia, nordul Africii, Ungaria. Lucrarea de doctorat, cu titlul ,,Studiul sistematic şi ecologic al diplopodelor proterandrice din fauna României'' a fost susţinută în 1968.

Profesorul Traian Ceuca a descris următorii taxoni noi pentru ştiinţă**:** o familie: *Hungarosomidae*, 3 genuri: *Romanosoma, Paraporatia, Napocodesmus*, 4 subgenuri: *Pseudomastuchus, Heteranthroleucosoma, Moldavobielzia, Spelaeoiulus*, 22 specii: *Polydesmus triacantos, P. brachydesmoides, Entomobielzia getica, E. varvarai, Napocodesmus endogeus, Romanosoma cavernicola, R. oltenica, R. bîrtei, R. odici, Hungarosoma inexpectata, Paraporatia racovitzai, Karpatophyllon dacicus, K. banaticum, K. carpaticum, Mastigophorophyllon bănărescui, M. aberatum, M. carpaticus, Anthroleucosoma spelaea, Stenophyllum semenicensis, Typhloiulus şerbani, T. unilineatus* şi *Glomeris pachitelopoda.* De asemenea, au mai fost citate 7 forme noi pentru fauna diferitelor ţări: Franţa, Tunisia, Bulgaria, Ungaria, Turcia. Întreaga colecţie de diplopode a profesorului Ceuca are valoare de patrimoniu şi a fost depusă la Muzeul Zoologic al Universităţii ,,Babeş-Bolyai'' din Cluj-Napoca.

În afară de pasiunea pentru studiul diplopodelor, în calitate de zoolog, s-a preocupat şi de studiul vertebratelor. Lucrările publicate în acest domeniu conţin date despre: modul de recepţionare al sunetelor de către şerpi, referinţe la cele trei exemplare de *Archaeopterix*, studii de anatomie comparată ale unor resturi de oase incinerate dintr-un mormânt dacic, precum şi concluzii legate de numărul animalelor şi al oamenilor pe care îl conţinea acesta, originea şi evoluţia vertebratelor, originea primatelor (cu referiri la *Tupaidae*), originea mamiferelor etc.

Cu toate vicisitudinile şi îngrădirile din perioada în care şi-a desfăşurat activitatea, profesorul Ceuca s-a impus ca un specialist de vârf pe plan european; astfel, a participat cu lucrări la cele mai prestigioase congrese internaţionale de miriapodologie: Paris, 1968, cu lucrarea ,,Peut-on parler de périodomorphose en absence de certains caractères secondaires", Bull. Mus. d'Hist. Nat. T. 41. Paris, 1969; Innsbruck, 1990, cu lucrarea ,,Quelques aspects sur la taxonomie, l'écologie, et zoogéografie des Diplopodes de la région Balcanique'' şi Paris, 1993, cu lucrarea „*Mastigophorophyllon* (Verh. 1897) et *Karpatophyllon* (Jawl. 1928), genres carpatiques (*Diplopoda Chordeumida*)''.

A publicat 73 de lucrări, dintre care 19 apărute în străinătate. Dar din păcate, lucrarea sa de referinţă**,** ,,Les Diplopodes de la Faune de Roumanie'', nu a putut vedea lumina tiparului în timpul vieţii autorului. Actualmente se fac eforturi ca această lucrare să apară la „Danicel''–Édition de l' Association Franco- Roumaine, France.

Profesorul Traian Ceuca a fost şi un erudit dascăl, care a format numeroase generaţii de naturalişti biologi. În cei 40 de ani de carieră universitară a predat un număr mare de cursuri universitare: zoologia vertebratelor, anatomia comparată a vertebratelor, anatomia omului, zoogeografie, zoologia nevertebratelor, tehnica preparării şi conservării materialului biologic.

A fost membru al Comitetului Internaţional de Miriapodologie (C.I.M.), cu sediul la Paris şi membru al Societăţii Germane de Zoologie (Deutsche Zoologische Gesellschaft), cu sediul la München.

Valoarea profesorului Traian Ceuca poate fi rezumată în spusele unui fost student de-al său, actual cadru didactic al facultăţii noastre: „Nu-l plângeţi că a murit, bucuraţi-vă că a trăit''.

DELIA CEUCA şi IOAN COROIU

RECENZII–BOOK REVIEWS

Universität Trier. Abteilung Bodenkunde (Trier University. Soil Science Department), **Skript zur Rekultivierung** (*Scripta on Recultivation*), Überarbeitete Version, Herbst, 1999 (Revised Version, autumn 1999), VII + 301 pages.

The book of „Scripta on Recultivation" is dedicated to description of the investigations performed, since the 80' by the Trier University through its Soil Science Department headed by Professor Dietmar Schröder, along the following four lines: 1. spoils from numerous strip brown coal mines, most of which are located within the Rhine and Wetterau Regions, in the Leipzig and Lausitz (Lusatia) Basins and recultivation of these spoils into arable or grass- or forestal lands; 2. pumice stone mine spoils in the Neuwid Basin and their recultivation into agricultural soils; 3. harbour muds from the Emden harbour and from the estuary of the Ems River and transformation of these muds into agricultural soils and 4. soils heavily contaminated with carcinogenic polycyclic aromatic hydrocarbons (PAHs) on the territory of a former coking and gas works in Saarbrücken and of a former iron works in Saarbrücken-Burbach and rehabilitation of these contaminated soils.

Both laboratory and field investigations were carried out.

The spoils, soils and harbour muds were submitted to mechanical, physical, micromorphological, chemical, biochemical (including enzymological), microbiological and zoological analyses. Redoxase and hydrolase enzyme activities were measured. Dehydrogenase, invertase and alkaline phosphatase activities were preferably determined. Substrate-induced respiration, microbial biomass and cellulose decomposition were the most frequently examined microbial parameters. Among the animals special attention was always paid to the earthworms. The results were presented in details based on their statistical evaluation. The recultivation technologies applied were also described in details. The economic value of the recultivated soils was also dealt with.

The book of „Scripta on Recultivation" consists of 66 papers (62 in German and 4 in English), each in form of a reprint or a photostat copy. Most of the papers (61) were published in the 1984-1999 period and 5 papers were under press in the autumn of 1999. At present, these five papers should also be taken as published. The 66 papers, comprising 473 printed pages with 171 figures and 131 tables in the text, were elaborated by 38 investigators. As individuals 22 of them (not listed below) contributed to a single paper, whereas 16 of them (listed below) are contributors to 2 or to more papers: W. Bartel, L. Hankes, J. Katzur, M. Kraatz, H. Kutsch and G. Weber (2 papers); M. Haubold and U. Sehy (3 papers); M. Haubold-Rosar and B. Schumacher (4 papers); N. Wermbter (6 papers); C. Emmerling, H. Schneider and M. Weyers (7 papers); R. Schneider (27 papers) and D. Schröder (54 papers).

The papers, according to their contents, can be grouped into five categories: original papers in journals (47 papers); original papers in proceedings of scientific meetings (5); review papers in edited works (9); dissertation abstracts (4) and excursion guide (1). Most of the original papers, namely 31 plus 7 papers appeared in two journals: Mitteilungen der Deutschen Bodenkundlichen Gesellschaft and Landwirtschaftliche Zeitschrift (Rheinland), respectively.

The book of „Scripta on Recultivation" can be considered a handbook as all the investigations described in it can serve as modells for the experts and decision makers interested in initiation or development of investigations aiming at the agri- or sylvicultural recultivation of mine spoils, transformation of harbour muds into agricultural soils and rehabilitation of soils contaminated with PAHs.

MIHAIL DRĂGAN-BULARDA
and STEFAN KISS

Tibor Szili–Kovács (Editor), **Proceedings of the Workshop on Managing Soil Quality – Using Microbial Resources** (12-13 September 2002, Budapest, Hungary), published in form of compact disk (CD) by the Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Budapest, in 2003, on IV + 94 pages including 51 figures and 17 tables in the text.

The workshop took place as a part of the COST (European Cooperation in the Field of Scientific and Technological Research) Action 831. Biotechnology of Soil: Monitoring, Conservation and Remediation.

The Proceedings comprise two reviews and 21 original papers. Both reviews deal with the activity (investigations and meetings) within the COST Action 831, in the period between October 1997 and September 2002. The first, one-page review was elaborated by the Editor and Dr. Oliver Dilly (Germany), vicechairman of the COST Action 831, whereas the second, 4-page review was written by Professor Anna Benedetti (Italy), chairwoman of the COST Action 831.

The names and countries of the 79 authors and coauthors and titles of the 21 original papers are listed below.

1. O. Dilly (Germany): *Microbial respiratory quotient as an indicator of nutritional conditions in agricultural and forest soils.*

2. D. Paşca, S. Kiss, M. Drăgan-Bularda, R. Crişan, V. Muntean (Romania): *Enzymological evaluation of therapeutic mud analogues obtained during incubation of peat mixed with a clay mineral or zeolitic tuff and inoculated with microorganisms from salt lakes.*

3. M. Pesaro, H. Bürgmann, F. Widmer, J. Zeyer (Switzerland): *Soil DNA content – a novel quantitative bulk parameter for description of microbiological soil characteristics.*

4. J.A. Pascal, C. García, T. Hernández, J.L. Moreno, M Ros (Spain): *The future of composts as biopesticides.*

5. M.T. Pereyra de la Iglesia, J. Domenech, B. Ramos, F.J. Gutierrez Mañero (Spain): *Screening for diesel-degrading rhizobacteria associated to* Piptatherum miliaceum *rhizosphere in a polluted soil.*

6. B. Stres, J.M. Tiedje (USA), I. Mahne, G. Avguštin (Slovenia): *Diversity of* nosZ *gene in two agricultural soils.*

7. K. Yrjälä, P.E. Galand, H. Fritze (Finland): *Diversity of methanogens in the light of ribosomal 16S- and functional MCR-gene occurrence in Finnish oligotrophic fen.*

8. I. Ignatiadis, C. Michel, F. Battaglia-Brunet, M.Bruschi, P. Bianco, E. Lojou, C. Tran Minh (France): *Development of an enzymatic amperometric biosensor using cytochromes C_3 for the fast quantification of chromate bioavailability in the environment.*

9. A. Anton, P. Máthé, G. Füleky (Hungary): *The effect of phosphorus fertilizer on the phosphomonoesterase activity of* Capsicum annuum L. *rizosphere.*

10. C. Trasar-Cepeda, M.C. Leirós, F. Gil-Sotres (Spain): *Sensitivity of soil biochemical properties to contamination with heavy metals.*

11. K. Svensson (Sweden): *The substrate-induced microbial biomass as a soil quality and fertility indicator in arable land.*

12. N. Riddech, S. Klammer, H. Insam (Austria), C. Mondini (Italy): *Comparison of the ability of GN and Eco microplates to discriminate microbial communities of different composting ages.*

13. J.L. Niqui-Arroyo, M. Bueno-Montes, J.J. Ortega-Calvo (Spain): *Biological and physico-chemical mechanisms involved in the bioavailability of polycyclic aromatic hydrocarbons in polluted soils.*

14. T. Szili-Kovács, K. Török, M. Halassy, R. Szabó (Hungary), D. Elhottová (Czechia): *Monitoring of restoration of sand grassland communities in abandoned fields under the manipulation of soil N availability.*

15. T. Takács, I. Vörös (Hungary): *Occurrence of indigenous AM fungi in calcareous sandy and chernozem soils and their effect on nutrient uptake of maize.*

16. A. Viterbo, O. Ramot, I. Chet (Israel): *Synergistic antifungal activity between two chitinases from the biocontrol agent* T. a s p e r e l l u m *T- 203.*

17. A. Prinčič, I. Mahne (Slovenia): *Responses of nitrifying bacteria to selective pressure of the changing environments – What we know and what we need to know.*

18. S.O. Petersen, K. Henriksen, G.K. Mortensen, P.H. Krogh, K.K. Brandt, J. Sørensen, T. Madsen, J. Petersen, C. Grøn (Denmark): *Recycling of sewage sludge and household compost to arable land: Fate of organic contaminants, and impact on soil fertility.*

19. J.K. Friedel, C. Kobel, M. Stemmer, W.J. Fitz, B. Freyer, W.W. Wenzel (Austria): *Advances in estimating soil microbial biomass and enzymatic activities in the rhizosphere.*

20. A. Halbritter, T. Mogyoróssy (Hungary): *Rhizosphere effect in a peat soil demonstrated via phospholipid fatty acid (PLFA) indication.*

21. G. Máthé-Gáspár, P. Máthé, A. Anton (Hungary): *Factors affecting the phosphomonoesterase activity of lignite mine spoils.*

One can suppose based only on the titles of these 21 papers and convincingly establish based on their full texts that the *Proceedings of the Workshop on Managing Soil Quality – Using Microbial Resources* are a very useful source of information for all students and experts whose professional activity is related to soils, especially for soil microbiologists and biochemists, including enzymologists and also for decision makers in problems of soil degradation and pollution and restoration of degraded and polluted soils.

DANIELA PAŞCA,
STEFAN KISS and
MIHAIL DRĂGAN-BULARDA

I s t v á n K i s s, **Az erodált talajok enzimológiája** (*Enzymology of Eroded Soils)*, Scientia Kiadó, Kolozsvár – Scientia Publisher, Cluj-Napoca, 2003, 160 pages with 34 tables in the text.

The book is a review of the enzymological investigations on eroded soils and also on slope and riparian soils very exposed to erosion. These investigations, originally described in 231 papers, had been carried out in 33 countries and, consequently, the book comprises 33 chapters. The order of countries was established based on the year in which the first paper on enzyme activities in eroded, slope or riparian soils has appeared. According to this year, the 33 countries (and chapters) present the following order: 1. Germany (1954); 2. Armenia (1965); 3. Romania (1966); 4. Russian Federation – Federal and Autonomous Republics: Russia (1970); Bashkiria (1979); Buryatia (1980); Udmurtia (1986); Evenkia (1999); 5. Byelorussia (1970); 6. Ukraine (1972); 7. Kirghizia (1974); 8. Azerbaijan (1975); 9. Uzbekistan (1975); 10. France (1976); 11. Belgium (1977); 12. Austria (1978); 13. Czech Republic (1978); 14. Georgia (Gruzia) (1980); 15. Bulgaria (1980); 16. Japan (1980); 17. Spain (1982); 18. India (1983); 19. Egypt (1984); 20. Moldavian Republic (1985); 21. Lithuania (1985); 22. Poland (1986); 23. Kazakhstan (1986); 24. New Zealand (1986); 25. Slovakia (1989); 26. U.S.A. (1991); 27. Costa Rica (1991); 28. Israel (1992); 29. Denmark (1993); 30. Canada (1994); 31. Peru (1994); 32. China (1995); 33. United Kingdom (1996).

The chapters comprise at least one of the subchapters entitled as follows: Soils eroded to different extent; Prevention of soil erosion; Improvement (rehabilitation) of eroded soils; Soils of pastures overgrazed and eroded to different extent; Improvement of overgrazed and eroded pastures; Slope soils; Soils of plateaus and depressions of regions having ondulating relief; Riparian soils.

The results of the investigations reviewed in the present book made it possible to draw the general conclusion that enzyme activities are sensitive indicators of the extent of soil erosion, of the efficiency of the methods applied for prevention of soil erosion and for the improvement (rehabilitation) of the eroded soils.

The present book is a valuable source of information not only for soil biochemists, microbiologists and technologists, but also for decision makers in problems of soil erosion.

As this book on enzymology of eroded soils is such a comprehensive and updated review which constitutes a novelty in the universal soil enzymological literature, we warmly recommend its translation into and publication in a world-wide spoken language.

KATALIN BARTÓK

Don Bradshaw, **Vertebrate Ecophysiology. An Introduction to Its Principles and Applications**, Cambridge Univ. Press, 2003, 287 pages.

The work is a manual, an introduction to the study of vertebrate ecophysiology. Its author is a Professor at the University of Western Australia, with an oustanding activity of over 30 years in the investigation of ecophysiology. This allows him to have an extremely complex functional image about animals, which is impressively reflected in the pages of this book. The vertebrates presented in the book live in habitats with extreme environmental conditions, anthropomorphically inhospitable: deserts or cold climates (arctic, antarctic). The information is exposed as case studies which present the working techniques and methods, the results of the research and their interpretation.

Many of the case studies refer to endangered animals in situations under which their internal environment is disturbed, and therefore the author structures his book in accordance with this fact. Thus, the book contains 8 chapters: 1. *Homeostasis: a fundamental organising paradigm in ecophysiology*; 2. *Stress: the concept and the reality* ; 3. *Basic methods used in ecophysiological studies* ; 4. *Turnover methodology: theory and practice* ; 5. *Case studies of stress: incidence and intensity*; 6. *Survival in deserts* ; 7. *Torpor and hibernation in cold climates* ; 8. *Marine birds and mammals*.

In the first Chapter, the factors that influence the state of internal environment are presented along with the regulating role of the endocrine and nervous system. At the very beginning of this Chapter, a special consideration for their contribution to the grounding of the concept of *homeostasis* is given to "the genius of the American physiologist" Walter Cannon, who invented the term *homeostasis* in 1929 and to the "great French physiologist" Claude Bernard, who in 1878 introduced the notion of *internal environment*. He was the first to suggest that animals regulate constantly their internal environment. As a sign of high esteem for the French physiologist, the French expression *milieu intérieur* is used throughout the book instead of the English *internal environment*. It is worth noting the author's remark that the internal environment actually varies within rather wide limits, especially in the passage phases from the juvenile to the adult state or in the reproductive period in the case of the females (after the embryo's implantation). Some pathological states, as for instance fever, presuppose a different homeostatic régime, which "resets" the upper and lower set-points.

The problem of animal stress in the natural habitat is treated mainly in Chapters 2 and 5, but the idea of stress crosses as a red line most pages of the book. Having pointed to the basic contribution of Hans Selye to the study of stress (in a nutshell: he identified three signs of the state of stress: adrenocortical enlargement, atrophy of the thymus and

lymphoid tissues and ulceration of the digestive tract, and distinguished three stages in the evolution of the state of stress: the stage of "alarm", "adaptation" and "exhaustion"), Don Bradshaw attempts at an operational definition of stress, being concerned with the identification and measurement of its intensity: … *the physiological resultant of demands that exceed an organism's regulatory capacities*. This resultant comes from a significant deviation of the internal environment from the state considered optimal, and which implies the surpassing of regulating responses, although they have been maximally activated.

Chapters 3 and 4 describe the most important methods used in ecophysiological research. These chapters are completed by 8 Appendices at the end of the book introducing the steps of an ecophysiological investigation. Out of the issues presented, we mention the ethical aspects that must be taken into consideration when experimenting with animals. In Chapter 6 one is acquainted with morphological, physiological and behavioural adaptations to desert environment of frogs, lizards, tortoises, rodents, jackrabbits, island wallabies, birds and camels, while Chapter 7 presents examples of mammals which take up adaptive states which allow them to survive under conditions of food shortage of low temperature.

The last chapter offers information about the ecophysiology of albatrosses, penguins, seals and whales. It should be mentioned that the author of the book demonstrates not only a high professional competence, but also a great sensitivity towards the animal world and towards nature in general. The book is recommended for advanced undergraduate and postgraduate students, for researchers in ecology, biodiversity and conservation. It is equally useful for university professors teaching ecophysiology and even for high school teachers.

PANTE GHERGHEL