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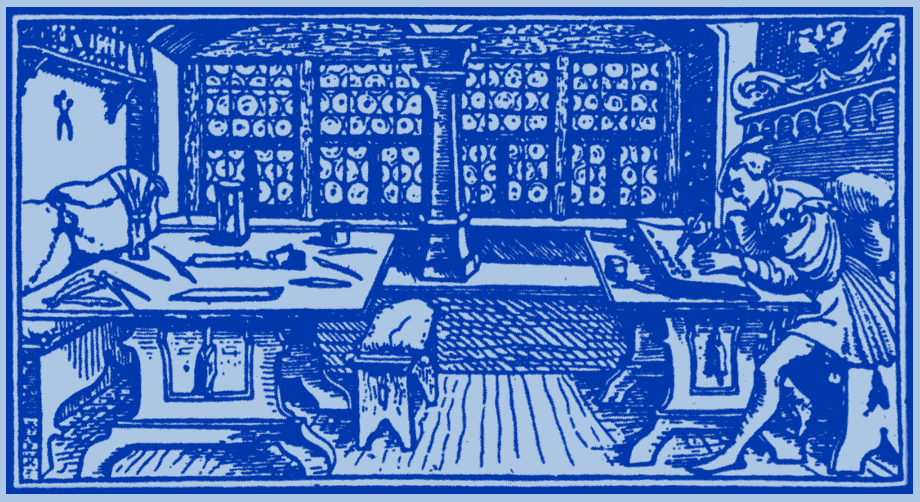

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SOIL ENZYME ACTIVITIES AS INFLUENCED BY EARTHWORMS

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SUMMARY. - This article is a review of the earthworm-related soil enzymological investigations described in the western and eastern literature mostly during the last 40 years. In the review, the literature data are grouped into 13 Sections entitled: 1. Comparison of enzyme activities in earthworm casts and underlying soil; 2. Comparison of enzyme activities in drilosphere and matrix soil; 3. Comparison of enzyme activities in soils containing and lacking earthworms, respectively; 4. Comparison of enzyme activities in soils with and without addition of earthworms; 5. Origin of earthworm cast enzymes; 6. Earthworm-related enzyme activities in cycling of plant nutrients in soils; 7. Enzyme activities and earthworms in soils as influenced by management practices; 8. Enzyme activities and earthworms as related to pesticide degradation in soils; 9. Enzyme activities and earthworms in pasture soils submitted to restoration after removal of their top layer used for landscape improvement; 10. Enzyme activities and earthworms in urban soils; 11. Enzyme activities and earthworms in mine spoils submitted to recultivation; 12. Enzyme activities in earthworm-worked dungs, composts, toxic crop residues and organic industrial wastes; and 13. Enzyme activities in soils treated with earthworm-worked manures.

Introduction. The first study showing that the earthworm casts are more enzyme-active than the underlying soil was published in 1957 [22]. The investigations along this line and in new directions have been amplified by many research groups and led to a rich literature on the relations between soil enzyme activities and earthworms. Some of these investigations were referred to in excellent review works on earthworms (*e.g.*[2, 30, 39, 45, 62]), but an updated review, considering the western and eastern literature with an equal emphasis, is lacking. This is why this review article was elaborated. The literature data reviewed are grouped into 13 Sections. Within Sections 6 and 12, subsections (3 and 4, respectively) are delineated.

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1. Comparison of enzyme activities in earthworm casts and underlying soil

In 1957, Kiss [22] reported that invertase activity was higher in the casts of *Lumbricus terrestris* than in the most enzyme-active underlying 0-2-cm soil layer and much higher than in the 5-10-cm layer. Two neighbouring areas, covered by dense and sparse vegetation, under a meadow and an arable land, on a neutral, clayey brown forest soil (located in the vicinity of the town of Dej, Transylvania, Romania) were studied (Table 1).

Table 1

Invertase activity in earthworm casts and underlying soil [22]

Studied area	Analysed material	Invertase activity*	
		$\Delta\alpha^0$	%
Meadow	Earthworm casts	2.70	177.6
	Soil from the 0-2-cm depth	1.52	100.0
	Soil from the 5-10-cm depth	0.70	46.0
Arable land	Earthworm casts	1.73	180.2
	Soil from the 0-2-cm depth	0.96	100.2
	Soil from the 5-10-cm depth	0.69	71.8

* Invertase activity, determined polarimetrically, is expressed as difference between optical rotations in reaction mixtures (prepared from 20 g air-dried casts or soil + 2.5 ml toluene as antiseptic + 10 ml 20% (weight/volume) sucrose solution + 50 ml distilled water) before and after 24 hours of incubation at 37^0C: $\alpha_0^0 - \alpha_{24}^0 = \Delta\alpha^0$. The percentage invertase activity was calculated by taking as 100% the activity measured in the 0-2-cm soil layer.

Hoffmann [19] sampled three layers (0-10-, 10-20- and 20-30-cm depths) from a sandy loam soil under meadow vegetation in the Weihenstephan area (Bavaria, Germany). The 0-10-cm layer consisted of earthworm casts and casts + soil mixture; cast-free soil could not be found in this layer. In the other two layers, three materials: casts, casts+soil mixture and cast-free soil could be separated. The results of the enzymological analyses are reproduced in Table 2.

Table 2 shows that the absolute activity of each enzyme was highest in the casts, lower in the casts+soil mixture and lowest in the cast-free soil. The absolute activities in each material decreased with decreasing organic matter content and with increasing soil depth. Based on these dependences of the absolute activities and also on the finding that the relative activities, excepting amylase activity, were not highest in casts at all soil depths, Hoffmann [19] has drawn the conclusion that the increased activities in the casts are due to microbial enzymes and, thus, the earthworms themselves do not contribute to a marked increase of the enzyme content in soil.

Table 2

Enzyme activities in earthworm casts and underlying sandy loam soil under meadow vegetation [19]

Depth of soil layer (cm)	Analysed material	Organic substance (%)	Enzyme activities*							
			Invertase		β-Glucosidase		Amylase		Urease	
			A.a.	R.a.	A.a.	R.a.	A.a.	R.a.	A.a.	R.a.
0-10	Casts	3.95	10.1	127.8	5.7	72.2	4.8	60.8	12.1	153.2
	Casts+ soil mixture	3.87	9.4	121.4	5.3	68.5	4.0	51.7	11.1	143.4
10-20	Casts	2.40	3.5	72.9	1.7	35.4	1.8	37.5	5.2	108.3
	Casts+ soil mixture	1.50	2.5	83.3	0.9	30.0	1.0	33.3	2.5	83.3
	Cast-free soil	0.98	1.8	91.8	0.4	20.4	0.7	35.7	1.7	86.7
20-30	Casts	1.77	2.7	76.3	1.5	42.3	1.4	39.5	1.3	36.7
	Casts+ soil mixture	0.67	1.1	82.0	1.3	97.0	0.5	37.3	0.9	67.2
	Cast-free soil	0.51	0.8	78.4	0.1	-	0.3	-	0.5	49.0

* Invertase, β-glucosidase and amylase activities are expressed in ml 0.1 N $Na_2S_2O_3$ equivalent to the amounts of reducing sugars and urease activity is expressed in ml 0.1 N H_2SO_4 equivalent to the amount of NH_3, produced from the enzyme substrates during incubation of reaction mixtures and reported to 2 g of analysed material (Absolute activity = A.a.) or to 1 g of organic substance (Relative activity = R.a.).

According to a report published in the Communications of the Agricultural University and Experiment Station in Gent (Belgium), Coucke [7] introduced *ca*. 12 earthworms (the species name is not given) into 2.5-kg samples of a garden soil of sandy loam texture placed in glass containers. Other samples of the same soil, at the same humidity, but without earthworm addition were the controls. The casts collected on the surface of soil treated with earthworms and the control soil were submitted to enzymological and microbiological analyses.

The results have shown that invertase activity was a little higher and amylase and cellulase activities were much higher in the casts than in the control soil, whereas urease activity was practically the same in the casts and control soil. Contrarily to the increased amylase and cellulase activities in the casts, the numbers of amylolytic, aerobic and anaerobic cellulolytic microorganisms were lower in the casts than in the control soil. This is why Coucke [7] considered that the increased amylase and cellulase activities in the casts resulted from the amylase and cellulase produced by the earthworms themselves.

Kozlovskaya and her collaborators [26-34] have determined enzyme activities in casts of *Lumbricus rubellus* and *Octalasium lacteum* and in the underlying peat soils and in casts of *Eisenia rosea* and the underlying soddy-podzolic soils. Investigations on earthworms and other soil invertebrates were carried out in 85 biogeocoenoses located in different European and Asian areas of the former USSR [27].

Casts of *L. rubellus* were more enzyme-active than the underlying peat soil. Thus, invertase and urease activities (expressed in mg of glucose and NH_3, respectively/g dry casts or soil/24 hours at 37^0C) were 80.00 and 3.66, and 8.50 and 1.05 in the casts and soil, respectively [29, 31, 32]. Mean values of cellulase activity (expressed in mg of glucose/g dry casts or soil/10 days at 37^0C) were also higher in *L. rubellus* casts (2.025) than in the peat soil (1.057) and in *O. lacteum* casts (1.909) than in the peat soil (1.080) [26-28, 31, 32, 34].

The enzyme activities underwent changes during "aging" of casts. Table 3 shows that protease activity was higher in the casts of each earthworm species than in the underlying soil. At the same time, protease activity was higher in the 1-month-old than in the 6-day-old casts of both *L. rubellus* and *O. lacteum*, but further aging of the *L. rubellus* casts led to a decrease in protease activity and, thus, in the 3-month-old casts, protease activity declined nearly to the activity of the underlying peat soil.

Table 3

Protease activity in earthworm casts and underlying soils [27, 33, 34]

Analysed material	Age of casts	Protease activity*
Lumbricus rubellus casts	6 days	9.797±0.155
	1 month	10.560±0.046
	2 months	9.594±0.077
	3 months	6.692±0.109
Peat soil	-	6.462±0.049
Octalasium lacteum casts	6 days	6.924±0.136
	1 month	12.900±0.120
Peat soil	-	5.266±0.127
Eisenia rosea casts	6 days	2.066±0.104
Soddy-podzolic soil	-	1.666±0.081

* Expressed in mg NH_2-N/g dry casts or soil/24 hours at 37^0C.

As the age-dependent increase then decrease of enzyme activity in casts are the results of microbial enzyme synthesis then destruction, the conclusions has been drawn that the earthworm casts (and excrements of other soil invertebrates) should be considered as centres of the microbial activity, of the biochemical synthesis and decomposition of organic substances [26-28, 30, 31, 34].

Sharpley and Syers [68] have determined phosphatase activity (by using *p*-nitrophenyl phosphate as substrate) in fresh earthworm casts and the underlying surface (0-5 cm) soil of silty loam texture. The casts of earthworms (dominantly *Allolobophora caliginosa*[*]) were collected from the soil surface at several sites close to undrained surface run-off plots in an experimental watershed (under permanent pasture) located adjacent to Massey University (Palmerston North, New Zealand).

The casts contained appreciably more total, inorganic and organic phosphorus than the underlying soil. Phosphatase activity was much higher in the freshly deposited (< 14 hours) casts than in the surface soil. Thus, this activity (expressed in μmoles of *p*-nitrophenol/g casts or soil/hour) was, after 1 and 5 days of incubation, 190 and 60 (after 1 day), and 60 and 40 (after 5 days) in casts and soil, respectively. It was also found that release of total P from fresh *casts* to 0.1 M NaCl at a solution: solid ratio of 50:1 did not change during the 5-day incubation at 16^0C. However, the amount of inorganic P released increased significantly and in parallel with the decrease in the amount of organic P released during the first 3 days of incubation, and these changes were related to and preceded by increased phosphatase activity. At the same time, little changes occurred not only in the amount of total P but also in the amounts of inorganic and organic P released from the *soil* under identical conditions of incubation.

Studying the seasonal variation of casting activity in the experimental watershed area specified above, Sharpley and Syers [69] have established that the amount of inorganic P released from freshly deposited (< 14 hours) casts to 0.1 M NaCl at a solution:solid ratio of 400:1 at 16 and 4^0C decreased progressively from a maximum in May to a minimum in August. In parallel, the amount of organic P released showed a reverse trend over the same May-August period. Consequently, the decrease in the inorganic P was attributed to a reduction in the conversion of organic to inorganic P because of the lower phosphatase activity as a result of declining soil temperature (the decrease being a little greater at 4 than at 16^0C). In contrast, seasonal changes in the release of inorganic and organic P to solution from the underlying soil (0-10 cm depth) were small at both 16 and 4^0C.

These investigations were referred to also in [73, 74].

Loquet *et al.* [42] and Loquet [41] have described investigations carried out on a *Lolio-cynosuretum* meadow permanent since at least 1840. It is situated in Cîteaux Monastery, at 30 km south of Dijon (France), on a nearly neutral, leached silty soil. The earthworms living here belong to 12 species, of which

[*] Syers *et al.* [72] note that the dominant earthworm species was *Lumbricus rubellus* and not *Allolobophora caliginosa* as suggested by Sharpley and Syers [68, 69].

Nicodrilus longus longus and *N. nocturnus cistercianus* are dominant. Earthworm casts and the 0-6-, 6-20-, 20-40-, 40-60- and 60-100-cm layers of the underlying soil were sampled several times in the March 1974-November 1976 period, for different analyses, including determination of invertase, urease, amylase and cellulase activities.

Invertase activity was higher in the casts than in the underlying soil, in which the activity progressively decreased with increasing soil depth. A single exception was recorded: in March 1974, the 0-6-cm soil layer was more invertase-active than were the casts.

Urease activity was also higher in casts than in soil, but amylase activity was very low and appreciable cellulase activity was lacking in both casts and soil, including the 0-6-cm soil layer.

Mulongoy and Bedoret [52] have determined enzyme activities in turret-shaped and granular casts presumably produced by the most numerous earthworm species in southern Nigeria, namely *Hyperiodrilus africanus* and *Eudrilus eugeniae (Eudrilidae)*, respectively, and in the underlying surface soils (0-15-cm layer).

Turret-shaped casts and underlying surface soils were sampled from four sites under different plant covers. Three sampling sites (designated A, B and C) are located at the International Institute of Tropical Agriculture in Ibidan (south-western Nigeria) and the fourth site (D) is located at the high rainfall substation of this Institute at Onne, near Port Hartcourt (south-eastern Nigeria). Site A is under *Leucaena leucocephala (Mimosae)*, site B - under non-leguminous vegetation in a secondary forest, site C - under *Treculia africana (Moraceae)* and site D - under *Pueraria phaseoloides (Papilionaceae)*.

Granular casts and underlying surface soil were sampled from a plot of *Treculia africana* (site C).

All samplings were done during the rainy season of 1985.

The results obtained (Table 4) clearly show that at each sampling site each enzyme activity was much higher in the earthworm casts than in the underlying surface soil. In general, no differences were found among turret-shaped casts or surface soils from different sites, but the turret-shaped casts and surface soil from site C were relatively low in acid phosphatase activity. β-Glucosidase, urease and acid phosphatase activities were not significantly different in the turret-shaped and granular casts sampled from the same site C, but the granular casts - in comparison with the turret-shaped casts - were more dehydrogenase - and alkaline phosphatase-active. Acid phosphatase activity exceeded the alkaline one in both casts and underlying soils.

Table 4

Enzyme activities in surface soil and earthworm casts from sampling sites A, B, C and D [52]

Analysed material	Sampling site	Enzyme activities*				
		Dehydro-genase	β-Gluco-sidase	Urease	Acid phosphatase	Alkaline phosphatase
Surface soil	A	192	38	6	99	27
	B	193	19	5	144	20
	C	145	11	4	78	27
	D	215	20	7	192	65
Turret-shaped casts	A	584	309	34	498	360
	B	404	134	16	540	124
	C	484	119	23	263	127
	D	265	69	23	528	137
Granular casts	C	614	129	21	335	202
LSD (5%)**		22	34	2	89	44

* Expressed in μg reaction product/g soil or casts/hour. Reaction products: triphenylformazan (dehydrogenase); ammonium (urease); *p*-nitrophenol (β-glucosidase, acid and alkaline phosphatases).

** LSD - Least significant difference. Means for granular casts are excluded.

Tiwari *et al.* [77] have determined dehydrogenase, urease and acid phosphatase activities in earthworm casts and underlying red sandy soil of laterite origin, at the Pineapple Research Station Nayabunglow (about 30 km north of Shillong, India). Samplings were done monthly, in the 15 April 1986-15 March 1987 period, during which five earthworm species (*Amynthas alexandri, Drawida assamensis, Megascolides antrophyes, Metaphire houlleti* and *Nelloscolex strigosus*) were recorded, *Drawida assamensis* being the dominant species.

Each activity was found at each sampling date to be higher in casts than in underlying soil. The trend of temporal variation in enzyme activities was similar in casts and soil. The maximum values were registered in July (dehydrogenase and acid phosphatase) and in May (urease), whereas each activity was lowest in January.

Tiunov [76] described enzymological studies related to the earthworms living in a spruce, a lime and a mixed forest (spruce, poplar, oak, lime) located on podzolic soils in the Moscow region (Russia). *Aporrectodea caliginosa* was found to be the dominant species in each of the three forests. *Lumbricus terrestris* was found in the lime and mixed forests.

Urease activity was determined in the spruce forest and mixed forest soils and in the casts of *A. caliginosa* cultured in these soils. The spruce forest soil was less urease-active than the mixed forest soil. Urease activity in fresh, 1- and 24-day-old casts exceeded the urease activity of the soil in which *A. caliginosa* was cultured. The cast urease activity in the mixed forest soil was higher than the cast urease activity in the spruce forest soil.

Matsumoto and Taniguchi [47] compared enzyme (phosphomono- and phosphodiesterase) activities in earthworm (*Pheretima communissima*) casts with those in the earthworm gut. Both phosphatase activities were much higher in the gut than in the casts. Thus, the activities measured at pHs 4.9, 6.6 and 8.2 and expressed in μmoles of *p*-nitrophenol/g material/hour gave the following values: 160.5, 92.28 and 120.3 in gut and 5.86, 7.23 and 6.40 in casts (phosphomonoesterase), and 5.74, 13.81 and 14.88 in gut and 1.07, 1.85 and 1.70 in casts (phosphodiesterase). The activities were also much higher in the gut than in the surrounding soil.

In another experiment, Matsumoto *et al.* [46] have found that phosphomonoesterase activity (expressed again in μmoles of *p*-nitrophenol/g material/hour) decreased in the order: 184.19 (foregut) > 58.33 (midgut) > 18.84 (hindgut) > 2.86 (casts). These investigators have also studied the effect of aging of casts on their phosphomonoesterase activity. Taking the activity in fresh casts as 100%, it decreased to 66, 56 and 43% after 1, 3 and 5 days of aging, respectively, and showed a slight increase (48%) after 7 days.

2. Comparison of enzyme activities in drilosphere and matrix soil

Drilosphere is a thin soil layer around the walls of the burrows of earthworms. It separates the burrows from the neighbouring, matrix soil, devoid of burrows.

The investigations carried out by Loquet *et al.* [42] and Loquet [41] for studying enzyme activities in earthworm casts and in the underlying soil were referred to in Section 1. On the same permanent meadow and in the same March 1974-November 1976 period, Loquet [41] has studied enzymologically the drilosphere, too.

A soil pit (1.4 m deep and 1.2 m wide) was examined and it was found that the earthworm (*Nicodrilus longus longus* and *N. nocturnus cistercianus*) burrows did not exceed 1 m in depth. For enzymological analyses, Loquet [41 collected the 2-mm wide drilosphere soil layer round the burrows at depths of 0-6, 6-20, 20-40, 40-60 and 60-100 cm; samples from the same depths of the matrix soil were also taken. Invertase, urease and dehydrogenase activities were determined.

The drilosphere enzyme activities changed rather irregularly in dependence of sampling depth and time and in comparison with activities in the matrix soil. For example, the ratio invertase activity in drilosphere/invertase activity in matrix soil was highest at the 60-100-cm depth in June 1975 and February 1976 and at the 6-20-cm depth in November 1975, April and November 1976. This activity was higher in the drilosphere than in the matrix soil at all depths in June 1975, but the reverse was true at depths of 20-100 cm in April 1976. In dependence of depth, invertase activity fluctuated in the drilosphere and progressively decreased in the matrix soil in February 1976.

Urease activity in drilosphere largely differed from that in the matrix soil at the 40-60-cm depth in June 1975, but the differences were small in June and November 1976.

The ratio between dehydrogenase activity in drilosphere and matrix soil at the four depths studied were 1.0, 2.5, 8.3 and 3.0, respectively, in June 1976.

As specified in a short report, Stehouwer *et al.* [70] have studied drilosphere and matrix soil at 7 depth intervals from 0 to 50 cm. Alkaline phosphatase activity, organic C and water-soluble organic C contents decreased with depth in both drilosphere and matrix soil. Concentrations in drilosphere were 2-3 times higher than in the matrix soil at the profile surface and up to 80 times higher at the depth of 50 cm.

Tiunov [75, 76] has determined enzyme activities in drilosphere of *Lumbricus terrestris* and *Aporrectodea caliginosa* and in matrix soil. The investigations were carried out in a lime and a mixed forest on podzolic soils in the Moscow region (see also Section 1). In the lime forest, *L. terrestris* drilosphere and matrix soils were collected from the 30-cm depth, whereas in the mixed forest, both *L. terrestris* and *A. caliginosa* drilosphere and matrix soils were taken from the 15-cm and 30-cm depths (*L. terrestris*) and the 10-20-cm depth (*A. caliginosa*).

Urease activity was 2-12 times higher in the drilosphere than in the matrix soil. It should be added that this activity was even lacking in the matrix soil of lime forest. The *A. caliginosa* drilosphere was more urease-active than the *L. terrestris* drilosphere. Protease activity was also higher (2-4 times) in the drilosphere than in the matrix soil. Contrarily, cellulase activity was often lower in the drilosphere as compared to the matrix soil.

3. Comparison of enzyme activities in soils containing and lacking earthworms, respectively

Atlavinyte *et al.* [1] conducted field experiments on a soddy-podzolic soil in the Širvintu district (northern Lithuania), in the 1975-1977 period. They used biometers, *i. e.* 2-m^2 microplots, in which the soil to depth of 50 cm was isolated from the surrounding soil by means of corrosion-resistant metal walls. The earthworms (*Allolobophora caliginosa* f. *typica*) were removed from a part of

biometers (biometers I) which remained free of earthworms during the 3-year experiments. In the other biometers (biometers II), the number of earthworms was maintained at an average of 150/m^2 by introduction of earthworms after sowing of winter rye in autumn 1975, barley in spring 1976 and red clover in spring 1977. Biometers I were sown with the same plants and at the same time as biometers II. Both biometers I and II were fertilised with ammonium nitrate, superphosphate and potash salt at rates (per ha) of $N_{60}P_{90}K_{90}$ (in 1975), $N_{110}P_{90}K_{90}$ (in 1976) and $P_{90}K_{90}$ (in 1977).

In August 1977 (3 years after the beginning of the experiments), soil samples were taken from the 0-13- and 13-26-cm depths for different analyses, including determination of invertase and protease activities.

Invertase activity (expressed in mg glucose/g dry soil) in the 0-13- and 13-26-cm soil layers was 32.7 and 26.7 in biometers I (no earthworms) and 32.4 and 25.0 in biometers II (with earthworms); the corresponding values for protease activity (expressed in mg NH_2-N/g dry soil) were 0.34 and 0.37, and 0.46 and 0.45, respectively. In other words, the earthworms did not affect invertase activity of soil, but led to increased soil protease activity. It should be added that under the influence of earthworms the crop yields increased (winter rye with 15%, barley with 27% and red clover with 45%).

Studying the earthworm *Pheretima communissima* in the soil of a reclaimed land near the seashore of the Osaka Bay (Japan), Matsumoto *et al.*[48] have found that invertase and urease activities were higher in the earthworm-worked soil and in the casts than in the surrounding soil lacking earthworms.

In another study, Matsumoto and Taniguchi [47] have found higher phosphomono- and phosphodiesterase activities in the upper layer of a soil rich in *Pheretima* earthworms than in a forest soil and a paddy soil in which no earthworms lived.

Tiunov [76] has collected soil samples from the 5-10-cm layer and earthworms (*Aporrectodea caliginosa*) in spruce, mixed and lime forests on podzolic soils in the Moscow region (see also Sections 1 and 2). The field-moist soils were passed through a 3-mm sieve, then placed into pots (1 kg/pot) and left without earthworms or inoculated with 3 earthworms/pot. For inoculation of each soil, the earthworms, collected from the respective soil, were used. The incubation, at 25-28% (weight/weight) soil moisture content, lasted 12 days. Before inoculation and incubation and after incubation, the soils were analysed for determination of their urease activity. In soil of each forest, urease activity was higher in the earthworm-containing variant than in that lacking earthworms. But the level of soil urease activity was dependent on forest type: the activity increased - in soils before inoculation and incubation as in those left non-inoculated or inoculated and incubated - in the order: spruce forest < mixed forest < lime forest.

4. Comparison of enzyme activities in soils with and without addition of earthworms

Subler *et al.* [71] conducted field experiments in two agroecosystems established in 1991 at the Ohio Management Systems Evaluation Area in Pike county (Ohio). The agroecosystems, each replicated (n=3) on 0.4-ha plots, were a corn-soybean rotation (CS) and a corn-soybean-wheat-hairy vetch cover-crop rotation (CSW) on predominantly silt loam and sandy loam soils. The corn in both systems was N-fertilised at the following rates (kg N/ha): 135 (as NH_3) + 44 (as liquid fertiliser, l.f.) in 1992 and 157 (as l.f.) in 1994 in the CS system; 56 (as NH_3) + 12 (as l.f.) + 28 (as manure) in 1991 and 105 (as l.f.) + ≈ 28 (as vetch) in 1994 in the CSW system. The liquid fertiliser contained 40% NH_4NO_3 and 30% urea, by weight.

In November 1993, earthworms were added (100/m^2) to enclosures (6.1 x 6.1 m) within plots of both agroecosystems. Other enclosures, to which no earthworms were added, served as controls. The enclosure walls, made of translucent corrugated plastic, were inserted *ca*. 5 cm into the soil and extended 25 cm above the soil. The added earthworms were collected from another area, namely from a no-till corn field in Columbus (Ohio) and were predominantly immature and adult *Lumbricus terrestris*.

In April 1994 (*i.e.* 5 months after addition of earthworms), some earthworm and control enclosures were used for counting earthworms. It was found that, in both CS and CSW systems, addition of earthworms led to less abundant surface-dwelling earthworms (collected above 15 cm) and most abundant deep-dwelling earthworms (collected below 15 cm) than in the control enclosures (to which, as mentioned above, no earthworms were added). Other earthworm and control enclosures were used for soil sampling at four depths: 0-5, 5-15, 15-30 and 30-45 cm. Besides a series of soil parameters, dehydrogenase activity was also determined.

In the CS system, dehydrogenase activity was insignificantly lower in the 0-5-cm soil layer and significantly ($P<0.05$) higher in deeper soil layers of the earthworm enclosure as compared to the control enclosure. Contrarily, in the CSW system, earthworm addition led to significant decrease in dehydrogenase activity at the soil surface (0-5 cm) and to insignificant changes in the deeper soil layers. These findings were interpreted as being the consequence of decreased abundance and activity of surface-dwelling earthworms in the earthworm enclosures.

The experiment performed by Ross and Cairns [61] and briefly described in Section 9 should also be mentioned here.

5. Origin of earthworm cast enzymes

It is a general opinion that the enzymes in earthworm casts originate from two sources: the microorganisms living in gut and casts and the earthworm tissues themselves. Table 5 lists literature data, according to which there are enzymes produced at least partly or not produced at all by tissues of the earthworm species studied.

Two other sources of cast enzymes can also be envisaged. If the enzymes present in the ingested food (*e.g.* plant residues) and in the ingested soil resist - as free proteins or as protein-humic complexes - to decomposition in the digestive tract, they will be ejected with the faeces. The experimental verification of these possibilities received no attention up to now, which seems to be attributed to the difficulties in elaboration of methodologies for such a verification.

Table 5

Production of enzymes in earthworms

Abbreviation for some earthworm genera: *A. - Allolobophora. D. - Dendrobaena. E. - Eisenia. L. - Lumbricus. O. - Octalasium (Octalasion)*

Enzyme	Earthworm	Examined material	Reference
1	2	3	4
Enzymes in earthworms produced, at least partly, by earthworm tissues			
Hydrolases			
Hydrolases participating in C-cycle			
Cellulase	17 species*	Extract from entire worms	78
Chitinase	12 species*	The same as above	78
Cellulase, chitinase	*L. terrestris*	Extract of foregut and hindgut wall	78
Invertase, maltase, cellobiase, melibiase, lactase, amylase, cellulase, chitinase	*D. octaedra*	Extract from homogenate of gut and gut content	54
Maltase, cellobiase, melibiase, lactase, amylase	*A. caliginosa*	The same as above	54
Laminarinase	*L. terrestris*, *L. rubellus*	The same as above	55
Amylase, cellulase, lichenase, chitinase, lipase	*L. terrestris*, *Allobophora* sp., *Pheretima* sp.	Extract from tissue of alimentary tract	37
Glycosidases	*L. terrestris*	Intestinal extract	40

Table 5 (continued)

1	2	3	4
Carbohydrases, lipases	*A. caliginosa*, *A. smaragdina*, *Allolobophora* sp., *L. rubellus*, *D. veneta*	Extract from digestive tract	44
Cellulase	*E. foetida*, *L. terrestris*, *O. tyrtaeum*	Cell-free extract from homogenate of entire worms	18
Cellulase	*E. fetida andrei*	Homogenate from gut wall and from gut wall+gut content	43
Cellulase	*D. vejdovskyi*, *D. octaedra*, *Dendrodrilus rubidus*, *L. castaneus*, *L. rubellus*, *A. caliginosa*, *A. rosea*, *O. lacteum*	Extract from homogenised gut wall (tissue)	79
α-Amylase, glucoamylase, laminarinase, xylanase, lichemase, C_x-cellulase, cellulase complex (exo- and endo-β-1,4-glucanase)	*D. octaedra*, *L. castaneus*, *L. rubellus*, *A. caliginosa*, *O. lacteum*	The same as above	80
Invertase, amylase, cellulase	*Lampito mauritii*, *Octochaetona surensis*, *Drawida willsi*	Gut-cleaned worms	8
Maltase, β-N-acetylglucosaminidase, amylase, carboxymethylcellulase, xylanase, laminarinase, galactomannanase	*Pontoscolex corethrurus*	*In vitro* tissue culture of gut wall	81
Glycolytic enzymes	*Millsonia anomala*	The same as above	35

Table 5 (continued)

1	2	3	4
Carbohydrases hydrolysing oligosaccharides (sucrose, maltose, cellobiose, laminaribiose, gentiobiose), heterosides (α- and β-glucoside, β-N-acetyl-glucosamine, β-mannoside, β-xyloside, β-galactoside) and polysaccharides (starch, cellulose, laminarin, mannan, galactomannan, pullulan, lichenin)	*Polypheretima elongata*	The same as above	36
Xylanase, acetylesterase, β-glucuronidase, α-arabinosidase, β-xylosidase	*E. andrei*	Extract from homogenate of entire, gut-cleaned worms	50
Hydrolases participating in N-cycle			
Urease	*Lampito mauritii*, *Drawida willsi*	Gut-cleaned worms	8
Protease	*L. terrestris*, *Allolobophora* sp., *Pheretima* sp.	Extract from tissue of alimentary tract	37
Protease	*A. caliginosa*, *A. smaragdina*, *Allobophora* sp., *L. rubellus*, *D. veneta*	Extract from digestive tract	44
Protease	*D. octaedra*, *L. castaneus*, *L. rubellus*, *A. caliginosa*, *O. lacteum*	Extract from homogenised gut wall (tissue)	80
Hydrolases participating in P-cycle			
Alkaline phosphatase	*Barogaster annandalei*	Different components of alimentary canal examined histoenzymologically	9

Table 5 (continued)

1	2	3	4
Alkaline phosphatase	*E. fetida*, *D. veneta*, *L. rubellus*, *A. caliginosa*	Fresh faecal material, containing acid phosphatase of microbial origin and alkaline phosphatase produced, at least partly, by the earthworms	62
Acid and alkaline phosphomono- and phosphodiesterase	*Pheretima communissima*	Foregut, midgut and hindgut	46, 47
Alkaline phosphodiesterase, acid and alkaline phosphomonoesterase	*L. terrestris*	Extract of homogenate from entire worms	58-60
Alkaline phosphomonoesterase	*E. andrei*	Extract of homogenates prepared during embryonic and postnatal stages of worms	57
Alkaline phosphomonoesterase	*A. andrei*	Extract of homogenate from midgut	56
Oxidoreductases			
Dehydrogenase, catalase, polyphenol oxidase, peroxidase	*E. nordenskioldi*	Extract from entire worms	25
Peroxidase	*O. tyrtaeum*, *L. terrestris*, *E. foetida*, *Pheretima hupiensis*, *A. chlorotica*	Cell-free extract from homogenate of entire worms	53
Peroxidase, catalase	*E. foetida*, *L. terrestris*, *O. tyrtaeum*	The same as above	18
Transferases			
Glutathione-S-transferase	*Pheretima posthuma*	Extract of homogenate from entire worms	17
Enzymes in earthworms not produced by earthworm tissues			
Hydrolases			
Hydrolases participating in C-cycle			
Trehalase, cellulase, pectinase, xylanase, chitinase	*E. rosea*	Extract from homogenate of gut and gut content	54
Trehalase, pectinase, xylanase, galactanase	*D. octaedra*	The same as above	54
Trehalase, pectinase	*A. caliginosa*	The same as above	54

Table 5 (continued)

1	2	3	4
β-N-acetylglucosa-minidase	*L. terrestris*	Intestinal extract	40
Cellulase, mannanase	*Pontoscolex corethrurus*	*In vitro* tissue culture of gut wall	81
Cellulase, mannanase	*Millsonia anomala*	The same as above	35
Hydrolases participating in N-cycle			
Urease	*Octochaetona surensis*	Gut-cleaned worms	8
Oxidoreductases			
Polyphenol oxidase, aldehyde oxidase	*E. foetida, L. terrestris, O. tyrtaeum*	Cell-free extract from homogenate of entire worms	18

* *A. caliginosa, A. chlorotica, A. icterica, A. longa, A. nocturna, (Bimastus eiseni), (D. mammalis), (D. rubica), D. subrubicunda, E. foetida, E. rosea, (Eiseniella tetraedra), (L. castaneus), L. rubellus, L. terrestris, O. cyaneum, O. lacteum.*
Five species, in which lack of material prevented the detection of chitinase, are placed in brackets.

Besides producing some own enzymes contributing to the enzyme content in casts, the earthworms stimulate the production of microbial enzymes, as demonstrated by Satchell *et al.* [64].

For culturing *Eisenia foetida* and *Dendrobaena subrubicunda* a medium was prepared from cellulose board shredded and mixed with distilled water in proportion of 1 to 9 and the resulting pulp was amended with phytin (calcium inositol hexaphosphate) in proportion of 1 to 40. Pulp (300 g) amended with phytin was placed over 10 g of fine acid-washed sand in plastic containers, to which 30 worms were then added. The cultures were kept at room temperature for 4 weeks and analysed weekly for determination of acid (microbial) phosphatase activity at pH 4 and of the amount of ATP considered as indicator of microbial biomass.

The results have shown that, in cultures of both earthworms, there was a significant correlation (r=0.93) between acid phosphatase activity and ATP content. Based on this finding the conclusion was drawn that the acid phosphatase activity observed reflects the effect of earthworm activity in increasing microbial biomass.

6. Earthworm-related enzymes activities in cycling of plant nutrients in soils

The investigations proving that the earthworm enzymes contribute to the cycling of plant nutrients in soils will be reviewed grouped according to the nature of the nutrient elements.

6.1. Nitrogen cycling. Syers *et al.* [72] have carried out investigations on 900-cm^2 plots in an experimental watershed under permanent pasture in New Zealand (see Section 1) and on an adjacent area. Casts of predominantly *Lumbricus rubellus* and underlying soil samples (from depths of 0-5 and 18-22 cm) were collected weekly in the April-October 1977 period and analysed for determination *e.g.* of exchangeable $NH_4^+ - N$ and $NO_3^- - N$ contents and urease activity. These three parameters were found to be appreciably higher in casts than in underlying soil. In casts, the $NH_4^+ - N$ content was always greater than the $NO_3^- - N$ content, but in casts incubated for 6 days at 4 and 16°C, the $NH_4^+ - N$ content continuously decreased during the incubation, while the $NO_3^- - N$ content decreased only during the first 4 days of incubation. Urease activity was highest at day 1, then continuously decreased in parallel with the decreasing $NH_4^+ - N$ content. All these changes were a little more intense at 16 than at 4°C. In the underlying soil, the duration and temperature of incubation had very little effect on the $NH_4^+ - N$ and $NO_3^- - N$ contents and urease activity.

These investigations were also referred to in [73, 74].

6.2. Phosphorus cycling. Sharpley and Syers' [68, 69] investigations, referred to in Section 1, have convincingly proved the role of earthworm cast phosphatase in conversion of organic P into plant-available, inorganic P. This role also results from the investigations described by Matsumoto and co-workers [46,47] and referred to in Sections 1 and 3.

Based on an experiment, in which 1.5-kg samples of a sandy soil, collected at Zwijnaarde (Belgium), were amended with fresh lettuce leaves without or with addition of two *Lumbricus terrestris* and maintained at 15-20°C for 5 weeks, Devliegher and Verstraete [10] have stated - commenting the results of the experiment - that "P-availability is increased in the presence of *L. terrestris* due to the production of phosphatases and the activation of microbial phosphatase production".

A similar conclusion was drawn by Ross and Cairns [61](see Section 9).

Ganeshamurthy *et al.* [14] studied a red and a black tropical soil (India) and the earthworm *Drawida assamensis*. No or ten worms per pot were added to 500-g soil samples, then the pots were kept, at constant soil humidity,

at 28^0C for 4 weeks. The soils were analysed weekly. The analytical results have shown that under the influence of earthworms the exchangeable P and SO_4-S contents increased significantly in both soils. The increases were explained by admitting that some of the ester-bound P and S were enzymatically hydrolysed to inorganic P and S thereby contributing to their availability.

6.3. Sulphur cycling. See the preceding paragraph.

7. Enzyme activities and earthworms in soils as influenced by management practices

Gehlen [15] determined dehydrogenase, catalase and alkaline phosphatase activities and number of earthworms in 16 soils, namely in four arable (cereals), four vegetable (cabbage, carrot, lettuce, potato etc.), four orchard (apple) and four vineyard soils, at seven localities in Nordrhein-Westfalen and Rheinland-Pfalz (Germany). On each soil, two neighbouring plots were selected for studies. One plot was under conventional management and the other plot was under organic-biological or biological-dynamic management. Soil samplings were done four times: in springs and autumns of 1984 and 1985.

All enzyme activities in soils of all cultures, at all sampling dates, were significantly ($P < 0.05$ or $P < 0.01$) higher under biological management than under the conventional one.

The number of earthworms in all arable soils (at all sampling dates), in all soils under vegetables (in autumn 1984, and in spring and autumn 1985), in all vineyard soils (in autumn 1985), as well as in some soils under vegetables (in spring 1984) and in some orchard soils (at all sampling dates) was higher under the biological than under the conventional management. In general, the enzyme activities were more intense and the earthworms were more numerous in the orchard soils than in the other soils.

Taking into consideration the results obtained in all soils at all sampling dates, it was established that the correlations among the three enzymes activities were positive and more pronounced (r=0.72-0.83) than those between each enzyme activity and number of earthworms (r=0.47-0.48).

Fraser *et al.* [12] determined protease, phosphatase and arylsulphatase activities as well as number and biomass of earthworms in samples of a stony silt loam soil collected from three experimental sites on the Winchmore Irrigation Research Station in the mid-Canterbury region (New Zealand).

The first site is a flood-irrigated, grazed pasture fertilised with super-phosphate at rates of 0 (control), 188 and 376 kg/ha annually for 37 consecutive years. The second site had been intensely cultivated with arable crops for the last 11 consecutive years. The third site was a wilderness nearby which had not been used for agriculture and was covered with native grasses, herbs and introduced species.

For enzymological analysis, the soil was sampled from depths of 0-5, 5-10 and 10-20 cm. The earthworm population (*Aporrectodea caliginosa, A. rosea, Lumbricus rubellus, Octalasion cyaneum*) were taken for analysis from the top soil layer (to a depth of approximately 30 cm).

Protease and arylsulphatase activities decreased and phosphatase activity increased with increasing soil depth in the soil of pasture and wilderness. The activities showed a nearly even distribution in the 0-20-cm layer of the arable soil.

The enzyme activities and the number and biomass of earthworms varied in dependence of sites (Table 6).

Table 6

Site-dependent increasing order of soil enzyme activities and earthworms
(compiled based on data from [12])

Soil enzyme activity and earthworms	Soil depth (cm)	Site*-dependent increasing order
Protease	0-5, 5-10 and 10-20	A<W<P(0)<P(188)=P(376)
Phosphatase	0-5	A<W<P(376)<P(188)<P(0)
	5-10 and 10-20	A<W<P(0)=P(376)=P(188)
Arylsulphatase	0-5 and 5-10	A<W<P(0)<P(188)=P(376)
	10-20	A=W<P(0)=P(188)=P(376)
Number of earthworms	0-30	A<W<P(0)<P(188)<P(376)
Biomass of earthworms	0-30	A<W=P(0)<P(188)<P(376)

* A - Arable. W - Wilderness, P (0) - Pasture (control), not fertilised. P (188) and P(376) - Pasture fertilised with 188 and 376 kg superphosphate/ha, respectively.

It is evident from this table that the site dependence of soil enzyme activities, especially protease activity and that of earthworms are similar.

The experiments conducted by Subler *et al.* [71] for studying soil dehydrogenase activity and earthworms in a corn-soybean and a corn-soybean-winter rotation were already dealt with in Section 4.

8. Enzyme activities and earthworms as related to pesticide degradation in soils

Park *et al.* [58-60] have studied *Lumbricus terrestris* obtained from stock cultures at the Indiana State University in Terre Haute (USA). For removal of gut content with its microbiota, the worms were purged by incubation for 24 hours on moist filter paper. Then, approximately 20 g of live earthworm biomass (3.5-5 g dry weight) was homogenised in 1:4 (weight/volume) 0.1 or 0.05 M Tris-HCl buffer (pH 8.8) with addition of no or 0.1% Triton X-100 and centrifuged at 13,000 *g* (6^0C) for 10 minutes. The supernatant (*i.e.* the extract from the homogenate of entire worms) was used as source of phosphodi- and phosphomonoesterase. Bis-(*p*-nitrophenyl) phosphate (BNPP) and *p*-nitrophenyl phosphate (NPP) served as enzyme substrates. These compounds are not insecticidal organophosphates, but their hydrolytic product, *p*-nitrophenol, is toxic to plants and animals.

It was found that the earthworm extract exhibited both phosphodi- and monoesterase activities, producing *p*-nitrophenol from both BNPP and NPP, which means that *L. terrestris* is able of toxic bioactivation of nitrophenyl phospho-ester xenobiotics.

Contrarily, another enzyme, found in earthworms, too, can participate not in toxic bioactivation of xenobiotics, but in their detoxification. This enzyme, glutathione-S-transferase (GST), the activation of which in the earthworm *Pheretima posthuma* was studied by Hans *et al.* [17], catalyses dealkylation, dearylation and dehalogenation of insecticides by their conjugation with glutathione, these reactions being the primary steps in detoxification.

Hans *et al*. [17] collected mature earthworms from the campus soil of the Industrial Toxicology Research Centre in Lucknow (India). The earthworms were introduced in potted soil, to which no insecticide (control) or aldrin, endosulphan or lindane was added in a concentration of 1 μg/g. The pots were kept at 25^0C at 70% relative humidity for 4 weeks. After 1, 2 and 4 weeks, GST was extracted from the earthworms, from the guts of which the soil was previously removed. Then, the worms were homogenised in 0.05 M saline phosphate buffer (pH 6.6). The homogenate was centrifuged at 1000 *g* for 5 minutes. The supernatant was the GST-containing extract. GST activity was determined using 1-chloro-2,4-dinitrobenzene as substrate.

The residual insecticide concentrations in earthworms and soil were also determined.

The extract from earthworms kept in the control soil exhibited GST activity, but this activity was significantly lower than that registered in extracts obtained from earthworms of the insecticide-treated soil. This means that each of the three insecticides tested has induced synthesis of GST in the earthworms. The insecticide-induced increase in GST activity was, generally, at a maximum after 1 week, then the activity declined. Thus, after 4 weeks of exposure to anyone of the three insecticides, GST activity declined to near the control values.

The increased GST activity was accompanied by glutathione conjugation-caused increased accumulation of each insecticide in earthworms and by a simultaneous decrease of the insecticide concentration in soil.

9. Enzyme activities and earthworms in pasture soils submitted to restoration after removal of their top layer used for landscape improvement

Ross and Cairns [61] have dealt with the restoration of a pasture soil (silt loam) at Judgeford, Wellington area (New Zealand). The top (0-15-cm) layer of this soil had been removed to be used for landscape improvement in urban areas. The remaining soil was rotary hoed to 15-cm depth to mix the AB and B_1 horizons. The resulting mixed subsoil was collected and packed into pots with each containing 24.9 kg oven-dry weight of soil.

The restoration experiment comprised addition of earthworms, *Allolobophora (Aporrectodea) caliginosa* (100 per pot) and sowing of ryegrass, *Lolium perenne* (100 seeds per pot). Lime and fertilisers (P, K, S, trace elements, and N as urea) were added at sowing and during the experiment. Four treatments were applied: 1. no earthworms and ryegrass (subsoil alone); 2. no earthworms, with ryegrass; 3. with earthworms, no ryegrass; 4. with earthworms and ryegrass. The pots were maintained in a glasshouse and watered regularly.

The experiment started in October 1978 and lasted some 13 months. The soil was analysed at approximately 4-monthly intervals. The results obtained in enzymological analyses are reproduced in Table 7.

One can deduce from Table 7 that in subsoil alone (treatment 1), xylanase, urease, phosphatase and sulphatase activities declined, but invertase activity increased during the experiment. The presence of earthworms resulted in increased cellulase and sulphatase activities. Generally, the presence of ryegrass enhanced all enzyme activities. The additional presence of earthworms further stimulated invertase, amylase, urease and phosphatase activities.

The conclusion was drawn that the earthworms - due to their stimulating effect on biochemical activities - contribute to the restoration of pasture productivity after topsoil removal.

Table 7

Influence of earthworms and ryegrass on soil enzyme activities [61]

Enzyme activity*	Duration (months)	Treatment**			
		Earthworms absent		Earthworms present	
		Ryegrass absent	Ryegrass present	Ryegrass absent	Ryegrass present
1	2	3	4	5	6
Invertase	4	170 aA	600 bA	140 aA	760 cA
	8	280 aB	810 bA	290 aB	1250 cB
	13	290 aB	1360 bB	380 aB	1570 bC
Amylase	4	36 aA	43 abA	61 abA	65 bA
	8	60 aA	78 aB	96 abB	130 bB
	13	43 aA	110 bC	55 aA	150 cB
Cellulase	4	7 aA	24 bA	23 bA	26 bA
	8	11 aB	27 bA	18 abA	31 bA
	13	6 aA	43 bB	8 aA	42 bB
Xylanase	4	15 aC	90 bA	17 aA	87 bA
	8	9 aB	110 bA	11 aA	140 bA
	13	5 aA	120 bA	10 aA	110 bA

Table 7 (continued)

1	2	3	4	5	6
Protease	4	19 aA	32 bA	21 abB	26 abA
	8	19 aA	28 abA	25 abB	32 bA
	13	14 aA	31 bA	12 aA	37 bA
Urease	4	190 aB	340 bB	180 aA	300 bA
	8	180 aB	240 bA	170 aA	290 cA
	13	160 aA	340 bB	150 aA	420 cB
Phosphatase	4	620 aB	800 bB	640 aB	840 bB
	8	540 bA	580 bA	460 aA	690 cA
	13	570 aA	910 bB	580 aB	910 bB
Sulphatase	4	84 aB	100 aB	95 aA	100 aA
	8	56 aA	74 bA	95 cA	80 bcA
	13	68 aA	79 abA	79 abA	87 bA

* Expressed in pmoles of reaction product/g dry soil/second. Products: "glucose" (invertase, amylase, cellulase); "xylose" (xylanase); "tyrosine" (protease); ammonium-N (urease); *p* - nitrophenol (phosphatase, sulphatase).

** a, b, c (effects of treatments) : any two means within a row not marked with the same letter are significantly different ($P < 0.05$).
A, B, C (effects of the duration of experiment): any two means within a column not marked with the same letter are significantly different ($P < 0.05$).

10. Enzyme activities and earthworms in urban soils

Based on the values of catalase, xylanase and urease activities in soils of 12 places and on the abundance of earthworms in soils of 42 places in Bonn-Bad Godesberg (Germany), Fründ *et al.* [13] found that none of the soil enzyme activities correlated significantly ($P > 0.05$) with the number and fresh biomass of earthworms. The most frequently found earthworms were *Lumbricus terrestris* and *Allolobophora caliginosa*. See also page 131 in [23].

Studying soils in the city of Dorsten, located at the northern rim of the Ruhr industrial area (Germany), Keplin and Broll [20] have determined dehydrogenase activity of the 0-8-cm soil layer and the number and dry biomass of earthworms (the earthworms with their gut content were dried at 65^0C for 24 hours). Seven lumbricid species were identified. Formerly, the studied soils were used as gardens, grassland and arable land. Soil dehydrogenase activity, like the number and dry biomass of earthworms, decreased from ancient garden land to grassland and arable land. Thus, in the most dehydrogenase-active soil (97 mg triphenylformazan/g soil) of the ancient garden, the number and dry biomass of earthworms were 224/m^2 and 19.4 g/m^2, respectively. The corresponding values in the least active formerly arable land (at present shrub woodland) were: 54 mg/g soil, 57/m^2 and 2 g/m^2, respectively. See also page 131 in [23].

11. Enzyme activities and earthworms in mine spoils submitted to recultivation

Müller *et al.* [51] studied 12 technogenic soils located in the Köln-Bergheim zone, within the surface-mined brown coal area in the Rhine region (Germany). These soils were recultivated after redeposition of mine spoils (loess) as dry materials 20 years previously. Since that time, four of them were used as grasslands, four as maple and hornbeam forests and four as arable lands.

Dehydrogenase activity was highest in the grassland soils, intermediary in the forest soils and lowest in the arable soils. Numerous earthworm burrows were observed in the grassland and forest soils, but only a few occurred in the arable soils. See also page 190 in [23].

In a similar study in the same surface-mined brown coal zone of the Rhine region, Schneider *et al.* [65] compared about 10- and 25-year-old recultivation spoil (loess) plots used as arable lands or forests. Dehydrogenase activity (expressed in μg triphenylformazan/5 g dry soil) in the 5-10-cm layer was ~ 100 in both 10- and 25-year-old arable spoil plots, and ~ 200 and ~ 320 in the 10- and 25-year-old forest spoil plots, respectively.

Total number of lumbricid earthworms (belonging to seven species) in the 0-10-cm soil layer of the 10-year-old arable and forest spoil plots was 60 and 141 /m^2, respectively, whereas in the same layer of the 25-year-old arable and forest spoil plots 223 and 339 earthworms/m^2, respectively, were recorded.

On the basis of these and other data, it was recommended that agricultural recultivation of brown coal mine spoils in the studied region should begin with forest recultivation; under such conditions, the arable soils will reach a "maturity stage" more rapidly. See also page 191 in [23].

By using coal mine spoils from the Nazarovo Basin (Siberia), Bezkorovainaya [5] carried out a pot experiment to study the role of earthworms in decomposition of litters produced by six tree species: aspen (*Populus tremula*), birch (*Betula fruticosa*), Siberian larch (*Larix sibirica*), Siberian pine (*Pinus sibirica*), Scotch pine (*Pinus sylvestris*) and Siberian spruce (*Picea obovata*). Each pot contained 2.5 kg of spoils. Litter (50 g dry weight) and five earthworms (*Eisenia* sp.) were placed on the surface of spoils. Pots with spoils and litter, but without earthworms were the controls. After moistening, the pots were exposed to hydrothermal conditions similar to the natural ones. The experiment lasted 3 years (1985-1987). The litters and spoils were submitted yearly to different analyses.

It was found that decomposition of each litter was stimulated in the presence of earthworms and, as expected, the decomposition degree was more advanced in deciduous than in coniferous litters. In the presence of earthworms, protease and urease activities of litters increased. The increase was highest in the urease activity of birch litter: it was 1.5-3-fold in comparison with the other litters and 2.5-fold compared with the control birch litter. See also pages 183-184 in [23].

12. Enzyme activities in earthworm-worked dungs, composts, toxic crop residues and organic industrial wastes

12.1. Dungs. Businelli *et al.* [6] have determined five hydrolase (amylase, acid and alkaline phosphatase, phosphodiesterase, arylsulphatase) activities as well as dehydrogenase activity in casts produced by *Lumbricus rubellus* from: cow and horse dungs (Sample 1), cattle and horse dungs (Sample 2), horse dung (Sample 3), cow and sheep dungs (Sample 4) and municipal waste compost (Sample 5).

Hydrolase activities were high and virtually the same in all samples. But dehydrogenase activity was low in Sample 5, which was attributed to the particularly high concentration of heavy metals, especially lead in the municipal waste.

The investigations of Benedetti *et al.* [3] indicate that during bioconversion of pig dung into a manure under the action of *Eisenia foetida*, the high protease and urease activities in the fresh dung gradually decrease.

12.2. Composts. In contrast to findings of Benedetti *et al.* [3], Seregina and Lysak [66] have found that urease activity, like invertase and dehydrogenase activities, exhibited some increases during composting of cattle dung, sewage sludge and dung+sludge mixture in the presence of added earthworms. It should be emphasised that such increases did not occur in the absence of earthworms. Catalase activity was low and remained practically unchanged during composting in both absence and presence of earthworms.

The wet vermicompost prepared from cattle dung, peat and straw (Lazarchik *et al.* [38]) exhibited high dehydrogenase and catalase activities.

Korotkova [24] carried out experiments for modelling vermicomposting of dung-peat mixture under climatic conditions of Siberia. Vermicomposting was conducted at 6-8^{0}C or at 20-25^{0}C. The vermicompost obtained at 6-8^{0}C was more protease- and catalase-active than that prepared at 20-25^{0}C.

For estimation of compost maturity, Forster *et al.* [11], soil scientists of the Bayreuth University (Germany), have measured dehydrogenase activity, arginine ammonification, respiration (CO_2 evolution) rate and several chemical parameters of six composts obtained from different parent materials, the composting technology and time being also different. Designation, parent materials and age of composts are specified below: S - barley and wheat straw, ~ 5 months; B - spruce and pine bark, 4 weeks; HB - hope rape from brewery waste and spruce bark, 1 year; Z - household and garden waste in the presence of *Eisenia foetida*, 6-9 months; O - household and garden waste (no earthworms added), ~ 18 months; and PS - paper dust, minicipal sewage sludge and willow shavings, 12 weeks.

It was found that for estimation of the maturity of these composts, the chemical data were contradictory. But a combination of the values of dehydrogenase activity and arginine ammonification was considered sufficient to estimate compost maturity and, implicitly, the consequences of applying a compost to soil. A high dehydrogenase activity and negative arginine ammonification indicate immaturity of composts, while low dehydrogenase activity and positive, but low arginine ammonification are characteristics of mature composts.

The values in Table 8 allow to establish the following increasing order of maturity of the six composts studied: B < PS < HB ≈ Z < S < O. It results from this order that compost Z, in which the household and garden waste had been worked by *E. foetida* during 6-9 months, was less mature than compost O, obtained from household and garden waste after composting without earthworms but during 18 months.

Table 8 also shows that respiration rate would give a mostly other order of compost maturity. It was pointed out that respiration rate did not contribute to the assessment of compost maturity.

Table 8

Dehydrogenase activity, arginine ammonification and respiration of composts [11]

Compost	Dehydrogenase activity*	Arginine ammonification**	Respiration***
S	2.06 b	13.5 cd	2.16 d
B	2.95 c	-18.5 a	2.19 d
HB	5.64 e	35.3 d	2.35 d
Z	4.63 d	78.4 e	1.49 b
O	0.83 a	5.3 bc	0.95 a
PS	5.89 e	-12.9 ab	1.81 c

* Expressed in mg triphenylformazan/g dry compost/24 hours.

** Expressed in μg $NH_4^+ - N$ / g dry compost/hour.

*** Expressed in mg CO_2/g dry compost/24 hours.

Means within columns followed by the same letter are not significantly different at the 1% level.

The three composts studied by Serra-Wittling *et al.* [67] were prepared from the organic fraction of the municipal solid household wastes in the town of Bapaume (France). The C1 compost was obtained by 6-week fermentation, followed by 7 months of maturation. For the C2 compost, the fermentation period lasted 10 weeks, followed by 3-month maturation. The LC2 compost was prepared as follows: after fermentation, a part of the C2 compost was lumbricomposted with *Eisenia andrei* for 2 months, then was subjected to 1-month maturation without earthworms.

Activities of seven hydrolases participating in the C- and N-cycles (cellulase, β-glucosidase, β-galactosidase, β-N-acetylglucosaminidase, protease, urease, amidase) and two oxidoreductases (dehydrogenase, peroxidase) were determined in the composts.

Protease activity was higher in the older C1 compost than in the younger C2. Urease and peroxidase activities did no differ significantly between these two composts. The other six activities gave higher values in C2 than in C1, up to 10 times higher for cellulase and amidase.

Little differences were registered in enzyme activities of C2 and LC2. Thus, cellulase, β-glucosidase, β-galactosidase, protease, urease and dehydrogenase activities were a little higher, while β-N-acetylglucosaminidase, amidase and peroxidase activities were a little lower in C2 than in LC2, but all differences were statistically insignificant. As C2 and LC2 were prepared from the same municipal wastes and had the same age (10 weeks + 3 months), one can deduce that the effect of *E. andrei* on enzyme activities in compost was not long-lasting.

Based on enzymological and chemical analyses of sewage sludge during its vermicomposting, Benítez *et al.* [4] have established that the ratio between dehydrogenase activity and water-soluble organic carbon content may be considered as an index which makes it possible to distinguish between hydrolytic and maturation phases in the sewage sludge composting process.

For vermicomposting a 1:1 mixture of two sewage sludges were used, namely an anaerobically digested sewage sludge from the wastewater treatment plant of a paper mill and an aerobically digested municipal sewage sludge. The mixture (1 kg fresh weight) was placed in 1-l cylindrical plastic container. The surface of the mixture was covered with 100 g (fresh weight) of vermicomposted sewage sludge to act as a microbial inoculum and to provide suitable conditions for earthworms. Then, ten *Eisenia foetida* were added to the vermicomposted material.

During the vermicomposting period (10 weeks), the moisture content of the mixture was maintained at 75-80% and the containers were kept in darkness at room temperature. Enzyme activities and chemical parameters of sludge and biomass of earthworms were determined weekly. After the vermicomposting period, the containers with compost and worms were left at room temperature for further 8 weeks to complete the stabilisation of organic matter. At the end of the 18th week, the enzymological and chemical analyses were repeated.

Four hydrolytic enzyme (β-glucosidase, urease, protease, phosphatase) activities and dehydrogenase (DHase) activity were determined. Of the chemical parameters analysed, the content of water-soluble organic carbon (WSC) should be emphasised.

The activities showed a decreasing tendency during the first 4-7 weeks of composting; then, they remained practically stable (constant) up to the end of the composting period and even at week 18.

Each enzyme activity correlated significantly ($P < 0.01$) with WSC. The correlations among enzyme activities were also significant ($P < 0.01$), excepting a single pair (urease-phosphatase) with insignificant correlation.

As DHase activity reflects the overall microbial activity and as it has been stable (constant) after 5 days of vermicomposting, Benítez *et al.* [4] used this activity, more precisely the ratio between this activity and WSC as an index which allows to distinguish two phases in the advancement of the vermicomposting process. The time - during which this index exhibits, after an initial increase, a decreasing tendency and the earthworm biomass shows an increasing trend - corresponds to the hydrolytic phase. During the next, maturation phase, the time-dependent changes in this index are not significant. Benítez *et al.* [4] have found that the hydrolytic phase lasted 7 weeks and the maturation phase comprised weeks 8-18. During the last 3 weeks of the composting period (weeks 8-10), the earthworm biomass did not increase and began to decrease.

12.3. Toxic crop residues. For disposal of cassava peel, a toxic crop residue, M b a [49] utilised the earthworm *Eudrilus eugeniae*. The bitter cassava (*Manihot utilissima)* is widely grown in Nigeria and other tropical countries for its large tuberous roots rich in food carbohydrate. The rind of the roots has a high cyanide content and, as cassava peel, forms a toxic waste.

Air-dried cassava peel samples (200 g each) were introduced to plastic pots with perforated bottoms and watered to field capacity. Eight sub-adult *E. eugeniae* were added to each pot. The developing worm cultures were watered every other day and amended with 100 g of air-dried cassava peel at monthly intervals. After 4 months, the worms and cocoons were separated by hand and the worm culture residue was submitted to different analyses, including measurement of dehydrogenase and acid phosphatase activities. For comparison, two controls were used: air-dried peel and decomposed peel (peel allowed to decompose without earthworms for 8 months).

The earthworm biomass, determined several times during the 4-month culturing, increased continuously with time.

The cyanide content in the worm culture residue was about 50% of that measured in the air-dried peel and was similar to that in the decomposed peel.

Dehydrogenase activity (expressed in mg 2,3,5-triphenyltetrazolium chloride reduced/100 g dry weight) and acid phosphatase activity (expressed in mg phenol/g dry weight) showed the order: 14.0 (air-dried peel) < 59.3 (decomposed peel) < 127.3 (culture residue) and 4.9 (air-dried peel) < 24.6 (decomposed peel) < 182.0 (culture residue), respectively. Thus, the earthworm-worked cassava peel became less toxic and more enzyme-active.

12.4. Organic industrial wastes. Satchell [62] and Satchell and Martin [63] used paper mill waste, rich in cellulose and poor in phosphate, for culturing *Eisenia foetida, Dendrobaena veneta, Lumbricus rubellus* and *Allolobophora caliginosa.*

The culture medium was prepared from 40 g of sterilised paper waste (pH 6.8; water content ≈ 85%) amended with 1 g phytin (calcium inositol hexaphosphate) serving as P source. Identical amounts of the medium (*i. e.* 40 g paper waste + 1 g phytin) were placed in small polythene tubs, then 20 earthworms were introduced in separate tubs for each worm species. No worm was added to the control medium.

The cultures were left for 24 days for the worms to work through the medium, and samples of the fresh faecal material and of the control medium were then collected for determination of phosphatase activity. It was found that phosphatase activity was higher in the faecal material of each worm species than in the control medium. When phosphatase activity was assayed in reaction mixtures buffered to provide a pH range of 2-10, two peaks could be registered in the faecal phosphatase activity, namely at about pH 3-5 and about pH 9-10, respectively. These findings were interpreted by the suggestion that the acid phosphatase in earthworm faeces is of microbial origin, whereas the alkaline phosphatase was produced, at least partly, by the earthworms themselves.

13. Enzyme activities in soils treated with earthworm-worked manures

A vermicompost prepared from cattle dung, peat and straw was used by Lazarchik *et al.* [38] for manuring soddy-podzolic soils. Pot and field experiments were carried out. In the manured soil, the green biomass of the test plants (*e. g.* lettuce) showed a 35-40% increase. Enzyme activities also increased in the manured soils.

Serra-Wittling *et al.* [67], who determined nine enzyme activities in the C1, C2 and LC2 composts (see Subsection 12.2), also determined the effect of these composts on enzyme activities in a loamy soil sampled from the 0-20-cm layer of a bare experimental plot at Grignon (France).

Fresh soil samples (50 g each) were amended with 10 or 30% of compost (weight/weight). Soil samples without compost and compost samples without soil (prepared from 25 g compost and 25 g inert sand) served for comparison. Then, all samples were moistened to 95% water-holding capacity and incubated in the dark at 28^0C for 189 days. During the incubation, the same nine enzyme activities were determined as those measured in the composts (see Subsection 12.2).

The results obtained may be summarised as follows.

The enzyme activities were higher in the composts than in the soil, except for urease which was similar in the composts and soil.

In the soil-compost mixtures, the enzyme activities were not additive.

The C-cyle enzyme (cellulase, β-glucosidase, β-galactosidase and β-N-acetylglucosaminidase) activities were not affected by addition of C1, but C2 and LC2, at 30% additions, increased these activities.

The N-cycle enzyme (protease, urease and amidase) activities in soil were stimulated by the 30% compost addition, but the increase was only transient for urease and amidase.

Dehydrogenase activity was the only activity which increased significantly in soil-compost mixtures as the rate of compost addition increased.

Peroxidase activity in soil increased at both compost addition rates, the increase being lower with C1 than with C2 and LC2.

No significant differences were observed in enzyme activities of the soil-C2 and soil-LC2 (lumbricompost) mixtures. In the absence of soil, a single important difference was found between C2 and LC2: after 189 days of incubation, urease activity was greater in C2 than in LC2.

Govedarica *et al.* [16] conducted a field experiment on a calcareous chernozem soil in Yugoslavia. Three 1-ha plots were used. The first plot was treated with 5 t of earthworm manure; the second received 15 t of green manure - rape (*Brassica napus oleifera*), and the third, serving as control, was not manured. The test plant was wheat. At the beginning, middle and end of the growing period, soil samples were taken from the 0-30- and 30-60-cm depths for enzymological and microbiological analyses.

Earthworm manuring increased dehydrogenase activity to a large extent and protease and urease activities to a lesser extent in the 0-30-cm layer at all sampling dates and in the 30-60-cm layer at two sampling dates. Green manuring increased dehydrogenase activity, as did earthworm manuring, but it had a negligible effect on protease activity and a strong decreasing effect on urease activity at both soil depths and at all sampling dates. In other words, the 5 t of earthworm manure enhanced more positively the enzymatic potential of soil than did the 15 t of green manure. Total number of microorganisms was also higher in the earthworm-manured than in the green-manured soil.

For studing remediation of oil-contaminated soils, Kireeva [21] carried out a field experiment. Microplots on a grey forest soil in Bashkiria (Russian Federation) were contaminated with 0, 8, 16 and 25 l of crude oil/m^2, then treated or not treated with Biohumus. Biohumus is a compost which was prepared from cattle, pig and horse dungs mixed with sawdust and chopped straw and inoculated with *Eisenia foetida*, then subjected to maturation for 6 months. Biohumus was applied several times at rates of 4-8 t/ha. The test plants were oats and barley. Enzyme activities were determined in the 0-20-cm soil layer 1 and 12 months after the Biohumus treatment.

Enzymological analysis of soil samples showed that invertase, phosphatase, dehydrogenase, catalase, peroxidase and polyphenol oxidase activities decreased and urease activity increased in parallel with the rate of crude oil contamination. Biohumus attenuated the negative effects of crude oil, increasing each enzyme activity in each plot. The only exception was urease activity as in the contaminated plots Biohumus diminished the oil-induced high urease activity.

Biohumus enhanced oil degradation. Grain yields of oats and barley, in both the second and third years, were significantly higher in the Biohumus-treated contaminated plots than in the untreated contaminated plots. The yields in the third year in plots contaminated with 8 and 16 1 of crude oil/m^2 and treated with Biohumus attained the yield obtained in the uncontaminated plots. But in the plots contaminated with 25 l of crude oil/m^2 and treated with Biohumus, the yields were about 60% of those of the uncontaminated plots. See also pages 32-33 in [23].

Concluding remarks. It is considered since Charles Darwin that the earthworms play an important role in soil fertility. The mechanisms by which the earthworms play this role is multiple as comprehensively reviewed by Syers and Springett [74].

Our present article dealt with only a single aspect related to contribution of earthworms to soil fertility. This, enzymological aspect was the objective of investigations performed by many research groups in a series of countries under different climatic conditions. The conclusion which can be drawn from the results of these investigations is that the activity of earthworms leads to increased enzymatic (catalytic) potential of soils, this potential having a major biological significance as the biochemical reactions in the living systems, including the soils, are catalysed by enzymes.

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NEW MORPHOMETRIC DATA AND GEOGRAPHICAL DISTRIBUTION OF CRICONEMATID SPECIES (*NEMATODA*: *CRICONEMATIDAE*) IN ROMANIA

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SUMMARY. - Twenty nematode species (*Nematoda*: *Criconematidae*) from several habitats in Romania are studied under the light microscope. Details of the main taxonomic characters for seven species (*Criconemoides annulatus, C. informis, C. morgensis, C. parvus, Mesocriconema kirjanovae, M. rusticum* and *Criconema demani*) are discussed and their morphometric data are given in tables. Distribution maps for all recorded species in Romania are provided and their preferences for certain habitats are underlined.

Ecological surveys of soil nematodes throughout Romania provided a rich variety of criconematids from over one hundred different study sites. So far, several species belonging to the *Criconematidae* have been reported from Romania [17, 19-21]. More recently, seven newly recorded species belonging to the genera *Mesocriconema* and *Ogma* were described and illustrated [22].

The present paper refers to new morphometric data, details on taxonomic characters of seven species and the geographical distribution of all known criconematid species in Romania.

Material and methods. Sampled sites containing criconematid species are briefly described in Table 1. Nematodes were extracted using the centrifugal method of de Grisse [8], killed and preserved in a 4% formaldehyde solution, mounted in anhydrous glycerin [24] and examined under the light microscope.

All measurements in Tables 2 and 3 are in μm; dimensions and ratios of the species are presented as mean and limit values.

The main taxonomic characters used are those underlined by de Grisse [6,7], Andrássy [1, 2], Raski and Luc [23], Loof [15] and Loof and de Grisse [16].

Results and discussion. Twenty nematode species belonging to the *Criconematidae* family were identified in the study sites. The main taxonomic characters of some species with large ranges of variations and the geographical distribution of all recorded species in Romania are presented below.

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Criconemoides annulatus Cobb in Taylor, 1936
(Fig. 1A; Table 2)

This species is characterised by numerous, finely crenate body annuli with 6-14 anastomoses present, large bodies and long spear. Submedian lobes present as submedian pseudolips (lip-like); vulva simple, closed, anterior vulva lip without lobes, spermathecae round to oval, filled with sperm; tail conical-rounded with three-lobed terminus. Morphometric data are presented in Table 2. Details on its intraspecific variation have already been discussed [18].

Distribution and habitat. The first report on this species in Europe, except for the Island of Spitzbergen [14], was from Romania [17]. It has a very restricted distribution in Romania (site no. 4) (Table 1, Fig. 1A) and was found in soil of a spruce forest at depths of 10 – 60 cm.

Criconemoides informis (Micoletzky, 1922) Taylor, 1936
(Fig. 1B; Table 2)

The present measurements (Table 2) are identical to the range of variability given in the literature [7, 11].

Submedian lobes lip-like; body annuli smooth to very finely crenate with few anastomoses; vulva simple and closed; tail conical. Almost all specimens from grasslands in Romania have large spermathecae filled with sperm, while specimens found in forest soils had either empty spermathecae or with few sperm or the spermathacae were not observed. Grassland soils seem to be more favourable for the development of fertile populations of this species than forest soils, possibly due to the high diversity of nutrients. This phenomenon was also observed and discussed by de Grisse and Loof [9].

Distribution and habitat. This species was found at the following sites: 1-3, 18, 22, 29, 30, 58, 60, 62, 85 and 106 (Table 1) in varying habitats from the sand dunes of the Danube Delta to the subalpine grasslands, also in mine spoil dumps under bioremediation (Fig. 1B). It was previously reported in some faunistic and ecological studies [17, 20, 21] and our data confirm its wide distribution in the Northern Hemisphere [10].

Criconemoides morgensis (Hofmänner and Menzel, 1914) Taylor, 1936
Syn.: *C. pseudohercyniensis* apud Popovici,1992
(Fig. 1A; Table 2)

Body cylindrical, robust, slightly curved ventrally; body annuli smooth to crenate with 1-14 anastomoses, two or three annuli successively connected; submedian lobes lip-like; vulva closed, anterior lip with two lobes; vagina straight; spermatheca filled with sperm; tail conical- rounded, with the last 2-3 folded annuli. Details on morphometric data are given in Table 2.

Distribution and habitat. This species was found at only one site, no. 101 (Table 1, Fig. 1A) and was reported from Romania [19] under the name *C. pseudohercyniensis*.

Criconemoides parvus Raski, 1952
(Fig. 1A; Table 2)

The presence of this species in Romania was first reported by Popovici [19]. More information on the only specimen (a female) found is given in Table 2.

Anterior end truncate, submedian lobes absent; body annuli without anastomoses, margins of annuli very finely crenate (more visible on tail). First annulus 7 μm in diameter, mid-body 24 μm width; esophagus 76 μm long; vulva closed; spermatheca filled with sperm; body wall bend at the level of vulva; tail rounded, 9 μm long.

Distribution and habitat. This species was found in sandy soil with high moisture content (site no. 101) (Table 1, Fig. 1A).

Mesocriconema crenatum (Loof, 1964) Andrássy, 1965
(Fig. 1C)

Morphometric and taxonomic details are described in [22].

Distribution and habitat. The species was found at site 73 (Table 1, Fig. 1C), in subalpine area.

Mesocriconema curvatum (Raski, 1952) Loof & de Grisse, 1989
(Fig. 1D)

Morphometric and taxonomic details are presented in [22].

Distribution and habitat. The species was found at sites no. 38, 45, 54 and 63 (Table 1, Fig. 1D).

Mesocriconema kirjanovae (Andrássy, 1962) Loof & de Grisse, 1989
(Fig. 1D; Table 3)

Head with small and rounded submedian lobes; lateral plates small but visible; stylet robust; vulva open, anterior vulva lip with two acute projections; vagina straight; spermathecae oval filled with sperm; tail conical pointed.

Morphometrics (Table 3) fall within the range given by Brzeski [4].

Distribution and habitat. The species was found only at site no. 102 (Table 1, Fig. 1D). It was previously reported from Romania [19].

Mesocriconema rotundicauda (Loof, 1964) Loof, 1989
(Fig. 1C)

Details concerning morphometrics and taxonomic characters are described in [22].

Distribution and habitat. The species was only found at site no. 73 in natural subalpine habitat (Table 1, Fig. 1C).

Mesocriconema rusticum (Micoletzky, 1915) Loof & de Grisse, 1989
(Fig. 2A; Table 3)

Submedian lobes large truncate. Annuli smooth; vulva open; vagina straight, tail rounded. Morphometric data are given in Table 3.

Distribution and habitat. M. rusticum is wide spread and was found in 30% of the study sites (no. 1-3, 5, 10, 12, 18-20, 22, 27-29, 39-44, 47, 48, 55, 64, 67, 74, 76, 79, 85, 92, 95, 101 and 102) (Table 1). It was found in both natural and disturbed habitats (Fig. 2A).

Mesocriconema solivagum (Andrássy, 1962) Loof & de Grisse, 1989
Syn. *Criconema dubium* apud Popovici, 1987
(Fig. 1C)

Details concerning morphometric data and taxonomic characters are given in [22].

Distribution and habitat. This species was collected at sites no. 1, 4, 64, 78 and 80 (Table 1, Fig. 1C). Two specimens (from sites no. 1 and 4), previously identified as *Criconema dubium* (de Grisse, 1967) by Popovici [17], belong to this species.

Mesocriconema xenoplax (Raski, 1952) Loof, 1989
(Fig. 2B)

Details concerning morphometric data and taxonomic characters are presented in [22].

Distribution and habitat. This species was found in sandy soil only in the Danube Delta (sites no. 101,103-105, 107 and 108) (Table 1, Fig.2B). These habitats experience very dry conditions during the summer and early autumn.

Xenocriconemella macrodora (Taylor, 1936) de Grisse & Loof, 1965
(Fig. 2B)

Morphometric data and taxonomic characters of specimens from Romania [17] fall within the range of the species variability given by de Grisse [7].

Distribution and habitat. This is the most common criconematid species in Romania (Fig. 2B), found in over 50% of the listed sites (no. 1, 5, 8, 10, 18, 19, 22-25, 27, 29-31, 33, 34, 36, 41-44, 46, 49, 50-53, 59, 60, 61, 64, 66, 69, 75-77, 81-

84, 87-95, 97-99) (Table 1). It was also previously reported from Romania [17, 20, 21]. The wide distribution of this species in Romania is in contrast with the statement by Escuer and Bello [10] that it prefers a Mediterranean climate.

Criconema annuliferum (de Man, 1921) Micoletzky, 1925
(Fig. 1A)

Measurements of specimens from Romania were previously reported [17] and fall within the range of variability given by de Grisse [7].

Distribution and habitat. This species is the second most common criconematid species in Romania (identified in 35% of the listed sites). This species shows preferences for natural habitats (forests and grasslands), from sites no. 1, 3, 6, 7, 9, 11, 12, 14, 15, 17, 21, 29, 30, 32-35, 41-43, 44, 48, 52, 53, 55, 61, 64, 69, 72, 76, 77, 80, 83, 84, 97-100) (Table 1, Fig. 1A).

Criconema demani Micoletzky, 1925
(Fig. 1B; Table 3)

The measurements (Table 3) fall within the range of the species variability already published [7].

Distribution and habitat. C. demani was mostly found in soil from forest ecosystems (sites no. 10, 56 and 71) (Table 1, Fig. 1B). Its presence in Romania was formerly reported [17, 20].

Criconema longulum Gunhold, 1953
(Fig. 1D)

Popovici [17, 20] already reported morphometric data and first notes on its distribution in Romania.

Distribution and habitat. C. longulum was identified only from sites no. 56-59 and 71 (Fig. 1D), representing timberline spruce forests and dwarf scrub in the subalpine area of the Carpathian Mountains (Table 1).

Criconema princeps (Andrássy, 1962) Raski & Luc, 1985
(Fig. 1C)

Morphometric data of this species from Romania and data on its relative abundance in different habitats were previously reported [17, 20, 21].

Distribution and habitat. C. princeps was found in small numbers, in 15% of the listed sites (no. 3, 5, 29, 32, 33, 53, 55, 58, 59, 61, 64, 69, 70, 72 and 83) (Table 1, Fig. 1C).

Ogma danubiale Andrássy, 1985
(Fig. 2C)

The characters and morphometrics of the specimens found in Romania correspond well with those of the original description [3]. Details on the specimens from Romania are presented in [22].

Distribution and habitat. *O. danubiale* from Romania was found in sand dunes in the Danube Delta Biosphere Reserve, at sites no. 103 and 106 (Table 1, Fig. 2C).

Ogma menzeli (Stefanski, 1924) Schuurmans-Stekhoven & Teunissen, 1938
(Fig. 2C)

Morphometrics and data on relative abundance of this species in Romania were previously reported [17, 20, 21].

Distribution and habitat. The species was found in 34% of the study sites viz. no. 8, 10, 12, 16, 22, 26-31,33, 35-37, 55,56, 74-77, 80, 82, 83, 86-88, 92, 95-99 (Table 1, Fig. 2C). It was found only in natural ecosystems from the Carpathians.

O. menzeli is widely distributed in Europe [2] and not restricted to the Atlantic area only, as reported by Escuer and Bello [10].

Ogma murrayi Southern, 1914
(Fig. 2C)

Morphometric data, the presence and relative abundance of this species from Romania were reported [17, 20, 21].

Distribution and habitat. This species was infrequently found in natural habitats such as beech, oak, spruce forests and grasslands (sites no. 6, 8, 13, 16, 55, 77 and 105) (Table 1, Fig. 2C).

Ogma zernovi Kirjanova, 1948
(Fig. 2C)

Ogma zernovi was described from Russia [13] and subsequently from Spain [12].

The specimens from Romania differ slightly from those previously reported and details are given in [22].

Distribution and habitat. The species was recorded only from habitats in the subalpine areas of the Retezat Mountains, at sites no. 60, 62, 64 and 65 (Table 1, Fig. 2C).

Table 1

Site description

Site No.	Region, Place	Altitude (m)	Geographical position	Plant association*
1	Bihor Mountains, Padiș	1250	46°34`N-22°43`E	*Festuco rubrae-Agrostetum*
2	Bihor Mountains, Poiana Ponor	1000	46°34`N-22° 43`E	*Lolio-Trifolietum repentis*
3	Bihor Mountains, Poiana Ponor	1050	46°34`N-22°43`E	*Hieracio rotundati-Piceetum*
4	Bihor Mountains, Someșul Cald	1100	46°35`N-22°50`E	*Hieracio rotundati-Piceetum*
5	Bihor Mountains, Padiș	1300	46°40`N - 22° 45`E	*Symphyto cordati-Fagetum*
6	Bihor Mountains, Arieșeni	800	47°28`N-22°43`E	*Festuco rubrae- Agrostetum*
7	Bihor Mountains, Smida	1000	46°39`N-23°01`E	*Violo declinatae-Nardetum*
8	Bihor Mountains, Călineasa	1200	46°30`N-22°55`E	*Hieracio rotundati- Piceetum*
9	Vlădeasa Mountain, Poiana Frânturii	1400	46°46`N-22°48`E	*Scorsonero-Festucetum nigricantis*
10	Vlădeasa Mountains, Stâna de Vale, Poiana Priscop	1400	46°46`N-22°45`E	*Scorsonero-Festucetum nigricantis*
11	Vlădeasa Mountains, Stâna de Vale	1000	46° 46`N-22°45`E	*Symphyto cordati-Fagetum*
12	Vlădeasa Mountains, Dealul cu trei Poieni	1640	46° 46`N-22°47`E	*Scorsonero-Festucetum nigricantis*
13	Vlădeasa Mountains, Zârna Valley	1250	46°45`N - 22°46`E	*Festuco rubrae- Agrostetum*
14	Vlădeasa Mountains, Drăgan Valley	1100	46°47`N- 22°43`E	*Scorsonero-Festucetum nigricantis*
15	Vlădeasa Mountains, Drăgan Valley,	1130	46°55`N-22°53`E	*Violo declinatae-Nardetum*
16	Vlădeasa Mountains, near chalet	1400	46°55`N-22°53`E	*Hieracio rotundati-Piceetum*
17	Vlădeasa Mountains, Preluca Rabului	1250	46°55`N-22°53`E	*Leucanthemo waldsteinii-Fagetum*
18	Trascău Mountains, Tureni Gorges	400	46°30`N-23°41`E	*Melico-Phleetum montani*
19	Trascău Mountains, Vălișoara Gorges	400	46°26`N-23°29`E	*Agrostio-Festucetum sulcatae*
20	Trascău Mountains, Intregalde Gorges	430	46°14`N-23°23`E	*Festuco rubrae - Agrostetum*
21	Trascău Mountains, Poșaga	675	46°26`N-23°27`E	*Asperulo capitatae-Seslerietum rigidae*
22	Trascău Mountains, Râmeț Gorges	440	46°19`N-23 °31`E	*Symphyto cordati-Fagetum*

Table 1 (continued)

Site No.	Region, Place	Altitude (m)	Geographical position	Plant association*
23	Trascău Mountains, Sălciua	1000	46°23′N-23°26′E	*Symphyto cordati-Fagetum*
24	Trascău Mountains, Poșaga, Scărița Belioara	700	46°26′N-23°27′E	*Symphyto cordati-Fagetum*
25	Trascău Mountains, Intregalde Gorges	435	46°14′N-23°23′E	*Carpino-Fagetum*
26	Trascău Mountains, Turzii Gorges	400	46°34′N-23°36′E	*Pinetum sylvestris-Seslerietosum*
27	Trascău Mountains, Turzii Gorges	410	46°34′N-23°36′E	*Carpino-Quercetum petraeae*
28	Metaliferi Mountains, Bucești-Vulcan	500	46° 11′N- 22° 50′E	*Festuco rubrae- Agrostetum*
29	Metaliferi Mountains, Bucești-Vulcan	550	46° 11′N- 22° 50′E	*Carpino-Fagetum*
30	Metaliferi Mountains, Roșioara Valley	800	46° 09′N - 23° 4′E	*Symphyto cordati-Fagetum*
31	Metaliferi Mountains, Dealul Mare	800	46° 16′N - 23° 04′E	*Carpino-Fagetum*
32	Metaliferi Mountains, Ghețar-Scărișoara	1050	46°26′N-23°17′E	*Telekio- Petasitetum*
33	Metaliferi Mountains, Muncel	700	46° 23′N - 23° 17′E	*Symphyto cordati-Fagetum*
34	Metaliferi Mountains, Zlatna	700	46° 07′N - 23° 13′E	*Symphyto cordati-Fagetum*
35	Gilău Mountains, Huza Valley	700	46°30′N-23°14′E	*Symphyto cordati-Fagetum*
36	Gilău Mountains, Iara Valley	800	46°30′N-23°14′E	*Leucanthemo waldsteinii-Fagetum*
37	Gilău Mountains, Muntele Mare, Capu Dealului	1300	46°30′N-23°14′E	*Hieracio rotundati-Piceetum*
38	Turda, Dealul Durgău, Cluj county	350	46°34′N-23°48′E	*Salicornietum europaeae*
39	Cluj, Fânețele Clujului Botanical Reserve	350	46°45′N-23°35′E	*Jurineo transsilvanicae - Stipetum pulcherimae*
40	Suatu Botanical Reserve, Cluj county	370	46°46′N-23°58′E	*Salvio nutantis-Festucetum rupicolae*
41	Baciu, Cluj county	590	46°48′N-23°31′E	*Carpino-Quercetum petraeae*
42	Câțcău, Secătura Valley, Cluj county	450	47°12′N-23°47′E	*Carpino-Fagetum*
43	Câțcău, Secătura Valley, Cluj county	450	47°12′N-23°47′E	*Carpino-Quercetum petraeae*
44	Aluniș, Cluj county	400	47°02′N-23°45′E	*Carpino-Quercetum petraeae*

Table 1 (continued)

Site No.	Region, Place	Altitude (m)	Geographical position	Plant association*
45	Ocna Sibiului, Sibiu county	400	46°52′N-24°03′E	*Artemisio-Festucetum pseudovinae*
46	Noroieni, Satu Mare county	150	47°46′N-22°52′E	*Quercetum robori-cerris*
47	Tg. Mureș, Mureș county	350	46°32′N-24°34′E	Apple orchard
48	Copșa Mică, Sibiu county	350	46°06′N-24°16′E	Arable land
49	Semenic Mountains, Canton Borloveni	1380	45°14′N-22°41′E	*Festuco rubrae- Agrostetum*
50	Mehedinți Mountains, Piatra Cloșani	330	45°07′N-22°43′E	*Festuco rubrae- Agrostetum*
51	Cernei Mountains, confluence of Roșu and Roșeț brooks	240	44°55′N-22°27′E	*Deschampsio flexuosae-Fagetum*
52	Cernei Mountains, Jelerău brook	500	44°55′N-22°27′E	*Phyllitidi-Fagetum*
53	Retezat Mountains, Gura Zlata, Poiana Lăncița	1050	45°33′N-22°52′E	*Festuco rubrae- Agrostetum*
54	Retezat Mountains, Dobrun Valley	1500	45°29′N-22°52′E	*Hieracio rotundati-Piceetum*
55	Retezat Mountains, Fața Retezatului	1830	45°24′N-22°55′E	*Campanulo-Juniperetum bruckenthalietosum*
56	Retezat Mountains, Fața Retezatului	1810	45°28′N-22°55′E	*Bruckenthalio-Piceetum*
57	Retezat Mountains, Fața Retezatului	1950	45°28′N-22°55′E	*Rhododendro myrtifolii-Pinetum mugi*
58	Retezat Mountains, Drăgșan,	1765	45°20′N-22°57′E	*Poetum mediae*
59	Retezat Mountains, Șcaua Scorota	1850	45°19′N-22°57′E	*Potentillo-Festucetum airoidis*
60	Retezat Mountains, Fața Iarului	1700	45°18′N-22°60′E	*Festucetum xanthinae*
61	Retezat Mountains, Scocul Iarului	1200	45°18′N-23°03′E	*Leucanthemo waldsteinii-Fagetum*
62	Retezat Mountains, Piatra Iorgovanului	1750	45°18′N-23°03′E	*Rhododendro myrtifolii-Pinetum mugi*
63	Retezat Mountains, Scorota cu Apă	1350	45°18′N-23°03′E	*Leucanthemo waldsteinii- Fagetum*
64	Retezat Mountains, Piule	1850	45°19′N-22°57′E	*Festucetum xanthinae*
65	Retezat Mountains, Piule	1900	45°18′N-23°03′E	*Rhododendro myrtifolii-Pinetum mugi*

Table 1 (continued)

Site No.	Region, Place	Altitude (m)	Geographical position	Plant association*
66	Retezat Mountains, Câmpușel	1150	45°18′N-22°60′E	*Phyllitidi-Fagetum*
67	Retezat Mountains, Câmpușel	1100	45°18′N-22°60′E	*Festuco rubrae-Agrostetum*
68	Retezat Mountains, Șeaua Scorota	1850	45°18′N-23°03′E	*Rhododendro myrtifolii-Pinetum mugi*
69	Parâng Mountains, Păpușa	2050	45°28′N-23°29′E	*Violo declinatae-Nardetum*
70	Parâng Mountains, near Câlcescu Lake	1850	45°21′N-23°33′E	*Violo declinatae-Nardetum*
71	Parâng Mountains, Coasta lui Rus	1800	45°25′N-23°22′E	*Hieracio rotundati-Piceetum*
72	Ciucaș Mountains, Roșu Mountain	1500	45°26′N-25°52′E	*Violo declinatae-Nardetum*
73	Harghita-Mădăraș Mountains	1750	46°35′N-24°23′E	*Campanulo- Juniperetum*
74	Harghita-Mădăraș Mountains	1650	46°35′N-24°23′E	*Hieracio rotundati-Piceetum*
75	Harghita-Mădăraș Mountains	1500	46°35′N-24°23′E	*Hieracio rotundati-Piceetum*
76	Harghita-Mădăraș Mountains, Filiou Valley	800	46°35′N-24°23′E	*Symphyto cordati-Fagetum*
77	Gurghiu Mountains, Gurghiu Valley	830	46°45′N-25°01′E	*Symphyto cordati-Fagetum*
78	Gurghiu Mountains, Sălard Valley	870	46°57′N-25°06′E	*Hieracio rotundati-Piceetum*
79	Căliman Mountains, Negoiul Românesc	1780	47°14′N-25°20′E	*Rhododendro myrtifolii-Pinetum mugi*
80	Căliman Mountains, Ilișoara Valley	1150	46°59′N-25°02′E	*Leucanthemo waldsteinii-Fagetum*
81	Bârgău Mountains, Măgura	950	47°22′N-24°54′E	*Festuco rubrae-Agrostetum*
82	Rodna Mountains, Pietrosul Rodnei	1560	47°25′N-24°54′E	*Hieracio rotundati-Piceetum*
83	Năsăud Plateau, Largă Valley	550	47°17′N-24°27′E	*Symphyto cordati-Fagetum*
84	Năsăud Plateau, Izvorul Negru Valley	1200	47°17′N-24°27′E	*Leucanthemo waldsteinii-Fagetum*
85	Rodna Veche, Bistrița- Năsăud county	700	47°25′N-24°46′E	Mine spoil dumps
86	Maramureș Mountains, Bârjaba Valley	1300	47°43′N-24°26′E	*Hieracio rotundati-Piceetum*
87	Igniș Mountain, Ferneziu, Firiza Valley	490	47°41′N-23°36′E	*Symphyto cordati-Fagetum*
88	Gutâi Mountain, Bolduț Valley	850	47°42′N-23°54′E	*Symphyto cordati-Fagetum*

Table 1 (continued)

Site No.	Region, Place	Altitude (m)	Geographical position	Plant association*
89	Gutâi Mountain, Roșia Valley	550	47°39′N-23°34′E	*Castaneo-Quercetum*
90	Lăpușel, Maramureș county	205	47°37′N-23°29′E	*Carpino-Quercetum petraeae*
91	Țibleș Mountains, Bradu Valley	680	47°29′N-24°04′E	*Symphyto cordati-Fagetum*
92	Țibleș Mountains, Suciu Valley	850	47°29′N-24°04′E	*Pulmonario rubrae-Fagetum*
93	Lăpuș Mountains, Leorda	800	47°30′N-24°01′E	*Symphyto cordati-Fagetum*
94	Lăpuș Mountains, Strâmbu-Băiuț	850	47°40′N-23°52′E	*Hieracio rotundati-Abietetum*
95	Oaș Mountains, Călinești-Oaș	350	47°54′N-23°18′E	*Carpino-Quercetum petraeae*
96	Giumalău Mountains, Putna Valley	940	47°29′N-25°24′E	*Hieracio rotundati-Piceetum*
97	Rarău Mountains, Codrul Secular Slătioara	1060	47°27′N-25°34′E	*Hieracio rotundati-Piceetum*
98	Rarău Mountains, Toanca Valley	1350	47°27′N-25°34′E	*Hieracio rotundati-Piceetum*
99	Rarău Mountains, Colbu Valley	1280	47°27′N-25°34′E	*Festuco rubrae-Agrostetum*
100	Ceahlău Mountains, Dochia Valley	850	46°47′N-25°41′E	*Symphyto cordati-Fagetum*
101	Danube Delta, Maliuc	50	45°13′N-29°04′E	Poplar plantation
102	Danube Delta, Caraorman	80	45°04′N-29°22′E	*Elymetum gigantei*
103	Danube Delta, Caraorman	80	45°04′N-29°22′E	*Quercetum pedunculiflorae*
104	Danube Delta, Enisala	250	42°52′N-28°50′E	*Quercetum pedunculiflorae*
105	Danube Delta, Letea	80	45°19′N-29°33′E	*Quercetum pedunculiflorae*
106	Danube Delta, Letea	80	45°19′N-29°33′E	*Elymetum gigantei*
107	Danube Delta, Sf. Gheorghe	35	44°52′N-29°36′E	*Elymetum gigantei*
108	Danube Delta, Gura Portiței	10	44°46′N-28°52′E	*Elymetum gigantei*

*C o l d e a [5].

Table 2

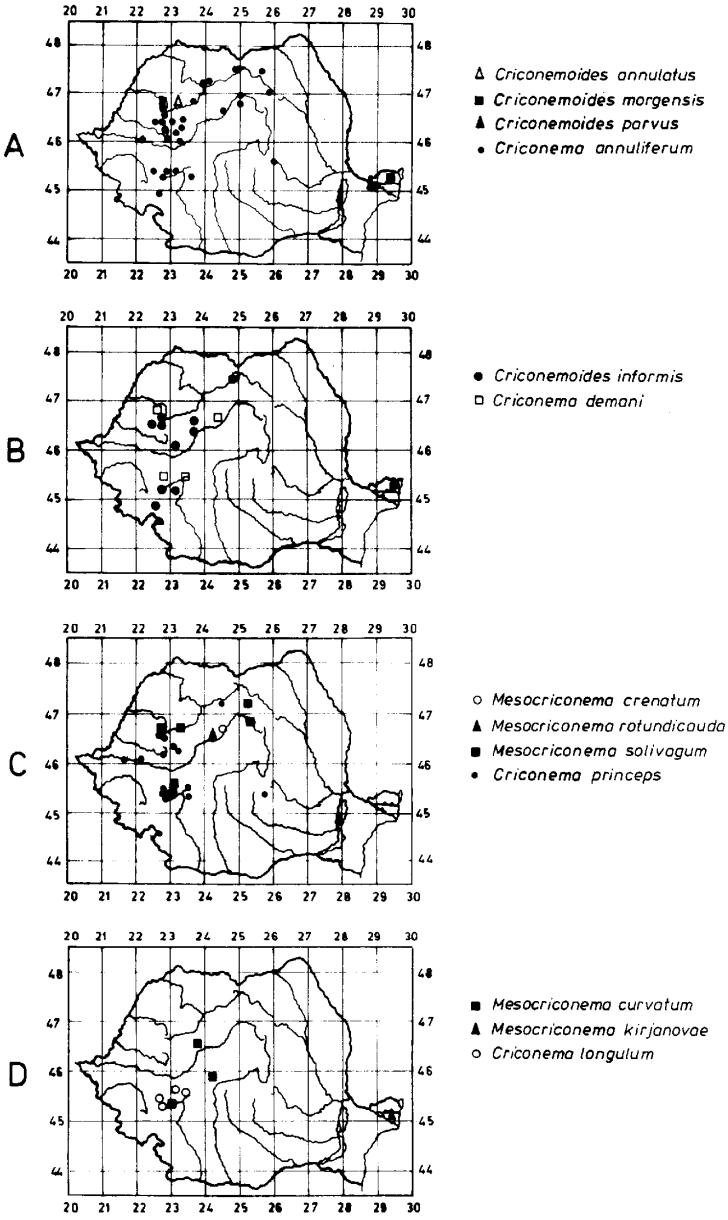
Measurements of Criconemoides annulatus, C. informis, C. morgensis and C. parvus

Characters	*C. annulatus*	*C. informis*			*C. morgensis*	*C. parvus*
	Bihor Mountains	Bihor Mountains	Metaliferi Mountains	Rodna	Danube Delta	Danube Delta
N	9 ♀♀	3 ♀♀	12 ♀♀	8 ♀♀	2 ♀♀	1 ♀
L	737 (683-902)	411 (356-482.5)	456 (401-492)	430 (372-530)	468-608	299
A	12.6 (11-15)	8.3 (7.7-8.9)	9.3 (8.2-10.6)	9.4 (7.4-12.6)	12.6-13.2	12.4
B	4.4 (3.5-5.2)	3.6 (3.3-3.8)	3.8 (3.3-4.2)	3.7 (3.2-4.5)	4.6-5.1	3.9
C	35.9 (26-45)	17.9 (17.5-18.3)	20.4 (13-29.5)	16.9 (10-20.2)	16.7-20.9	33.2
V	95 (94.3-95.8)	89 (88.6-90)	91(89.4-92.5)	90 (88.4-92.1)	92.9-93.2	95
St	107 (100-112)	67.5 (60-75)	70 (64-74)	67.5 (67-71)	54-66	35
R	162 (158-172)	57 (55-59)	60 (55-64)	59 (54-72)	122-124	173
RSt	29 (24-36)	10 (9-12)	11(10-12)	11 (9-12)	17-22	23
ROes	45 (39-55)	16 (14-17)	17 (15-19)	17 (15-19)	30-33	49
Rex	45 (44-48)	18 (17-19)	19 (17-20)	19 (17-20)	33-36	50
RV	9 (8-10)	7 (7-8)	7 (6-8)	8 (7-9)	10-11	12
Ran	6 (4-7)	5 (4-5)	4 (4-6)	5 (4-6)	8-9	9
Rvan	1 (0-3)	2 (1-2)	1 (0-2)	1 (1-2)	1	2
VL/VB	0.9 (0.8-1.1)	1.2 (1.1-1.2)	1.1 (0.9-1.2)	1.1 (0.8-1.4)	1.1-1.2	0.7
VL/St	0.3 (0.2-0.4)	0.6	0.6 (0.5-0.7)	0.6 (0.4-0.7)	0.6	0.3
St%L	15 (12.5-18)	16 (15.5-17)	15.3 (13.4-17.2)	16 (13-18)	10.8-11.5	12
St%Oes	62 (56-69)	59.5 (56-63)	58 (54-65)	59 (48-79)	50-57.5	46
CP%St	77 (72-82)	76 (75-78)	77 (75-81)	79 (74-81)	68.5-71	80

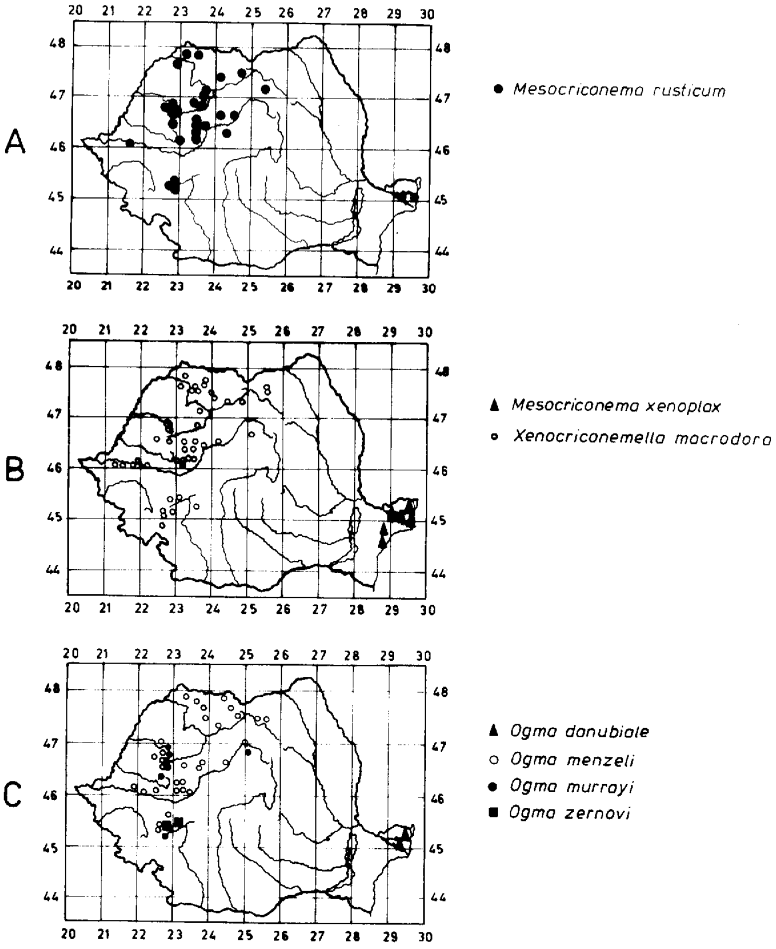
Table 3

Measurements of Mesocriconema kirjanovae, M. rusticum and Criconema demani

Characters	*M. kirjanovae*	*M. rusticum*				*C. demani*	
	Danube Delta	Rodna Veche	Tureni Gorges	Fânețele Clujului	Danube Delta	Harghita Mountains	Parâng Mountains
n	4 ♀♀	2 ♀♀	5 ♀♀	2 ♀♀	11 ♀♀	1 ♀	7 ♀♀
L	392 (372-420)	442-400	417 (371-490)	436-536	401 (350-440)	394	401 (377-428)
a	9.5 (7.8-10.8)	9.8-8	10.7 (9.3-11.6)	10.9-12.1	9.2 (7.4-11.9)	10.8	9.8 (9.1-10.4)
b	4 (3.6-4.4)	4	4.2 (3.8-4.9)	3.6-4.1	3.9 (3.8-4.3)	4	3.9 (3.5-4.4)
c	14.5(12.9-16.8)	25.2-26.9	20 (15.3-22.8)	29.7-33.5	26.4 (20.1-33.6)	10	12.4 (10.1-15)
V	90 (88.6-90.4)	93.2-94.4	93 (92-93.4)	93.8	93 (92-94)	86	87 (85-90)
St	57 (53-61)	55-62.5	53 (50-55)	63-84	55 (50-59.5)	63	61(58-63)
R	83 (81-84)	96-104	101 (94-109)	101-82	91(87-97)	75	74 (69-76)
RSt	15 (12-17)	14	17 (15-18)	15	15 (13-16)	14	12 (11-14)
ROes	24 (23-25)	26-27	26 (25-29)	22-28	26 (23-28)	19	19 (18-20)
Rex	25	30-31	29 (27-31)	23-29	26 (26-27)	20	15 (13-19)
RV	11 (10-11)	7-8	8 (8-9)	7	7 (6-9)	14	14 (11-15)
Ran	8 (7-9)	5-6	7 (6-7)	4	5 (3-6)	10	10 (9-11)
RVan	2 (1-2)	1	1 (0-1)	2	2 (1-3)	3	4 (3-5)
VL/VB	1.3 (1.2-1.4)	1-1.1	0.9 (0.8-1)	1.1	1.1- (1.0-1.2)	1.9	0.6 (0.5-0.7)
VL/St	0.6 (0.6-0.7)	0.5	0.5 (0.5-0.6)	0.4	0.6 (0.5-0.7)	0.9	0.4 (0.4-0.5)
St%L	14 (13-16)	12.4	13 (11-14)	14-16	14 (13-15)	16	15 (14-17)
St%Oes	57 (50-68)	50	53 (51-55)	52-66	55(51-58)	64	61 (57-64)
CP%St	76 (77-79)	75-77	76 (74-78)	74-75	77 (75-79)	84	83 (81-85)



F i g. 1 A - D. *Distribution maps of* Criconemoides, Criconema and Mesocriconema *species in Romania (geographical degrees).*



F i g. 2 A - C. *Distribution maps of* Mesoriconema, Xenocriconemella *and* Ogma *species in Romania (geographical degrees).*

General remarks on criconematid distribution in Romania. The *Criconematidae* species from Romania were the most prevalent in grasslands (32 % of sites), followed by deciduous forests (28 %) and coniferous forests (17.5 %).

The most frequently found species were *X. macrodora* (51.5 %), *C. annuliferum* (35 %), *O. menzeli* (34 %) and *M. rusticum* (31 %) (see also Figs.1 and 2) and C. *informis* and *X. macrodora* were the most widely distributed species in Romania.

Mesocriconema crenatum, M. rotundicauda and *Ogma zernovi* were found only in the subalpine areas of the Carpathians, while *M. kirjanovae, M. xenoplax, C. morgensis, C. parvus* and *O. danubiale* were found only in the sandy soils from the Danube Delta Biosphere Reserve.

Conclusions. 1. Twenty criconematid species from several habitats in Romania are identified in 108 study sites.

2. Details on the main taxonomic characters for seven species (*Criconemoides annulatus, C. informis, C. morgensis, C. parvus, Mesocriconema kirjanovae, M. rusticum* and *Criconema demani*) are discussed and their morphometric data are given in tables. The ranges of their variability are important for taxonomic studies of the *Criconematidae* family.

3. Distribution maps for all recorded species in Romania are provided. Their prevalence in grasslands and deciduous forests is noted. The most frequently found species are *Xenocriconemella macrodora*, *Criconema annuliferum*, *Ogma menzeli* and *Mesocriconema rusticum*. *Criconemoides informis* and *X. macrodora* are the most widely distributed species in Romania.

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ECOLOGY OF TERRESTRIAL ISOPODS IN THE NATURE RESERVE SCĂRIŢA-BELIOARA, ROMANIA

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SUMMARY. - In the Scărița-Belioara Nature Reserve, located in the Gilău – Muntele Mare Massif, we have collected terrestrial isopods using pitfall traps, in four ecosystems: meadow, rocky area (open ecosystems), spruce and beech forests. Six species of terrestrial isopods live in these ecosystems, of which four species are sylvan (*Protracheoniscus politus*, *Trachelipus wächtleri*, *T. arcuatus* and *Armadillidium carniolense*) and two are praticolous species (*T. nodulosus* and *A. versicolor quinqueseriatum*).

In the open ecosystems of the Nature Reserve, the prohibition of grazing has favoured the expansion of the herbaceous layer, especially in the meadow, which caused, at the soil surface, the formation of a microclimate resembling that in the litter layer of the forests. Under these conditions, the species of sylvan isopods have extended their spread outside the forests, presenting large populations in the meadow and the rocky areas where they are the dominant species. In the meadow there live three sylvan species (*P. politus*, *T. wächtleri* and *A. carniolense*) and a praticolous one (*T. nodulosus*). In the rocky area there live four sylvan species (*P. politus*, *T. wächtleri*, *T. arcuatus* and *A. carniolense*) and two praticolous ones (*T. nodulosus* and *A. versicolor quinqueseriatum*). In the meadow the dominant species is *T. wächtleri* (84.3%), and in the rocky area *A. carniolense* (73.9%). In the spruce and the beech forests there live only sylvan species (*P. politus*, *T. wächtleri* and *A. carniolense*). The presence of sylvan species in open mountain ecosystems where grazing is prohibited shows that these species are not strictly sylvan. Their distribution in hilly and plane zones only in forests is determined by the microclimate that is formed under the litter layer, a microclimate that presents small variations of humidity and temperature.

The sex ratio in the isopod populations of the Nature Reserve varies between 20-80% and 40-60% males: females. The value of the specific density and equitability is low in the meadow, rocky area and the spruce forest, due to the great differences that exist among the sizes of the populations of isopod communities from the three ecosystems.

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The research on the ecology of terrestrial isopods was focussed mainly on the populations of terrestrial isopods that live in grassland, meadow ecosystems and in the forests from hilly and plane zones [1-8,10-12, 14, 15]. We have studied the isopod communities and the distribution of species in relation to the ecological factors that exist in these types of ecosystems. The research concerning the terrestrial isopod fauna from mountain ecosystems is less extended. In Romania there are mountains on a large surface of the country, thus this type of research will contribute to a better analysis of the ecology and biology of isopod species.

The research has been carried out in 1996 in the Scărița-Belioara Nature Reserve. The Reserve is located in the Gilău-Muntele Mare Massif, in the Apuseni Mountains at an altitude over 1200 m. The annual average temperature is 4.2-5^{o}C, and the rainfall varies between 850-900 mm. The geological substratum is made of limestone covered by brown and rendzinous soil.

The terrestrial isopod samples have been collected in four types of ecosystems: meadow, rocky area, spruce and beech forests.

The **meadow** is located on a plateau with an inclination of 5 degrees. The herbaceous plants are tall (40-60 cm) and cover 90-100% of the soil surface, grazing being prohibited in the Nature Reserve. A relatively thick layer of detritus, composed of decaying herbaceous plants, covers the surface of the soil. The detritus layer maintains the water in the soil and prevents excessive warming of the soil surface during summer. A microclimate is formed at the surface of the soil, resembling that located under the litter layer of the deciduous forests from hilly and plane areas.

The **rocky area** is located on slopes with an inclination of 35-40 degrees. The soil layer is thinner and discontinuous, being interrupted by outcrops of rock. The herbaceous vegetation has a lower density compared to the plateau meadow. In this area there also are isolated trees. There exists a greater ecological diversity, with many microhabitats, due to the diversity of the microrelief, the thickness and the discontinuity of the soil, the inclination and the aspect of the slopes, the presence of isolated trees etc.

Spruce forest mixed with juniper shrubs. In the forest there are many clearings with abundant herbaceous vegetation. The reduced density of the trees has favoured the formation of a herbaceous layer also in the forest. The litter layer is composed of fallen spruce leaves and decaying herbaceous plants.

The **beech forest** is made up of trees that are 70-80 years old. The density of the trees is high, and the herbaceous layer is very scarce. A thick and uniform layer of litter, made almost exclusively of fallen beech leaves covers the surface of the soil.

Materials and methods. The isopods were collected using pitfall traps. In each ecosystems we placed 7 pitfalls. The pitfalls have been placed at the end of May, and emptied three times, once every month. The captured animals were conserved in 70% alcohol and studied in the laboratory. We have captured a total number of 1785 individuals. We have studied the distribution of species in the Nature Reserve, the numerical abundance and relative abundance, sex ratio, diversity and equitability.

The ecological indices of quantity have been statistically calculated.

The relative abundance has been calculated using the formula $A_r=n_i/N\cdot100$, where n_i = number of individuals of species i, N = total number of individuals.

To calculate the diversity we have used the Shannon-Weaver index: $H'= -\Sigma pi\cdot\log pi$, where pi = the proportion of the individuals of species i, and log is the decimal logarithm. The equitability has been calculated using the following formula: $e=H'/H_{max}$; $H_{max}=\log S$, where S represents the total number of species in a biocoenosis.

Results and discussion. The isopods that we have collected belong to six species (Table 1), of which four species are sylvan (*Protracheoniscus politus*, *Trachelipus wächtleri*, *T. arcuatus* and *Armadillidium carniolense*) and two species are praticolous (*T. nodulosus* and *A. versicolor quinqueseriatum*).

The distribution of species in the four ecosystems is varied. The size of the populations in a biocoenosis also varies greatly (Table 1).

In the meadow, which is an open ecosystem (without trees), three sylvan species (*P. politus*, *T. wächtleri* and *A. carniolense*) and only one praticolous species (*T. nodulosus*) represent the isopod fauna. From the numerical point of view the dominant species is *T. wächtleri* (a sylvan species), the population of wich represents 86.4% of the isopod fauna of the studied zone. On the contrary, the other two sylvan species (*P. politus* and *A. carniolense*) have a very low numerical representation, which suggests the fact that the values of the ecological optimum of these species are different from the ecological optimum of *T. wächtleri*, although in many coniferous and deciduous forests the three species coexist and are represented by large populations [9, 13].

In the rocky area all the six species are present, but in different proportions. The ecological diversity found here is also reflected in the structure of the isopod communities. The sylvan species *T. wächtleri* and *A. carniolense* are again numerically dominant, in spite of the fact that this also is an open ecosystem. The two praticolous species are also present here in a small number, proving that there are few microhabitats that provide them with optimum life conditions.

Table 1

Distribution, numerical abundance, relative abundance and sex ratio for the species of terrestrial isopods in the studied Nature Reserve

Station	Parameters*	Species						Total amount of izopods/station		X / trap / station
		P.politus	*T.nodulosus*	*T. wächtleri*	*T. arcuatus*	*A. carniolense*	*A. versicolor quinqueseriatum*	Number of individuals	%	
Meadow	n	7	98	605	0	9	0	719	40.3	102.7
	a	1.0	14.0	86.4	0	1.28	0			
	b	0.9	13.6	84.3	0	1.2	0			
Rocky area	n	49	4	88	4	451	14	610	34.2	87.2
	a	7.0	0.57	12.57	0.57	64.42	2.0			
	b	8.1	0.65	14.4	0.65	73.9	2.3			
Spruce forest	n	40	0	361	0	33	0	434	24.3	62
	a	5.7	0	51.57	0	4.71	0			
	b	9.22	0	83.18	0	7.6	0			
Beech forest	n	5	0	10	0	7	0	22	1.2	3.2
	a	0.71	0	1.4	0	1.0	0			
	b	22.72	0	45.47	0	31.81	0			
Total number of individuals		101	102	1064	4	500	14	1785		
% / species		5.6	5.6	59.8	0.2	28.0	0.8			
Sex ratio M/F (%)		20/80	40/60	40/60		40/60				

* n - Number of individuals/species;
a - Average (X) of individuals/ trap;
b - Relative abundance/species/ station.

In the two types of forests we found the same communities of sylvan species (*P. politus, T. wächtleri* and *A. carniolense*), but with much more numerous populations in the spruce forest, due to the more abundant herbaceous layer and a great number of glades. Analysing the distribution of the six species, we observe that the sylvan species *P. politus, T. wächtleri* and *A. carniolense* are present in all four ecosystems, which suggests that the boundaries of their ecological valences are broader compared to those of the other species. *T. nodulosus* is present in both open ecosystems (meadow and the rocky area), and *T. arcuatus* and *A. versicolor quinqueseriatum* live only in the rocky area.

The presence of sylvan species in the open ecosystems at the Scărița-Belioara Nature Reserve, which is located in a mountainous area, suggests that these species are not strictly sylvan. Their distribution, limited to forest ecosystems from hilly and plane zones, is determined mainly by the constant and moderate temperature values and the lack of excessive decrease of humidity during summer, conditions which exist only under the litter layer of the forests, and not in the open ecosystems encountered here.

The values of the numerical abundance (Table 1) are related to the sizes of the populations existent in an ecosystem. In the Scărița-Belioara Nature Reserve, *P. politus* and *A. carniolense* have more numerous populations in the rocky area and the spruce forest, *T. wächtleri* in the meadow and the spruce forest, *T. nodulosus* in the meadow. *T. arcuatus* and *A. versicolor* have a low numerical abundance, which suggests that in the examined area the values of the environmental factors are at the boundary of the ecological optimum of the two species.

The relative abundance, calculated for the communities of isopod species from each ecosystem, indicates the numerical dominance of *T. wächtleri* in the meadow and the spruce forest (84.3 and 83.18%, respectively), and of *A. carniolense* in the rocky areas (73.9%). We consider that for the beech forest the values of the relative abundance are not relevant, due to the small number of isopods collected here (22 individuals). From the total number of individuals which we have captured, 59.8% belong to *T. wächtleri* (1064 individuals), 28.0% - *A. carniolense* (500 individuals), 5.6% - *P. politus* (101 individuals), 5.6% - *T. nodulosus* (102 individuals), 0.2% belong to *T. arcuatus* (4 individuals) and 0.8% to *A. versicolor quinqueseriatum* (14 individuals). For the species with populations which are numerically reduced, the general ecological factors in the studied ecosystems are less favourable, and their presence in small populations is probably due to the small number of microhabitats in which the ecological factors are maintained within the optimum range.

In order to evaluate the abundance of the terrestrial isopod fauna in the four ecosystems, we have also calculated the average number of isopods captured with each trap, taking into consideration the total number of isopods

sampled in each ecosystem. The most numerous isopod populations were found in the meadow (X=102.7), then in the rocky areas (X=87.2) and the spruce forest (X=62.2). We are able to conclude that in the open mountain ecosystems, where grazing is prohibited, there are optimum ecological conditions for the isopod populations, including the sylvan species, and these populations are much more numerous compared to the isopod populations that live in forests located in mountainous areas.

The sex ratio has been calculated for four species, from which we have collected over 100 individuals (Table 1). For all the populations of the species that we have studied, we found that the females were predominant: 60-80%. The great percentage of females in the populations of the ecosystems from the Nature Reserve constitutes an advantage for the species by increasing their reproductive potential. In the mountainous areas, the low temperatures and the late spring frost increase the mortality rate of the isopods at young ages.

For the isopod communities that live in the meadow and the rocky area the values of the diversity and equitability index are lower (Table 2). Although in these ecosystems there live more species compared to the spruce or beech forest, with a number of three species of terrestrial isopods living in each ones (Table 1). These low values indicate the numerical dominance of only one species the population of which has a major contribution to the biological activities that take place in the biocoenosis.

Table 2

Values of the Shannon-Weaver diversity index (H') and equitability index (e) in the different types of ecosystems

Index	Type of ecosystem			
	Meadow	Rocky area	Spruce forest	Beech forest
H'	0.22292	0.37172	0.24374	0.46005
e	0.37026	0.47767	0.51090	0.96422

Conclusions. 1. In the Scărița-Belioara Nature Reserve, located in the Gilău-Muntele Mare Massif, at an altitude over 1200 m, there live six species of terrestrial isopods: four sylvan species (*P. politus, T. wächtleri, T. arcuatus* and *A. carniolense*) and two praticolous species (*T. nodulosus* and *A. versicolor quinqueseriatum*).

2. In open ecosystems (meadow and rocky areas) the predominant species are the sylvan ones. Their presence here suggests that in mountainous open ecosystems the climate conditions are similar to those existent in the litter layer of the forests from hilly and plane zones, where these species are frequent.

3. The presence in mountainous open ecosystems of species that are generally considered sylvan indicates the fact that these species are not strictly sylvan, and their distribution only in the forests of hilly and plane zones is determined by the temperature and humidity that here are within the optimal range.

4. The highest value of the numerical and relative abundance has been recorded for *T. wächtleri* in the meadow and the spruce forest, and for *A. carniolense* in the rocky areas. From the total number of captured individuals (1785), 59.8% belong to *T. wächtleri*, 28.0% - *A. carniolense*, 5.6% - *P. politus*, 5.6% - *T. nodulosus*, 0.8% - *A. versicolor quinqueseriatum*, 0.2% - *T. arcuatus*.

5. The sylvan species are numerically predominant in mountainous open ecosystems where grazing is prohibited. It is necessary to extend the prohibition of grazing in mountain areas, in order to protect the epigeous fauna.

6. Females (60-80%) mainly represent the populations of terrestrial isopods from the Scărița-Belioara Nature Reserve.

7. The values of the diversity and equitability express the existent differences regarding the size of the populations of the terrestrial isopod communities, differences which are emphasized in the meadow, rocky areas and in the spruce forest.

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INFLUENȚA VÂRSTEI ASUPRA COMPORTAMENTULUI ŞI CAPACITĂȚII DE REPRODUCERE LA *MAMESTRA BRASSICAE* L. (*LEPIDOPTERA: NOCTUIDAE*) ÎN CONDIȚII DE LABORATOR

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SUMMARY. - Influence of Age on Mating Behaviour and Reproductive Capacity of Mamestra brassicae L. (Lepidoptera: Noctuidae) under Laboratory Conditions. Under laboratory conditions, at 22°C, an optimum calling behaviour of *Mamestra brassicae* L. was recorded in the females of 2-5 days and the percentage of female calling gradually decreased with age increase. On the other hand, an age increase ascertained an increase of the calling duration, the number of calling bouts and an early initiation of calling. At 24°C, in the females of one day, 53.6% of them showed a clear calling behaviour. An optimum of successful matings was obtained for adult moths of 2-5 days and the number significantly decreased at the age greater than 10 days. In the mating tests with adult moths of the same age or of different age, the data obtained showed that the presence of young sexually mature males was necessary for achievement of successful matings and great fecundity. The oviposition pattern dependend on female reproductive status. In mated females the length of oviposition period gradually decreased with age increase; the percentage of eggs laid in the first day after copulation increased, too. In the virgin females a typical egg retention behaviour with adaptative value was recorded. Fecundity, as total number of eggs laid and eggs from ovaries, was influenced by adult age. The optimum fecundity was recorded in females of 3-6 days and the presence of young males was necessary. The fertility of eggs was greatest in the females of 2-5 days and decreased with age increase.

Vârsta este unul din factorii interni (fiziologici) cu semnificație deosebită în modelarea comportamentului de reproducere la insecte, dar alături de ea şi alți factori din această categorie (ritmul circadian, hrana, numărul de împerecheri, expunerea la mediatori chimici, densitatea populației, hormonii) influențează

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comportamentul general. Fiecare secvență în parte este controlată neuro-endocrin, dar o serie de factori ecologici modifică modelul specific asigurând valoare adaptativă pentru specie. La nivelul reproducerii, comportamentul de chemare (eliberarea feromonului sexual) este o secvență declanşatoare, dar între ea şi cele care se succed (curtarea, acuplarea, ovipozitarea) există o strânsă dependență.

Cercetările referitoare la influența vârstei adulților asupra reproducerii prezintă atât o importanță teoretică cât mai ales una practică, în relație cu introducerea şi apoi extinderea metodelor şi procedeelor biotehnice şi biologice în monitoring, management sau combatere la specii de insecte cu importanță economică sau științifică. Studii aprofundate au fost făcute în special la lepidoptere [1, 6-9, 11, 12, 21].

Mamestra brassicae L. este o specie importantă în agricultură, dar în ceea ce priveşte influența factorilor ecologici asupra comportamentului s-au studiat în special factorii externi. Influența vârstei a fost analizată doar la nivelul comportamentului de chemare [20] şi asupra împerecherii în relație cu suşa crescută în laborator şi generația din care a provenit materialul biologic [18].

Lucrarea prezintă rezultatele obținute în perioada 1980-1994 cu privire la influența vârstei adulților de *M. brassicae* asupra chemării, împerecherii, ovipozitării, fecundității şi fertilității. Studiile au fost făcute la diferite linii de creştere în laborator şi număr mare de generații.

Material şi metode. *Creşterea speciei în laborator*. Adulții folosiți în experimentări au provenit din populații de larve crescute în laborator (23 ± 1^{o}C; 16:8 ore regim fotoperiodic; UR > 70%) pe diete artificiale [16, 19], în condiții specifice şi după "metoda cutiilor Petri şi a sistemului celular" [17]. Menținerea heterogenității la suşele din laborator a fost asigurată prin control dirijat al creşterii larvelor şi al selecției adulților pentru reproducere, cu material biologic provenit din 5 linii de creştere (LF, LG, C, LV, V) [17]. In fiecare an au fost aclimatizate şi introduse în laborator alte linii noi. Pupele, separate pe sexe, s-au menținut la 22^{o}C şi întuneric continuu, emergența adulților s-a înregistrat zilnic (în unele situații de două ori/zi), iar adulții au fost puşi în vase de sticlă sau cuşti speciale, în funcție de varianta experimentală. In interior s-a introdus hârtie de filtru ca suport şi soluție de glucoză 20%, ca sursă de hrană.

Condițiile de experimentare. Observațiile directe asupra secvențelor comportamentale sau vasele de împerechere s-au făcut în scotofază, în intervalul care coincide cu perioada circadiană a activității de reproducere la această specie [14, 15], valorile unor factori climatici esențiali fiind: temperatură constantă - 22 ± 1^{o}C; fotoperioada de 16:8 ore lumină:întuneric; umiditatea relativă >70%. Pentru femelele de 0-2 zile, comportamentul de chemare s-a studiat şi la 24^{o}C. Montarea experiențelor a fost făcută în fotofază.

Influența vârstei asupra comportamentului de reproducere. Comportamentul de chemare a fost studiat prin metoda observării directe cu stenografierea elementelor comportamentale şi înregistrare pe casetofon [14, 15]. Studiul s-a făcut pe femele de 0, 1, 2....15 zile, observate individual sau în grup. Chemarea în relație cu vârsta femelelor a fost caracterizată prin periodicitate, % chemare, durata chemării, ora medie a perioadei de chemare, ora medie a perioadei de inițiere a chemării, modelul de chemare şi numărul de reprize de chemare/femelă. Datele finale reprezintă valori medii pentru un număr mare de femele (Fig. 1). Acest număr a depăşit frecvent valoarea optimului de informație necesar în prelucrarea matematică a datelor, iar femelele au provenit din diferite suşe crescute în condiții similare. In aceleaşi condiții s-a studiat şi comportamentul de curtare. S-a înregistrat periodicitatea, durata, ora medie a perioadei de activitate. S-au studiat împerecherile multiple dependent de vârsta adulților şi capacitatea de supraviețuire. In acest caz s-au folosit perechi de adulți, iar numărul mediu cumulat de spermatofori a fost evaluat după 1, 2, 3....15 zile din momentul în care s-au format perechile cu adulți de 0 zile. Evaluarea numărului de spermatofori s-a făcut după metoda descrisă pentru acest tip de cercetări [17]. In studiul împerecherii, inițial s-a folosit câte o singură pereche de adulți/vas, dar ulterior s-au folosit două perechi, constatând că procentajul de acuplări reuşite şi fecunditatea au fost mai mari (chiar semnificativ pentru unele linii de creştere în laborator). In acest studiu datele reprezintă valori medii ale testelor de acuplare, diferențele fiind analizate într-un alt studiu. In cadrul comportamentului de ovipozitare în relație cu vârsta adulților s-a analizat modelul de depunere al ouălor, perioada de ovipozitare, durata şi eclozarea.

Pentru primele două secvențe, chemare şi împerechere, s-a înregistrat ora medie a perioadei de inițiere a chemării (respectiv împerecherii), reprezentând ora medie (în exprimare zecimală) a intervalului de timp dintre prima şi ultima femelă care au inițiat chemarea la nivelul unei populații observate în aceleaşi condiții (respectiv, intervalul dintre prima şi ultima acuplare). Ora medie a perioadei de chemare (respectiv de împerechere), în exprimare zecimală [22], s-a estimat după relația:

$$x_H = \frac{[\Sigma(t_i \cdot \frac{n}{t_i})]}{N}$$

(t_i = ora observării, la nivelul unei scotofaze de 8 ore, notată de la 0 la 8; n/t_i = numărul de femele în chemare, respectiv acuplate, în momentul observării; N = numărul total de femele în chemare, respectiv de perechi, la nivelul variantei experimentale).

Durata medie a chemării, fără a lua în considerare reprizele de chemare (intervalul de timp în care a existat o postură tipică de chemare, separată de o altă postură printr-o activitate locomotoare în care chemarea nu a fost evidentă), s-a estimat după relația:

$$D_C = \frac{[\sum(D_i \pm 15)]}{N}$$

(D_i = durata individuală de chemare (min.); ± 15 = se adaugă sau se scad 15 minute în funcție de postura femelei respective în momentul observării la intervale de 15 minute; N = numărul total de femele în chemare). Similar s-a evaluat şi durata împerecherii.

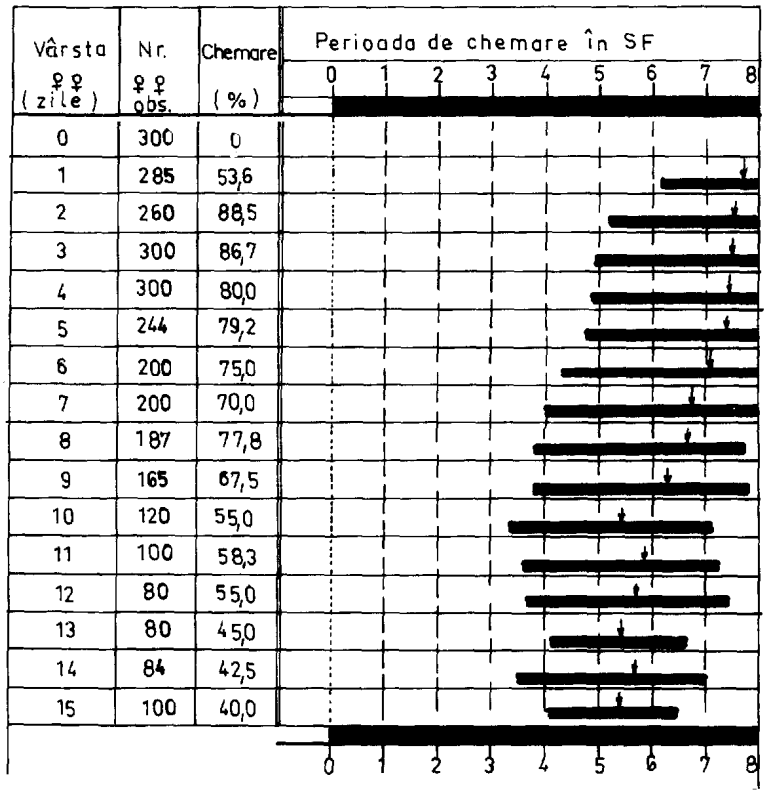
Influența vârstei asupra fecundității şi fertilității. Fecunditatea a fost acceptată ca număr total de ouă/femelă (ouă depuse + ouă existente în ovare la moartea femelei), iar fertilitatea se referă la ouăle viabile din care au eclozat larvele. De asemenea, s-a înregistrat şi % de pontă sterilă. Numărul de ouă din ovare a fost înregistrat la moartea femelelor prin disecții, iar clasificarea s-a făcut după metoda descrisă [17].

Prelucrarea datelor. Datele obținute au fost prelucrate statistic. Spre deosebire de teste experimentale similare unde informația este furnizată de număr relativ mic de variante sau repetiții, datele din acest studiu reprezintă valori medii pentru un număr foarte mare de adulți pentru a atenua variația individuală a răspunsului şi a modificării comportamentului în condițiile creşterii insectelor în laborator. Pentru analiza variației s-a folosit Duncan's New Multiple Range Test (D'sNMRT; P=0,05), cu transformarea inițială a şirului de date în log (x+1).

Rezultate şi discuții. *Vârsta femelelor şi comportamentul de chemare*. Rezultatele obținute sunt prezentate în Fig. 1. Intervalul optim pentru eliberarea feromunului sexual s-a înregistrat pentru femelele de 2-4 zile, nivelul menținându-se ridicat şi la femelele de 5-8 zile. S-a observat comportament de chemare şi la femelele de o zi. Datele confirmă studiile citologice efectuate asupra structurii şi dezvoltării glandei feromonale la această specie [10].

Pentru *M. brassicae* nu s-a observat un comportament de chemare la femelele proaspăt emerse (0 zile), iar în alte investigații nici pentru femelele de o zi. Procentul a crescut şi curba a fost similară pentru femelele de 3-5 zile [20]. Referitor la *M. brassicae*, studii citologice şi fiziologice ale glandei feromonale [10] au arătat că la femelele de o zi celulele glandei feromonale sunt formate, eliberare de feromon sexual are lor şi în prima zi, dar maximul s-a inregistrat în zilele 2-3. Datele noastre sugerează existența la *M. brassicae* a unui comportament de chemare (cu predominarea chemării slabe) şi la femelele de o zi. De altfel modele similare au mai fost evidențiate şi pentru alte specii (*Lacanobia suasa, Autographa gamma, Agrotis ipsilon*), autorii sugerând că la femelele

proaspăt emerse există frecvent un comportament de "chemare slabă" [21, 22] şi chemarea este evidentă la femelele de 2-3 zile. Factorul care induce cele mai pronunţate modificări la nivelul localizării perioadei de chemare este temperatura [15]. De aici rezultă şi diferenţele obţinute de diferiţi autori. Modelul comportamental de sinteză şi eliberare a feromonului sexual este dependent de specie şi este determinat genetic fiind sub control neuro-endocrin. Există însă o serie de factori interni şi externi care modifică acest comportament. La specia *Choristoneura rosaceana* modelul de chemare a fost corelat cu vârsta şi modificat de influenţa temperaturii constante sau fluctuante [4].



F i g. 1. *Influenţa vârstei asupra nivelului şi periodicităţii comportamentului de chemare al femelelor de* Mamestra brassicae.

Banda neagră reprezintă scotofaza de 8 ore, iar benzile mai subţiri reprezintă locali-zarea perioadei de chemare (săgeţile indică ora medie a perioadei de chemare).

Perioada de chemare la această specie este localizată la sfârşitul scotofazei [14]. Odată cu creşterea vârstei chemarea s-a inițiat mai timpuriu, iar ora medie a perioadei de chemare la nivel populațional s-a deplasat spre începutul scotofazei. Acesta este un comportament cu valoare adaptativă pentru specie, oferind şanse mai mari, pentru acuplare, femelelor mai în vârstă în competiția lor cu cele tinere. O semnificație similară, în relație cu creşterea vârstei, o are creşterea duratei chemării şi a numărului de reprize de chemare / femelă (Tabel 1).

Tabel 1

Influența vârstei adulților de Mamestra brassicae asupra comportamentului de chemare în condiții de laborator

Datele reprezintă valori medii pentru testele efectuate la diferite linii de creştere şi generații, în perioada 1983-1993

Vârsta ♀♀ (zile)	N	Ora medie a chemării*		D_{CH}**	NR_{CH}***
		I_{CH}	P_{CH}		
1	820	6,28	7,68	86,4 a	3,6 a
3	760	5,27	7,46	92,8 b	4,2 b
5	565	5,04	7,29	99,7 b	4,6 bc
7	540	4,21	6,78	115,6 c	5,1 cd
9	420	4,08	6,24	121,3 cd	5,7 de
11	384	4,01	5,92	128,9 d	6,1 ef
13	280	4,37	5,41	116,6 c	6,4 f

* **I_{CH}** - Ora medie pentru perioada de inițiere a chemării la nivel populațional. P_{CH} - Ora medie a perioadei de chemare. Faza de întuneric (scotofaza) de 8 ore este notată de la 0 la 8, cu exprimare zecimală a orei [22].

** **D_{CH}** - Durata chemării. Aceeaşi literă indică diferențe nesemnificative în funcție de vârstă, pentru acelaşi parametru (D'sNMRT; P=0,05).

*** **NR_{CH}** - Numărul de reprize de chemare/femelă în chemare.

Cercetările arată că există două grupe de specii: a) cele la care modelul de chemare a variat dependent de variația vârstei [5, 6, 20, 21]; b) specii la care modelul de chemare nu a variat corelat cu vârsta [1, 2].

In cazul primei categorii, la specia *Dioryctria abietella* cca. 20% dintre femelele observate au inițiat chemarea încă din ziua 1. Pentru vârsta de 2-10 zile nu au existat variații mari (pentru vârsta de 9-10 zile chemarea a fost chiar mai mare ca în rest) [5]. Pentru specia *Agrotis ipsilon* pe intervalul 1-4 zile, odată cu creşterea vârstei, au crescut procentul de femele în chemare, durata chemării, numărul de reprize de chemare (calling bouts)/femelă în chemare, iar chemarea s-a inițiat mai timpuriu, fapt care arată că femelele mature sunt mai competitive decât cele tinere [21]. Modelul de chemare al speciei *Chilo suppressalis* a variat de asemenea cu

vârsta [6]. Numărul de femele în chemare a fost mare şi aproximativ egal pentru vârsta de 1-5 zile, după care a scăzut semnificativ. Numărul de reprize de chemare şi lungimea acestora a crescut uşor la femelele mai bătrâne (pe intervalul 1-5 zile), dar pentru intervalul de peste 6-8 zile a scăzut din nou.

Caracteristic pentru grupa a doua este specia *Laspeyresia pomonella*, la care nu au existat diferenţe privind %chemare, ora medie a perioadei de chemare şi durata chemării pe intervalul vârstei de 0-6 zile [2]. Ca şi la alte specii, în paralel cu creşterea vârstei chemarea s-a iniţiat mai timpuriu, iar durata chemării a crescut. Trebuie precizat că observaţiile au fost făcute pe grupe de femele de 0-1; 1-2; 2-3; 3-4; 4-5; 5-6 zile (totuşi la cei 3 parametri amintiţi au existat diferenţe semnificative între femelele de 0-1 zile şi celelalte grupe). La *Grapholitha molesta*, femelele proaspăt emerse (1-9 ore) nu au avut un comportament de chemare, dar pe intervalul 1-6 zile modelul a fost similar [1].

Relaţia dintre împerechere (acuplare) şi vârsta adulţilor. Datele sunt prezentate sintetic în Fig. 2. Un model comportamental similar cu cel observat pentru chemare la *M. brassicae*, s-a observat şi în cazul împerecherii. Eliberarea feromonului sexual (chemarea) este secvenţa care declanşează răspunsul masculilor. In urma derulării a două faze caracteristice (răspunsul la distanţă şi curtarea) are loc împerecherea. Există un sincronism în timp al modelelor comportamentale, acuplarea fiind caracterizată şi ea de o iniţiere mai timpurie odată cu creşterea vârstei, în paralel cu creşterea duratei (Tabel 2).

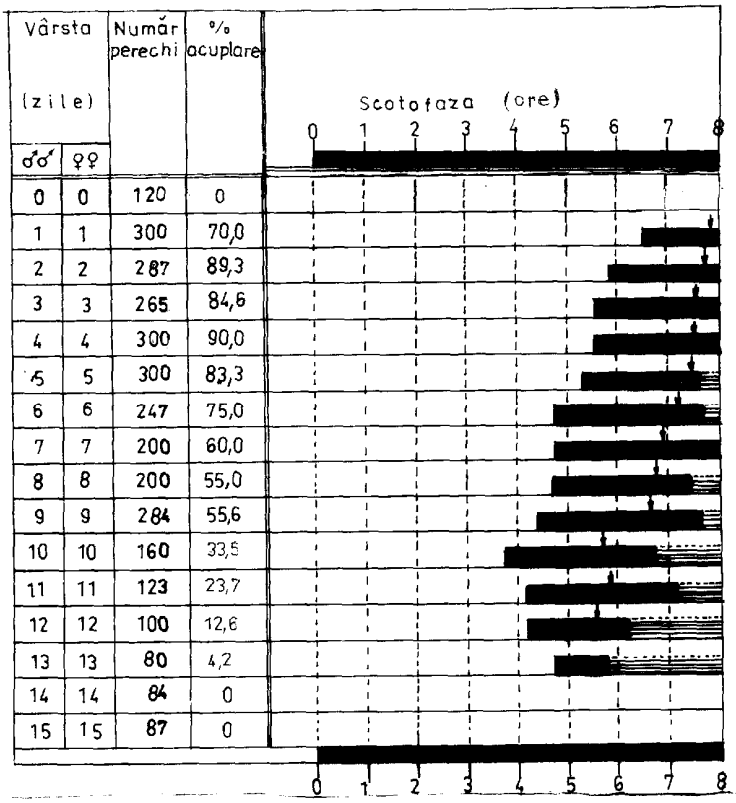
Tabel 2

Influenţa vârstei adulţilor de Mamestra brassicae asupra comportamentului de împerechere în condiţii de laborator

Datele reprezintă valori medii pentru rezultatele obţinute în toate experimentele efectuate la diferite linii de creştere şi generaţii (1983-1993)

P*	Vârsta adulţilor (zile) (♂:♀)														
	1:1	**1:3**	**3:1**	**3:2**	**3:3**	**5:1**	**5:3**	**7:1**	**7:2**	**1:5**	**3:5**	**1:7**	**2:7**	**1:9**	**3:9**
I_{AC}	6,61a	6,38a	6,44a	6,18b	5,77bc	5,32cd	5,18c	5,02cd	4,92d	6,32a	6,28b	6,08b	6,16b	5,92b	4,98d
P_{AC}	7,62a	7,64a	7,78a	7,52a	7,24b	7,22b	6,39c	6,18c	5,38d	7,65a	7,12b	7,18b	7,28b	6,32c	5,98e
D_{AC}	68,2a	76,1b	68,4a	56,3c	68,5a	64,8a	76,8b	82,2b	70,6a	108,5d	94,8e	84,2b	78,5b	101,8d	118,34f

* P - Parametrii analizaţi. **I_{AC}** - Ora medie pentru perioada de iniţiere a acuplării la nivel populaţional. **P_{AC}** - Ora medie a perioadei de acuplare. **D_{AC}** - Durata acuplării (în minute). Faza de întuneric (scotofaza) de 8 ore este notată de la 0 la 8, cu exprimare zecimală a orei [22]. Aceeaşi literă indică diferenţe nesemnificative între variante, în cadrul aceluiaşi parametru (D'sNMRT; P=0,05).



F i g. 2. *Influența vârstei adulților de* Mamestra brassicae *asupra împerecherii în condiții de laborator*.

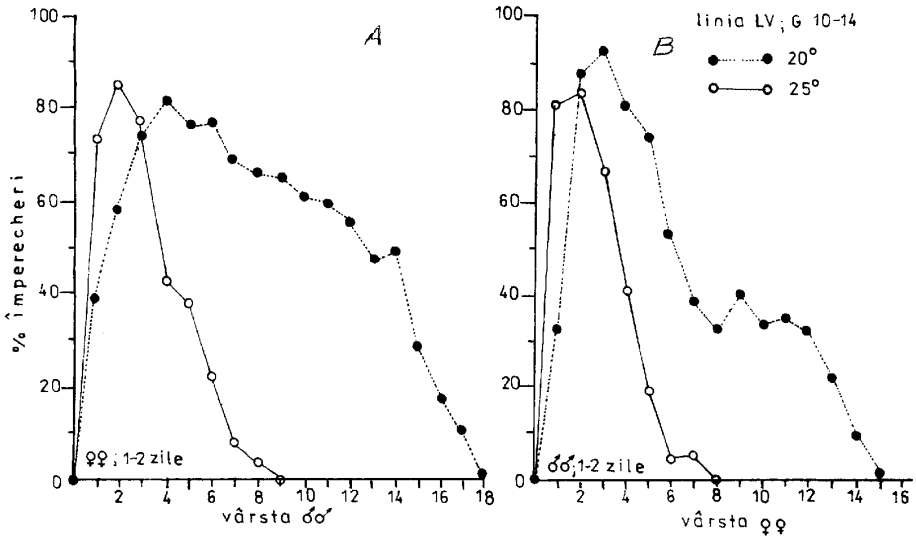
Banda neagră reprezintă scotofaza de 8 ore, iar benzile mai subțiri reprezintă localizarea perioadei de împerechere (săgețile indică ora medie a perioadei de împerechere).

Pe lângă sincronismul evidențiat între secvențele de chemare - curtare - acuplare, cercetările efectuate la speciile de lepidoptere, unde există o comunicare evidentă prin feromoni sexuali, s-a dovedit clar că masculii sunt deja pregătiți pentru acuplare şi chiar manifestă un comportament caracteristic chiar înainte de eliberarea feromonului de către femelă (în acest caz perioada lor de activitate este mai largă) [15]. Aceasta are o valoare adaptativă deosebită pentru specie. Ca şi în cazul chemării, împerecherea este influențată de vârsta adulților.

La *M. brassicae* cercetările noastre referitoare la comportamentul de reproducere al diferitelor linii de creştere în relaţie cu generaţia au arătat că împerecherea poate fi influenţată doar de generaţie, între linii nefiind diferenţe semnificative pentru o aceeaşi generaţie şi vârstă a adulţilor [18]. In schimb, temperatura este un factor extern deosebit de important care poate modifica puternic un model obţinut în relaţie cu vârsta (Fig. 3). Trendul curbelor referitoare la nivelul împerecherii în relaţie cu vârsta s-a modificat semnificativ datorită scurtării duratei de viaţă sub influenţa temperaturii ridicate. Din Fig. 3 se desprinde şi un alt aspect interesant. Astfel, se pot observa modificările în evoluţia trendului curbelor în funcţie de sex, mai pronunţat pentru intervalul vârstei de 6-12 (14) zile. Se poate constată că o împerechere este reuşită în condiţiile în care cuplul este format în special cu femele tinere, mature sexual.

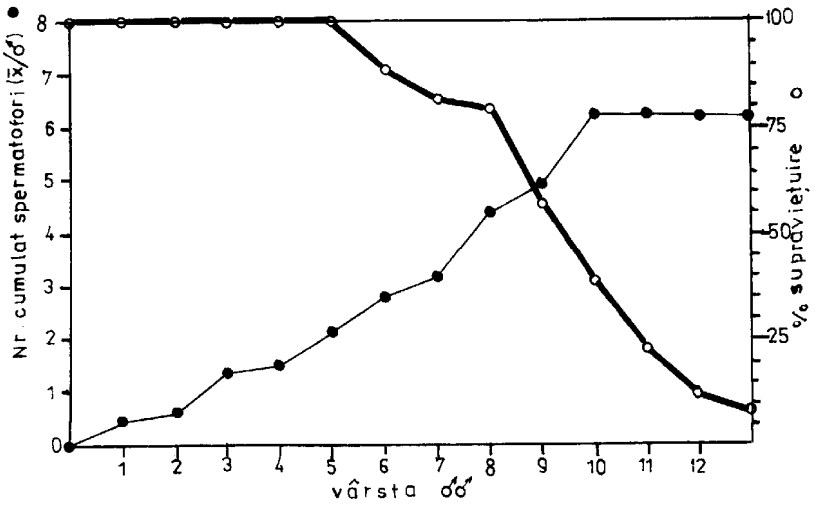
Odată cu creşterea vârstei a crescut numărul de spermatofori transmişi în secvenţa de împerechere, corelat cu scăderea procentajului de supravieţuire (Fig. 4).

Un model comportamental similar cu cel de faţă obţinut la *M. brassicae*, pentru toate secvenţele reproducerii a fost evidenţiat la diferite nivele de aprofundare şi la alte specii de lepidoptere cu activitate nocturnă.



F i g. 3. *Influenţa vârstei masculilor (A) şi a femelelor (B) în funcţie de temperatură, asupra împerecherii la* Mamestra brassicae, *în condiţii de laborator*.

In testele experimentale, masculii sau femelele de diferite vârste s-au pus la împerecheat cu femele, respectiv masculi, de 1-2 zile. N = minim 200 perechi/vârstă. Linia de creştere (suşa) LV, generaţiile 10-14.

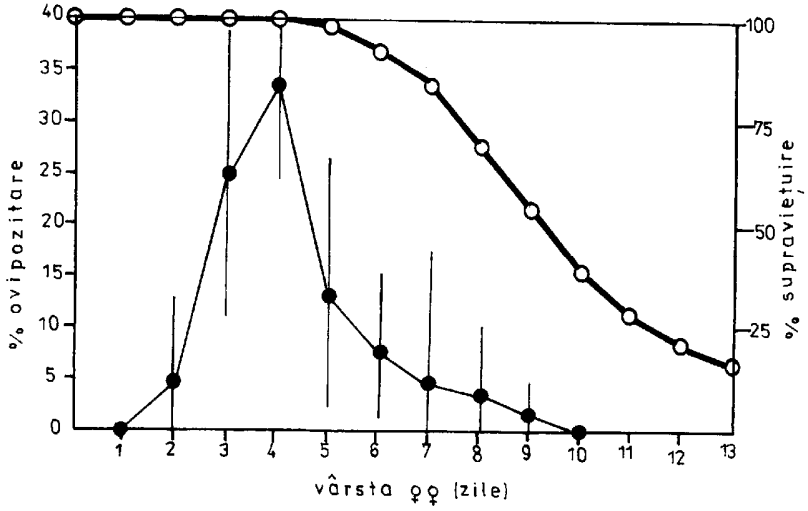


F i g. 4. *Numărul cumulat de spermatofori transmişi de masculii de* Mamestra brassicae *pe toată durata de viață şi relația cu evoluția nivelului de supraviețuire*.
Perechi de adulți puşi la împerecheat la vârsta de 0 zile.

In cadrul acuplării în relație cu vârsta adulților semnalăm cazul speciei *Spodoptera littoralis* [7] unde există diferențe în ceea ce priveşte vârsta la care cele două sexe ating maturitatea sexuală. In ambele cazuri femelele au fost atractive şi la vârsta de o zi, dar masculii numai la vârsta de 2-3 zile. O valoare maximă a fost atinsă pentru vârsta de 3 zile a ambelor sexe. La *Earias insulana* acuplarea a fost absentă la vârsta de o zi a celor două sexe, indiferent de combinațiile făcute, iar valoarea cea mai mare s-a obținut pentru adulții de 4 zile [8].

In ceea ce priveşte împerecherile multiple, numărul acestora în general creşte odată cu creşterea vârstei. Modelul este însă foarte diferit de la o specie la alta, dependent de durata de viață a adulților şi factorii interni şi externi implicați în experiment. La *S. littoralis* masculii care au trăit maxim 26 zile au transferat în medie 5,2 spermatofori, iar numărul mare al acestora (4, 5 şi 6) s-a înregistrat pe intervalul vârstei de 14-26 zile [7]. Un model similar a fost obținut şi la specia *E. insulana* [8].

Ovipozitarea şi vârsta adulților. La femelele de *M. brassicae*, acuplate şi care au depus pontă fertilă, ovipozitarea s-a inițiat chiar din a doua zi după împerechere. In condițiile noastre de experimentare, numărul mediu cel mai mare de ouă depuse în zilele succesive de ovipozitare s-a înregistrat la femelele de 4 zile, cu un procentaj destul de mare şi pentru femelele de 3 zile (Fig. 5). Odată cu înaintarea în vârstă, nivelul de ovipozitare s-a diminuat progresiv.



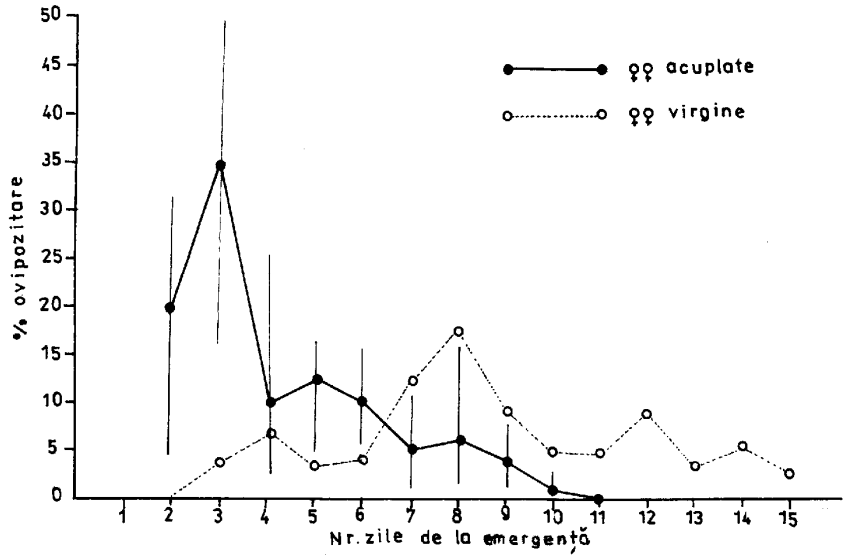
F i g. 5. *Dinamica ovipozitării şi supravieţuirea la femelele acuplate de* Mamestra brassicae.

Adulţi puşi la împerechere la vârsta de 0 zile. Datele reprezintă valori medii, iar liniile verticale marchează intervalul de variaţie pentru 36-64 serii experimentale din diferite linii de creştere şi generaţii (1983-1993).

Există diferenţe semnificative între modelul de ovipozitare al femelelor acuplate care depun pontă fertilă şi femelele virgine (Fig. 6). Conform datelor prezentate în figură, decalajele sunt pronunţate la nivelul de maxim şi terminarea ovipozitării. Fenomenul a fost atenuat în ceea ce priveşte iniţierea ovipozitării, deşi dominant femelele virgine au iniţiat mult mai târziu ovipozitarea, rata zilnică a fost redusă şi a durat mai multe zile corelat şi cu o durată de viaţă mai mare a acestora. Fenomenul de reţinere al ouălor are valoare adaptativă deosebită şi caracterizează şi alte specii.

Ca durată de timp în care a avut loc ovipozitarea, modelul şi procentajul de femele care au ovipozitat au fost corelate cu vârsta femelelor (Fig. 7). Femelele tinere acuplate (2-3 zile în momentul iniţierii ovipozitării) au depus numărul cel mai mare de ouă în ziua a doua după acuplare şi ovipozitarea a durat mai multe zile. Pentru femelele cu vârsta de la 3 zile în sus, procentul maxim de ouă depuse s-a înregistrat în ziua imediat următoare acuplării, iar durata ovipozitării s-a redus treptat, asociat cu creşterea vârstei. Şi la alte lepidoptere nocturne a existat un model asemănător, dar variabil în funcţie de specie şi dependent de o serie de factori interni şi externi. La toate speciile împerecherea stimulează ovipozitarea

care creşte progresiv pe perioada maturității sexuale [3], după care descreşte treptat. Femelele virgine depun ouăle la o rată mică, după o perioadă de retenție, dar frecvent rata creşte odată cu creşterea vârstei [11]. In medie o femelă împerecheată depune de cel puțin două ori mai multe ouă decât una virgină. La alte specii femelele neîmperecheate ovipozitează zilnic, dar tot la o rată semnificativ mai mică decât cele acuplate şi toate ouăle sunt sterile [9].



F i g. 6. *Reprezentarea comparativă a dinamicii ovipozitării femelelor virgine şi acuplate de* Mamestra brassicae, *în condiții de laborator*.
Alte explicații - ca în Fig. 5.

Fecunditatea, fertilitatea şi vârsta adulților. In acest gen de cercetări efectuate la insecte, fecunditatea este acceptată ca numărul total de ouă/femelă (ouă depuse pe toată durata de viață + numărul de ouă existente în ovare la moartea femelelor). Dominant în lucrări se ține cont cel mai mult de numărul de ouă depuse, bazat pe faptul că între cele două componente există o relație strânsă [17]. Astfel, fecunditatea femelelor acuplate a fost semnificativ ridicată în perioada optimă a maturității reproductive. Diminuarea procentajului de supraviețuire se corelează atât cu diminuarea numărului de ouă depuse cât şi cu golirea ovarelor. Femelele cu durată de viață lungă, care au depus un număr mare de ouă au avut, la moartea acestora, ovarele goale (sau cu un număr foarte redus de ouă) (Tabel 3).

Tabel 3

Influenţa vârstei adulţilor de Mamestra brassicae asupra numărului de spermatofori transmişi, fecundităţii şi fertilităţii, în condiţii de laborator

Datele reprezintă valori medii pentru testele efectuate în perioada 1983-1993

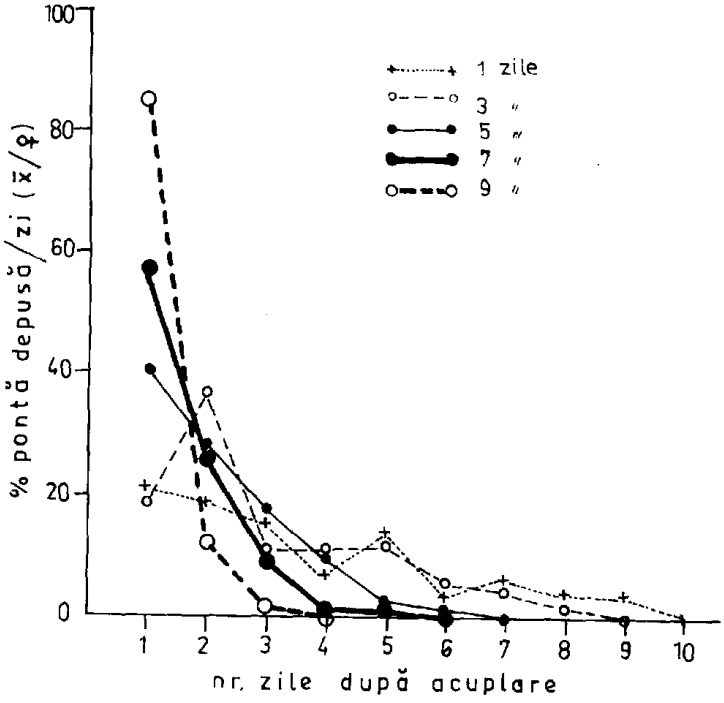
Vârsta*		Fecunditatea ($\bar{x}$ ouă/♀)			Număr spermatofori***	%f****
♂♂	♀♀	Număr de ouă depuse (± AS)	Ouă/ovare**			
			Imature	Corionate		
1	1	870,9 ± 28,4	+; o	0; I	4,8	86,4
3	3	1680,5 ± 118,2	o; +	0	3,1	92,5
5	5	1412,8 ± 168,8	+	I	2,4	96,4
7	7	1108,4 ± 174,6	+	I; II	1,6	88,3
9	9	1005,5 ± 214,4	++	II; III	0,7	72,8
11	11	982,3 ± 112,2	+++	III	0,5	74,5
13	13	706,8 ± 164,2	+++	IV	0,7	62,3
1	1	964,8 ± 38,4	o; +	0	5,8	82,7
1	2	1612,5 ± 116,2	o	0	4,1	96,3
1	3	1512,7 ± 84,5	o; +	0; I	5,1	95,2
3	1	1528,4 ± 138,9	o	0	3,2	93,4
3	2	1584,2 ± 176,2	o	0	2,1	92,5
3	3	1418,7 ± 212,8	o; +	0; I	2,4	96,9
5	1	1226,5 ± 174,1	o; +	I	2,3	94,1
5	2	1104,6 ± 132,8	+	0; I	1,5	86,2
5	3	1074,8 ± 224,5	+	I	1,1	91,4
7	1	1229,5 ± 158,3	+; ++	I; II	0,6	88,2
7	2	1176,5 ± 170,0	++	I	0,8	84,1
7	3	986,4 ± 175,5	+++	I	0,4	86,4
1	5	1312,1 ± 324,2	o; +	I	3,1	90,4
2	5	1176,3 ± 286,5	+	0; I	2,6	91,2
3	5	1342,2 ± 324,8	o; +	I	1,4	88,3
1	7	1014,2 ± 346,7	o; +	I; II	1,6	86,5
2	7	987,5 ± 322,2	+	II	1,8	82,4
3	7	1078,4 ± 284,9	++	II	1,4	79,8
1	9	879,6 ± 322,1	+	II; III	1,4	74,7
2	9	912,5 ± 294,8	+	III	1,2	78,6
3	9	848,4 ± 345,4	+++	III; IV	0,9	76,2

* Cifrele reprezintă vârsta la care adulţii s-au pus la împerecheat.

** Evaluare la moartea femelelor (notarea reprezintă minim 50% din femele, pentru ouă imature şi mature [18]). Ouă imature: **o** - absent; **+** - foarte puţine, izolate (până la 20); **++** - multe; **+++** - foarte multe. Ouă mature (corionate): **0** - ouă absente; **I** - ouă puţine (1-50); **II** - nr. mic de ouă (51-100); **III** - nr. mare de ouă (101-150); **IV** - ouă multe (>150 - frecvent abdomen plin).

*** Număr de spermatofori transmişi pe durata de viaţă.

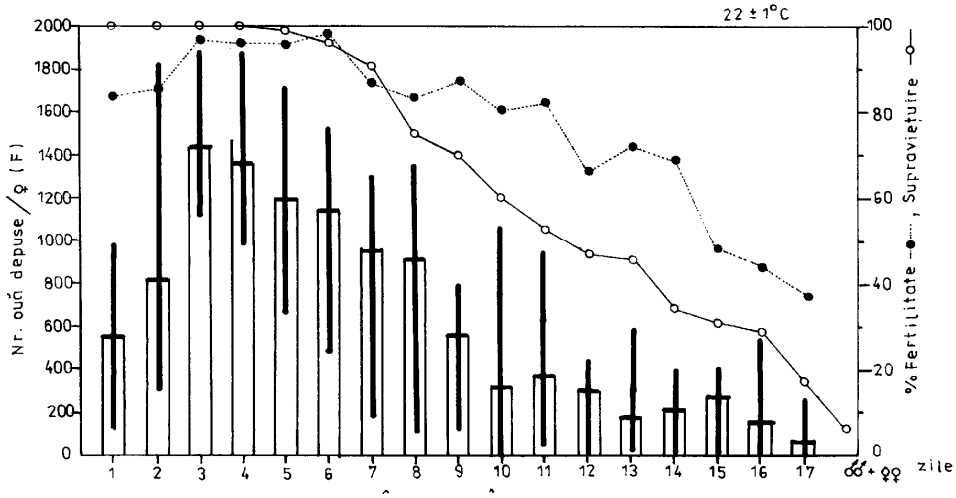
**** %f - Procentul de fertilitate a ouălor depuse.



F i g. 7. *Modelul de ovipozitare la* Mamestra brassicae, *pentru femelele acuplate, în funcție de vârsta la care au fost puse la împerecheat.*

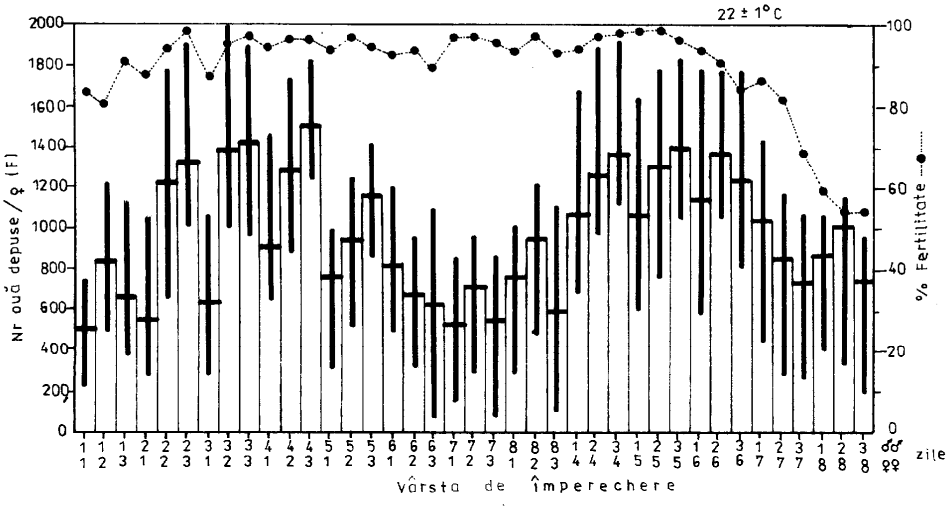
Datele reprezintă valori medii, iar liniile verticale marchează intervalul de variație pentru 12-18 serii experimentale din diferite linii de creştere şi generații (1990-1993).

In condițiile în care s-au pus la împerechere adulți de aceeaşi vârstă, numărul cel mai mare de ouă depuse s-a înregistrat pentru vârsta de 3-6 zile (Fig. 8). Şi femelele cu vârsta de împerechere de 2 zile au depus un număr mare de ouă, dar s-a constatat un interval de variație foarte larg. Incepând cu vârsta de acuplare de 7 zile, fecunditatea s-a redus progresiv odată cu scăderea procentajului de supraviețuire. Un trend similar a avut şi curba fertilității. Referitor la ponta sterilă se impun unele observații. Astfel, un număr variabil de ouă sterile (depuse) au marcat fecunditatea practic pe toată perioada de ovipozitare. Numărul lor a crescut odată cu creşterea vârstei, dar ouă sterile au fost prezente şi la femelele tinere (1-2 zile). In procentaj de 0,1-3,6% au fost evidențiate şi la femelele de 3-6 zile.



F i g. 8. *Influența vârstei asupra fecundității și fertilității la* Mamestra brassicae *în condițiile variantelor experimentale de împerechere cu adulți de aceeași vârstă*. Datele reprezintă valori medii, iar liniile verticale marchează intervalul de variație pentru 74-96 serii experimentale din diferite linii de creștere și generații (1987-1993).

Modelele fecundității şi fertilității au avut o evoluție interesantă în condițiile în care s-au pus la împerechere adulți de vârste diferite (Fig. 9). Numărul cel mai mare de ouă depuse a fost înregistrat în condițiile în care s-au împerecheat masculi de 2-4 zile cu femele de 2-3 zile. Nivelul s-a menținut ridicat şi pentru masculii de o zi împerecheați cu femele de 2-3 şi chiar de 8 zile. În schimb, creşterea vârstei masculilor, pe intervalul 5-8 zile, a indus o diminuare a fecundității chiar în condițiile împerecherii cu femele de 2-3 zile. Un alt aspect interesant se referă la fertilitate. Pentru un nivel al fecundității relativ acelaşi, fertilitatea s-a menținut ridicată când împerecherile au fost făcute între masculi bătrâni şi femele tinere, dar a scăzut semnificativ în cazul acuplărilor dintre adulți tineri şi femele bătrâne. Rezultatele obținute confirmă faptul că fertilitatea este strict dependentă de vârsta femelelor. Pentru adulții tineri creşterea procentajului de pontă sterilă este asociată cu acuplări nereuşite sau cu o capacitate mai redusă de fertilizare a spermei. Reducerea capacității de fertilizare se diminuează semnificativ odată cu creşterea vârstei, astfel că şi la femelele mature sexual, care au ovipozitat numai ouă fertile şi în număr mare, la bătrânețe a crescut numărul de ouă sterile.

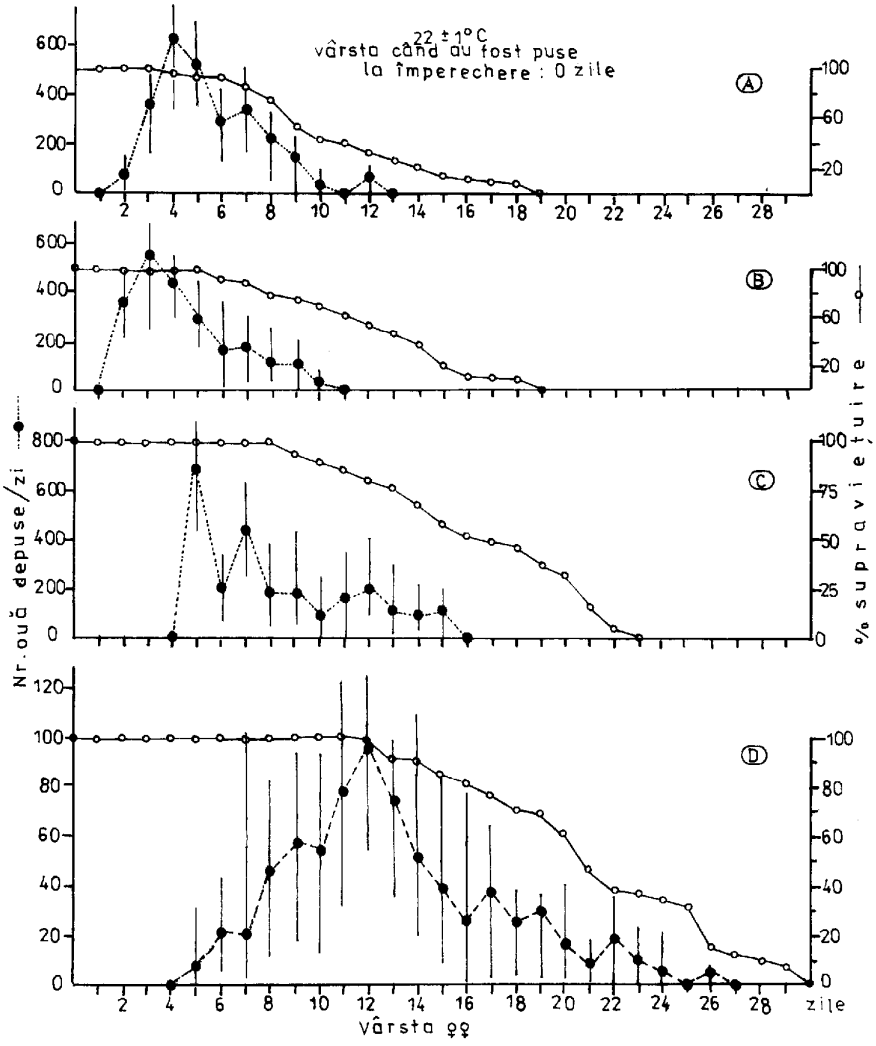


F i g. 9. *Influența vârstei asupra fecundității şi fertilității la* Mamestra brassicae *în condițiile variantelor experimentale de împerechere cu adulți de vârste diferite.*
Alte explicații - ca în Fig. 8.

Dacă nivelul fertilității este asociat cu calitatea femelelor, fecunditatea este influențată şi de calitatea masculilor. Astfel, un nivel ridicat al fecundității a implicat neapărat prezența adulților tineri şi maturi sexual, chiar în condițiile împerecherii cu femele mai bătrâne.

Modelul de ovipozitare şi nivelul fecundității femelelor de *M. brassicae* a fost parțial influențat şi de comportamentul de împerechere (Fig. 10). Nu au fost diferențe între femelele cu o singură împerechere şi cele cu 2-3 acuplări, pe toată durata de viață. In schimb, dacă prima acuplare a avut loc după 4-5 nopți, s-a modificat trendul curbei de ovipozitare odată cu creşterea duratei de supraviețuire la nivel populațional. Pentru femelele neacuplate modelul se aseamănă cu cel din Fig. 6.

Un comportament asemănător cu cel prezentat pentru *M. brassicae* a fost descris şi la alte lepidoptere, dar există particularități specifice chiar dacă el se aseamănă mult la noctuide [12]. La *S. littoralis* evoluțiile numărului de ouă depuse şi supraviețuirea au fost corelate atât cu vârsta cât şi cu rata de împerechere [7]. La *Mythimna separata* numărul de ouă/femelă a fost mic (10-250) pentru vârsta de 1-5 zile şi mare (820-900) la femelele de 20 zile [13]. In schimb, la *Bombyx mori* a existat o corelație semnificativ negativă (r = -0,928) între fertilitate şi vârstă [11].



F i g. 10. *Modelul de ovipozitare şi evoluţia numărului de ouă depuse de femelele de* Mamestra brassicae *pe toată durata de viaţă, în funcţie de statutul reproductiv.*

Adulţi puşi la împerecheat la vârsta de 0 zile. **A** - O singură acuplare/durata de viaţă (N=420). **B** - 2-3 acuplări/durata de viaţă (N=380). **C** - 2 împerecheri, prima acuplare după 3-4 nopţi (N=240). **D** - Femele virgine (N=464). Valori medii din testele experimentale făcute pe material biologic din diferite linii de creştere şi generaţii (1987-1993). Liniile verticale indică intervalul de variaţie.

Cunoaşterea particularităților comportamentale ale unor specii importante din punct de vedere economic, asociat cu prolificitatea şi longevitatea lor şi în funcție de valorile factorilor ecologici prezintă valoare practică deosebită în vederea elaborării unor modele predictive în cadrul acțiunilor de monitoring şi management.

Concluzii. 1. Un comportament optim de chemare (eliberarea feromonului sexual) la *M. brassicae* a fost evidențiat pentru femelele de 2-5 zile. La un regim termic de 24^{o}C, 53,6% din femele au prezentat o postură de chemare şi la vârsta de o zi. La 22^{o}C, peste vârsta de 5 zile procentajul de femele în chemare s-a redus progresiv odată cu creştere vârstei (aceasta a indus şi o creştere a duratei chemării, a numărului de reprize de chemare şi o inițiere mai timpurie a chemării).

2. In ceea ce priveşte periodicitatea a existat o similaritate între chemare şi acuplare. Procentul cel mai mare de acuplări reuşite s-a obținut pentru femelele de 2-5 zile, numărul s-a redus semnificativ peste vârsta de 10 zile şi au fost absente peste 13 zile, vârstă la care 45% din femele erau totuşi în chemare. Rezultatele obținute în testele de împerechere cu adulți de aceeasi vârstă şi de vârste diferite au evidențiat că vârsta masculilor este esențială în realizarea acuplărilor reuşite.

3. Modelul de ovipozitare a fost diferit la femelele împerecheate care au depus pontă fertilă şi cele virgine. In primul caz, odată cu creşterea vârstei a crescut şi procentajul de pontă depusă în prima zi după acuplare, iar durata de ovipozitare s-a redus progresiv. Pentru femelele virgine s-a observat un comportament de reținere al pontei (cu valoare adaptativă pentru specie), ovipozitarea s-a întins pe durată de timp aproape dublă şi la o rată zilnică mică.

4. Fecunditatea nu a fost influențată de numărul de acuplări, dar vârsta a fost un factor intern cu o semnificație mare. In condițiile în care împerecherea a fost analizată pe perechi de adulți de aceeaşi vârstă, fecunditatea cea mai mare s-a obținut pentru femelele de 3-6 zile. In varianta cu adulți de vârstă diferită, nivelul cel mai mare al fecundității s-a înregistrat pentru combinația dintre masculi de 1-4 zile cu femele de 1-8 zile. Datele sugerează că realizarea acuplărilor reuşite este dependentă de prezență masculilor tineri.

5. Fertilitatea pontei a fost mare pentru adulții de 2-5 zile şi a scăzut mult odată cu creşterea vârstei. Prezența unui procentaj mai mare de pontă sterilă s-a obținut şi în cazul adulților de 1-2 zile, când ovipozitarea a avut loc în ziua imediat următoare după acuplare.

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USING OF ALLELOMORPHIC FEATURES IN IDENTIFYING THE TWO SPECIES BELONGING TO THE GENUS *BOMBINA* (*ANURA: DISCOGLOSSIDAE*) FROM TRANSYLVANIA

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SUMMARY. - The paper analyses the allelomorphic features of seven *Bombina* populations, living in Transylvania (Romania) and in Hungary between 150 and 1700 m altitude, in order to establish which of them are the most important for the determination of belonging to *bombina* or *variegata* species, or for the determination of *bombina*-like or *variegata*-like hybrids. The analysed material consists of 259 toads (122 adult males, 110 adult females and 27 juveniles) belonging to the genus *Bombina*. The populations found in the plain areas belong to *bombina* species: Marghita with 98.18 % of the features and Parassapuszta with 94.57 % of the features. The most hybrid populations were found in the hilly regions: Cluj with 71.15% and Ciuc with 69.65% of the features belonging to *B. variegata*. The most *variegata* population was not found at the highest altitude as was expected (Soarbele – 1400-1550 m above sea level), but in Câmpuşel at 1000 m altitude, with 93.42% of the features belonging to *variegata*. Two features are the most important for species determining: the length of the leg (the tibio-tarsal articulations are/are not in connection) and the presence/absence of the dorsal spots.

M é h e l y [9] was the first to report in 1905 the natural hybridisation between two European species of fire-bellied toads, *Bombina bombina* (L.) and *Bombina variegata* (L.).

Artificial hybrids were obtained in laboratory and heterospecific pairs in amplexus were observed in field [10], but fertility of hybrids was unknown and the morphology of F1 hybrids did not resemble most intermediate forms found in nature [13]. Several authors, among them M i c h a l o w s k i [10,11], S t u g r e n [16], L á c [4] and M a d e j [7] conducted studies of morphological variation in the contact zone of their ranges. Furthermore, the lowland and the mountain populations of *Bombina* have different mating calls [5], which traditionally have been regarded as effective barriers to gene exchange in many amphibian species.

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It has been suggested that the mixture of species characters is due to interspecific crosses and backcrosses [10,12]. S t u g r e n and V a n c e a [17] sustain that interspecific crosses explain the mixture of species-specific characters when both species occur together (sympatrics), or are at least ineffectively isolated reproductively, when living in different neighbouring habitats. Moreover, the phenotypical heterogeneity with toad populations, the admixture of characters of the other species can not be entirely explained by the assumption of interspecific crosses.

The newly developed molecular techniques could answer the question of hybridisation. The application of protein electrophoresis demonstrated that the fire-bellied toads and yellow-bellied toads do interbreed west of Cracow [18].

Molecular evidence suggests that these taxa have been diverging for 3-5 million years [8]. During this period, their behaviour, life history and morphology diverged extensively, and many of these differences are thought to be adaptations to their respective habitats. Briefly, *B. variegata* lays larger eggs that develop more quickly [15], and also has thicker skin and longer legs [7]. The more quiescent behaviour of *B. bombina* tadpoles is likely to give them a survival advantage over *B. variegata* in the presence of invertebrate predators found in ponds [3].

S z y m u r a and B a r t o n [19] examined the pattern of genotype frequencies - in particular, cline shape and linkage disequilibra among marker loci - to estimate the strength and nature of selection which maintains the hybrid zone in southern Poland. They cited the hybrid zone in *Bombina* as a typical example of a tension zone. At the same time, they suggested two lines of evidence for the occurrence of endogenous selection against hybrids: embryonic mortality and morphological aberrations. However, their evidence was limited to a single study with small samples, and the conjecture of lowered fitness from such phenotypic aberrations is doubtful [2].

The structure of narrow hybrid zones depends above all on the balance between selection and recombination. Selection and recombination typically maintain the distinct adaptations and coadaptations of the pure taxa. Those are typically maintained by selection favouring different genotypes in different environments, by selection against hybrids or by combination of both. Yet, the dispersal in hybrid zones creates mixed populations in which recombinations can act. If the selection is not too strong, a wide range of recombinants is produced. The break-up of parental gene combinations then frees individual genes from the effects of the selected loci with which they were originally associated: the effective selection acting on each gene becomes weaker, and so the hybrid zone becomes wider. With sufficient hybridisation, particularly fit recombinant genotypes can establish themselves, further broadening the hybrid zone, or even allowing the creation of hybrid taxa. However, if selection is sufficiently strong, then the integrity

of the two gene pools can be maintained despite the continuous production of hybrid genotypes. A strong barrier to gene flow will be maintained because individual alleles will be trapped in a largely unrecombined genetic background. Barriers to gene exchange can be strengthened by genetic variation for habitat preference [6]. These authors demonstrate the effect of habitat heterogeneity and a habitat preference on the genetic structure of the hybrid zones between fire-bellied and yellow-bellied toads.

The problem of hybridisation and the characteristics of this hybrid zone between *B. bombina* and *B. variegata* are not yet clarified. Therefore, we have analysed the allelomorphic features of seven populations of *Bombina* genus, between 150 to 1700 m altitude in order to establish which of them are the most important for the determination of belonging to *bombina* or *variegata* species, or for the determination of *bombina*-like or *variegata*-like hybrids.

Material and methods. The analysed material consists of 259 toads (122 adult males, 110 adult females and 27 juveniles) belonging to the genus *Bombina*. The specimens were collected in 10 different geographical populations as follows (see also: Fig. 1):

I. **Săcărâmb**: 19 individuals (16 males, 3 females); the population is from Hunedoara county, 20 km north-east from Deva in the Metaliferi Mountains, at 950 m altitude;

II. **Câmpuşel**: 45 individuals (18 males, 27 females), from the Retezatul Calcaros Mountains (Hunedoara county), at 1000 m altitude;

III. **Soarbele Valley,** at 1400 m altitude, and **Tăul Soarbele,** at 1550 m altitude, in the Retezatul Calcaros Mountains (Hunedoara county): 32 specimens (22 males, 10 females);

IV. **Ciuc region**: 48 individuals (27 males, 21 females), in Harghita county: **Şoimeni** - 12 km north-east to Miercurea-Ciuc at 750 m altitude, and **Armăşeni,** at 20 km south-east from Miercurea-Ciuc at 750 m altitude;

V. **Cluj region**: 44 individuals (22 males, 14 females, 8 juveniles) resulted from **Baci Valley**, 7-8 km west from Cluj-Napoca (Cluj county), at 350 m altitude, and **Fânațele Clujului,** 10 km north from Cluj, 450 m altitude;

VI. **Petreu**: 48 individuals (8 males, 21 females, 19 juveniles), 3 km south-west from Marghita (Bihor county), at 150 m altitude;

VII. **Parassapuszta**: 23 individuals (9 males, 14 females) from the Ipoly Valley, the Börzsöny Mountains, Hungary, at 200 m altitude.

The *Bombina* specimens were studied morphologically according to the method of S t u g r e n [16]. This method consists of analysing 9 allelomorphic characters, but we considered only 5 of these and further 3 features were added (Table 1). The proportions of the characters were expressed as percentages: 0% for pure *Bombina bombina* and 100% for pure *Bombina variegata*. The results were compared to the theoretical features of pure *Bombina bombina* (0 %) and *B. variegata* (100%) populations (Table 2).

Table 1

The allelomorphic features of the two species of genus *Bombina* living in Romania (after[16] modified)

No.	Allelomorphic feature	*B. bombina*	*B. variegata*
1.	Colour of the light spots on the lower surface of the body	Red, orange, yellowish	Yellow
2.	Colour of the upper part of the thumb and of the tips of the toes (right and left)	Black	Light
3.	Relation between the light tarsal and sole spots	Separated	United
4.	The proportion between head length (HL) and head width (HW)	HL>HW	HL<HW
5.	Patterns of the lateral and lower surface of the body	White spots around the lateral and ventral warts	Lateral and ventral warts without white spots around
6.	Patterns of the upper surface of the body	Regularly disposed dark tubercles	Scattered dark tubercles
7.	Dorsal warts	Flat, sleek, lenticular	Cone-shaped, rugged
8.	Tibio-tarsal articulations when the femur and the tibia are perpendicular	Not touching	Touching

The results were statistically analysed: before using the tree diagram analysis, the differences between *Bombina* populations were checked by the analysis of variance (ANOVA and Duncan tests).

Results. The variation of characters is summarised in Table 2. The population of Săcărâmb is the purest *Bombina variegata*, with 93.42% of the characters belonging to "*variegata*". The head length is larger than the head width, this being a typical feature for the yellow-bellied toad. In the majority of specimens, the dorsal tegument has visible tubercles and the dorsal warts are cone-shaped. The specimens do not show any white spots around the lateral and ventral warts. In 78.91% of them the yellow spots reach the tip of the toes. In *Bombina variegata*, the posterior leg is longer than in *B. bombina* [12,14] because of its more terrestrial life. In the toads of Săcărâmb the tibio-tarsal articulations are connected in 84.21%, showing a larger leg.

Table 2

The percentage of the eight allelomorphic features in the seven studied populations of *Bombina*, in comparison to the theoretical pure *Bombina bombina* and *B. variegata* populations

Char.1 – Char.8: the number of the respective allelomorphic feature as in *Table 1*

Populations	Char.1 (%)	Char.2 (%)	Char.3 (%)	Char.4 (%)	Char.5 (%)	Char.6 (%)	Char.7 (%)	Char.8 (%)	Total (%)
Pure *B.v.*	100	100	100	100	100	100	100	100	100
Săcărâmb	100	84.21	100	100	78.94	84.21	100	100	93.42
Câmpuşel	100	75.09	93.75	95.53	97.41	73.8	100	100	92.18
Soarbele	100	21.87	81.25	100	87.5	37.5	100	100	78.51
Ciuc region	91.66	68.75	100	0	100	91.66	83.33	100	71.15
Cluj region	100	34.09	100	0	100	29.54	93.18	100	69.65
Marghita	0	10.41	0	0	0	4.16	0	0	1.82
Hungary	0	34.78	0	0	0	8.96	0	0	5.43
Pure *B. b.*	0	0	0	0	0	0	0	0	0

The population of Câmpuşel shows 92.11 % *Bombina variegata* characters. The head is wider than longer and the dorsal spots are weakly formed in 75.89 %. The corn-shaped dorsal warts are pointed at 93.75% of specimens and lenticular in three females. F u h n [1] shows that *Bombina variegata* females' spins can be underdeveloped. There are no white spots on the belly and the yellow spots of the tarsal and sole regions are united. The yellow spots reach the tip of the toes in 97.41 % of the specimens and in 73.8 % of them the tibio-tarsal articulations are connected.

The populations of Soarbele Valley and Tăul Soarbele have *Bombina variegata* characters in 78.51 % of individuals. The head is typically "*variegata*" being more wide than long. The dorsal tegument has pronounced spots even kidney-like in most specimens (*B. bombina* character), only in 21.87 % the dorsal spots are totally absent (*B. variegata* character). The corn-shaped dorsal warts are pointed in 81.25 % of the specimens.

In Ciuc region the populations have *B.variegata* characters in 71.15 %. The heads are typical *B. variegata*. In 68.75 % the dorsal spots are missing, the remainder having kidney-like spots and small ones; the dorsal warts are pointed. These populations have lateral and ventral warts surrounded by white spots. The yellow ventral spots are united (tarsal and sole) in 83.33 % of specimens. The tibio-tarsal articulations are connected in most specimens (91.67 %), which prove the adaptations to a more terrestrial habitat.

The populations of Cluj region have in 69.65 % "*variegata*" characters. The analysed specimens have clearly visible and kidney-like dorsal spots in most of the specimens. The tibio-tarsal articulations are connected only in 29.54 %, the remainder having shorter legs. There are white spots in the ventral and lateral tegument and the yellow-orange spots between tarsal and sole are united.

The *B. bombina* population close to Petreu (Marghita, Bihor county) has in 1.82 % *B. variegata* characters. The specimens' heads (*B. bombina* character) are longer than wide. The dorsal tubercles are obvious, almost all specimens have two kidney-like spots. The lateral and ventral warts are flat and surrounded by white spots. The leg is shorter and the tibio-tarsal articulations are connected in only 4.16% of the toads. The colour of the ventral spots is dark orange; the tarsal and sole spots are not united. The ventral tegument is predominantly black.

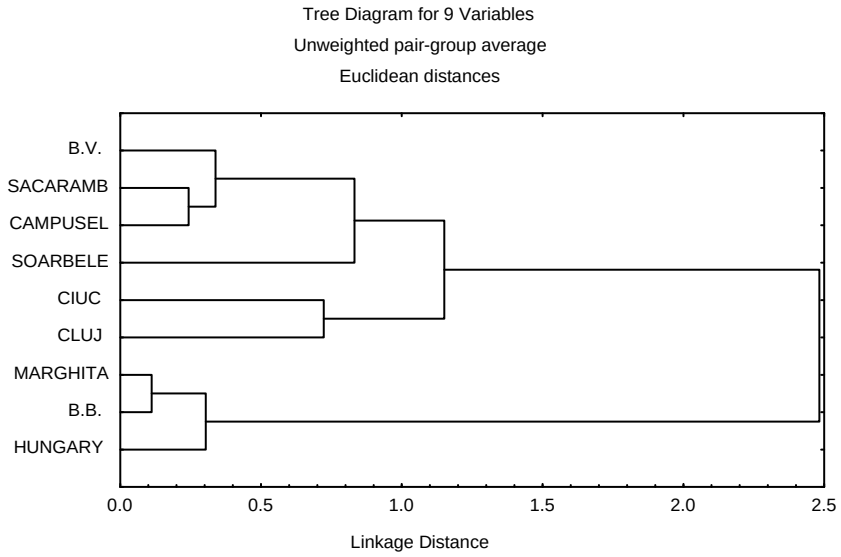
The population of Parassapuszta (Hungary) has 5.43 % characters of *B. variegata*. The dorsal tubercles are *B. variegata*-like in 34.78% and the tibio-tarsal articulations are connected only in a few specimens (8.69%). The colour of the ventral spots is orange, the tarsal and sole spots are separated. The lateral and ventral tegument is predominantly black and has warts surrounded by white spots.

For the comparison of the *Bombina* populations, we used the tree diagram analysis (Table 3 and Fig. 1).

Table 3

Values of the Duncan test following the ANOVA test (F=31.001; df=63: p< 0.0001), showing the significant statistical differences among the *Bombina* populations

	Pure *B.v.*	Săcărâmb	Câmpuşel	Soarbele	Ciuc	Cluj	Marghita	Hungary	Pure *B.b.*
Pure *B.v.*									
Săcărâmb	0.2361								
Câmpuşel	0.1462	0.7183							
Soarbele	**0.0277**	0.2483	0.3842						
Ciuc	**0.0257**	0.2346	0.3707	0.9328					
Cluj	**0.0100**	0.1238	0.2120	0.6332	0.6701				
Marghita	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0001**	**0.0001**			
Hungary	**0.0000**	**0.0000**	**0.0000**	**0.0001**	**0.0001**	**0.0001**	0.8432		
Pure *B.b.*	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0001**	0.9232	0.7834	



F i g. 1. *Tree diagram of the seven* Bombina *populations for the eight analysed features.*

Conclusions. 1.Two of the seven studied populations belong to *Bombina bombina*: the population of Marghita (98.18 % of the features) and the population of Parassapuszta - Hungary (94.57 % of the features).

2.The populations of Săcărâmb and Câmpuşel belong to *B. variegata* and have 93.42 % and, respectively, 89.58% characters of *B. variegata*.

3.The population of Soarbele though living near Câmpuşel at a higher altitude exhibits fewer characters of *B. variegata* (76.16 %) than that of Câmpuşel (93.42 %), a fact yet unexplained.

4.The populations of Ciuc and Cluj regions are natural hybrid populations between *Bombina bombina* and *Bombina variegata.* In this populations the "*variegata*" characters are predominant (71.15% and 69.65%, respectively).

5.The belonging to *Bombina bombina* or *B. variegata* can be established on the basis of two characters: the length of the leg (the tibio-tarsal articulations are/are not in connection) and the presence/absence of the dorsal spots. The dendrogram analysis of the two features, confirming this statement, proves to be similar to the dendrogram of all the features.

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BIOMETRIC STUDY OF THE SHREWS (*SORICIDAE, INSECTIVORA*) IN TWO HILLY ZONES OF THE SOMEŞUL MIC BASIN (ROMANIA)

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SUMMARY. - Researches were carried out in the Gilău and Bonțida hilly zones of the Someşul Mic basin. In both zones, six shrew species were recorded: *Sorex araneus, S. minutus, Neomys fodiens, N. anomalus, Crocidura leucodon* and *C. suaveolens.* Ten body and skull measurements, important from biometric and taxonomic points of view, were made on each individual. The differences between the adult and subadult age groups were found to be significant ($P < 0.05$) in most of the ten biometric traits determined, but the differences between the males and females were significant only in some biometric traits, those of the males being greater. Exceptionally, none of the ten biometric traits of males and females was significantly different in the *Crocidura suaveolens* population from the Bonțida zone.

In the two studied zones, the populations of *Sorex araneus* and *S. minutus* are morphologically similar to the populations from the northern part of Romania and Central Europe, while the *Crocidura leucodon* and *C. suaveolens* populations are similar to those from different regions of Romania and Central Europe.

There are only few data published on the insectivore mammals living in the north-western part of Romania [4, 9, 13]. This is why we have initiated researches, especially biometric ones, concerning the shrew populations from this part of Romania.

Materials and methods. The researches were carried out in the hilly zones around the localities Gilău and Bonțida in the Someşul Mic basin. Gilău is situated at $46^0$44' North latitude and $23^0$22' East longitude at an altitude of 420 m above sea level, and this zone represents the north-eastern gate of the Apuseni Mountains. Bonțida is situated at $46^0$54' North latitude and $23^0$51' East longitude at an altitude of 350 m above sea level, on the western bottom of the Transylvanian Plain.

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The shrews were caught with live traps in several types of ecosystems with different plant associations: *Salicetum albae, Carpino-Fagetum sylvaticae, Quercetum robori-petreae*, pasture with brush vegetation (*Crataegus monogyna, Cornus mas, Rosa canina, Pyrus pyraster, Malus sylvestris, Viburnum lantana*) and cultivated lands. All the caught individuals were measured and weighed. Ten body and skull measurements, important from biometric and taxonomic points of view, were made: body length (BL), tail length (TL), hindfoot length (HFL), body weight (BW), condylobasal length (CBL), breadth of brain case (BBC), interorbital constriction (IOC), height of brain case (HBC), mandible length (ML) and mandible height (MH). The biometric traits were registered in mm, excepting body weight which was recorded in g. For each biometric trait the following statistical parameters were considered: mean ($\bar{x}$), standard deviation (SD), minimum and maximum values (Min-Max). Significance of the differences between the biometric values of the adult and subadult age groups and of those of males and females was calculated by using Student's *t* test.

Results and discussion. In both hilly zones, six shrew species were caught [1]. The species and the number of the caught individuals were the following: *Sorex araneus* 34, *S. minutus* 11, *Neomys fodiens* 4, *N. anomalus* 3, *Crocidura leucodon* 47 and *C. suaveolens* 33.

In Tables 1-4, referring to *Sorex araneus, S. minutus, Crocidura leucodon* and *C. suaveolens*, NS and S indicate nonsignificant and significant ($P<0.05$) differences, respectively, between the age groups (adults vs. subadults; A/SA) and between the sexes (males vs. females; M/F). The results obtained for *Neomys fodiens* and *N. anomalus* were not taken into consideration because of the small number of the caught individuals belonging to these two species.

Table 1 shows that, in both Gilău and Bonțida populations of *Sorex araneus*, significant differences were found between the adult and subadult individuals concerning three biometric traits: body length, hindfoot length and interorbital constriction. The differences between the biometric traits of males and females of both *S. araneus* populations were significant only in the height of brain case and mandible length.

The values of biometric traits, especially those of body and condylobasal lengths and body weight of the *S. araneus* individuals from the Gilău and Bonțida zones are greater than those indicated for *S. araneus* living in other regions of the country [4, 6-8]. The biometric values of the *S. araneus* individuals caught by us are close to those recorded for the Central European *S. araneus* populations, but the maximum value of the condylobasal length is greater [10, 11]. The body and hindfoot length values are lower and the maximum value of condylobasal length is greater as compared to values registered in the East European *S. araneus* populations [2].

Table 1

Statistical parameters of the biometric traits of Sorex araneus

Biometric trait	Zone and number of the caught individuals							
	Gilău (26)				Bonțida (8)			Gilău+ Bonțida (34)
	$\bar{x}$ ±SD	Min-Max	A/SA	M/F	$\bar{x}$ ±SD	Min-Max	A/SA	$\bar{x}$ ±SD
BL	67.635 ±2.379	63.5-70.9	S	NS	62.738 ±7.965	52.1-77.9	S	66.482 ±4.711
TL	43.923 ±2.453	39.7-48.6	NS	NS	42.000 ±3.179	36.6-46.6	NS	43.471 ±2.718
HFL	12.250 ±0.432	11.4-13.2	S	NS	11.863 ±1.087	10.3-13.3	S	12.159 ±0.648
BW	7.196 ±1.014	6.0-9.5	NS	NS	5.675 ±1.322	4.4-7.7	S	4.838 ±1.257
CBL	19.831 ±0.682	18.2-21.3	NS	NS	19.025 ±0.688	18.3-20.0	S	19.641 ±0.757
BBC	8.577 ±0.657	7.5-9.9	NS	NS	8.775 ±0.900	7.4-9.8	NS	8.623 ±0.507
IOC	4.158 ±0.495	3.3-5.0	S	NS	3.988 ±0.464	3.4-4.6	S	4.188 ±0.486
HBC	5.692 ±0.373	5.0-6.2	NS	S	5.5 ±0.2	5.2-5.9	NS	5.647 ±0.347
ML	9.792 ±0.682	8.2-10.9	NS	S	9.825 ±0.557	9.0-10.5	NS	9.800 ±0.647
MH	4.515 ±0.211	4.1-4.9	S	NS	4.613 ±0.280	4.3-5.1	NS	4.538 ±0.228

The males of *Sorex minutus* have significantly greater body length than the females, whereas the differences between the other biometric traits are nonsignificant (Table 2).

The values of biometric traits of the Gilău and Bonțida populations of *S. minutus* are close to those recorded for the Suceava region [3] and are lower than those mentioned for other regions of Romania [4, 8]. Our results are similar to those described for the Central European *S. minutus* populations, excepting the condylobasal length, the minimum value of which is lower, and the mandible height, the minimum value of which is greater in the *S. minutus* individuals caught by us [10, 11]. In comparison with the *S. minutus* individuals from East Europe, those captured by us have greater limit values of the body length and much greater limit values of the condylobasal length [2, 5, 12].

Table 2

Statistical parameters of the biometric traits of Sorox minutus

Biometric trait	Zone and number of the caught individuals					
	Gilău (9)			Bonțida (2)		Gilău+ Bonțida (11)
	$\bar{x} \pm SD$	Min-Max	M/F	$\bar{x} \pm SD$	Min-Max	$\bar{x} \pm SD$
BL	52.044 ±3.661	47.5-58.1	S	53.450 ±1.768	52.2-54.7	52.300 ±3.317
TL	39.178 ±2.427	33.2-41.2	NS	39.400 ±1.273	38.5-40.3	39.218 ±2.209
HFL	10.556 ±0.456	9.9-11.2	NS	10.050 ±0.212	9.9-10.2	10.464 ±0.461
BW	3.511 ±0.744	2.7-4.5	NS	2.950 ±0.071	2.9-3.0	3.409 ±0.703
CBL	11.867 ±2.528	9.5-15.3	NS	16.650 ±0.212	16.5-16.8	12.736 ±2.977
BBC	7.278 ±0.222	7.0-7.7	NS	7.550 ±0.212	7.4-7.7	7.327 ±0.237
IOC	3.256 ±0.151	3.0-3.5	NS	3.150 ±0.071	3.1-3.2	3.236 ±0.143
HBC	4.267 ±0.180	4.0-4.5	NS	4.750 ±0.071	4.7-4.8	4.354 ±0.254
ML	5.611 ±1.358	4.4-7.8	NS	7.950 ±0.212	7.8-8.1	6.036 ±1.541
MH	3.2 ±0.1	3.0-3.3	NS	3.150 ±0.071	3.1-3.2	3.191 ±0.094

One can see from Table 3 that the differences between the adult and subadult individuals of *Crocidura leucodon* are significant in seven biometric traits (Gilău population) and in four traits (Bonțida population). In the Gilău population, the males have significantly greater body length, hindfoot length and breadth of brain case than the females, but in the Bonțida population, only two biometric traits (tail length and height of brain case) are significantly greater in the males than in the females.

Table 3

Statistical parameters of the biometric traits of Crocidura leucodon

Biometric trait	Zone and number of the caught individuals								
	Gilău (24)				Bonțida (23)				Gilău+ Bonțida (47)
	$\bar{x} \pm SD$	Min-Max	A/SA	M/F	$\bar{x} \pm SD$	Min-Max	A/SA	M/F	$\bar{x} \pm SD$
BL	64.821 ±8.467	53.8-77.8	S	S	68.609 ±7.240	56.3-81.3	S	NS	66.674 ±8.040
TL	34.754 ±3.089	28.7-39.4	NS	NS	32.922 ±3.369	27.4-40.0	NS	S	33.857 ±3.325
HFL	11.333 ±0.653	10.5-12.8	S	S	11.296 ±0.861	9.6-12.6	NS	NS	11.315 ±0.754
BW	6.229 ±2.459	2.5-11.0	S	NS	7.643 ±2.971	3.0-13.5	NS	NS	6.921 ±2.801
CBL	19.179 ±0.412	18.4-20.0	NS	NS	19.043 ±0.614	18.1-20.0	S	NS	19.113 ±0.519
BBC	9.200 ±0.389	8.7-9.9	S	S	9.078 ±0.288	8.7-9.8	NS	NS	9.140 ±0.345
IOC	4.608 ±0.259	4.2-5.0	S	NS	4.596 ±0.146	4.3-4.9	NS	NS	4.602 ±0.209
HBC	5.292 ±0.376	4.8-5.8	S	NS	5.191 ±0.241	4.8-5.7	NS	S	5.243 ±0.318
ML	9.823 ±0.324	9.3-10.6	S	NS	9.930 ±0.370	9.2-10.9	S	NS	9.878 ±0.348
MH	4.575 ±0.227	4.3-5.2	NS	NS	4.517 ±0.308	3.9-5.1	S	NS	4.547 ±0.269

The variability limits of the biometric trait values in the studied two *C. leucodon* populations are wider than those recorded for other *C. leucodon* populations in the country [4, 5, 8]. The values of body and condylobasal lengths of the *C. leucodon* individuals caught by us are greater and the variability limits of body weight are wider than those described for Central European *C. leucodon* populations [10, 11]. In the Gilău and Bonțida populations of *C. leucodon*, the body and hindfoot are shorter and the condylobasal length is greater in comparison with the East European *C. leucodon* populations [2, 12].

Significant differences were found between the adult and subadult *Crocidura suaveolens* individuals in eight biometric traits (Gilău population) and five traits (Bonțida population). The differences between the biometric traits of males and females were significant in two traits (body weight and mandible length), while no significant difference was found between the biometric traits of males and females of the Bonțida population (Table 4).

Table 4

Statistical parameters of the biometric traits of Crocidura suaveolens

Biometric trait	Zone and number of the caught individuals								
	Gilău (16)				Bonțida (17)				Gilău+ Bonțida (33)
	$\bar{x} \pm SD$	Min-Max	A/SA	M/F	$\bar{x} \pm SD$	Min-Max	A/SA	M/F	$\bar{x} \pm SD$
BL	55.844 ±3.442	50.7-64.2	S	NS	58.982 ±4.299	52.5-66.1	S	NS	57.461 ±4.163
TL	34.538 ±1.602	32.8-38.4	S	NS	32.624 ±1.591	29.6-34.5	NS	NS	33.552 ±1.847
HFL	10.569 ±0.665	10.0-11.6	S	NS	10.453 ±0.501	9.7-11.1	NS	NS	10.509 ±0.580
BW	4.188 ±0.609	3.2-4.9	S	S	3.594 ±0.766	2.7-5.1	S	NS	3.882 ±0.747
CBL	16.525 ±0.686	15.9-17.9	S	NS	16.712 ±0.598	15.2-17.1	S	NS	16.621 ±0.639
BBC	8.156 ±0.405	7.6-8.8	S	NS	7.706 ±0.590	6.3-8.2	NS	NS	7.924 ±0.551
IOC	3.813 ±0.280	3.2-4.1	NS	NS	4.076 ±0.315	3.8-4.8	S	NS	3.948 ±0.323
HBC	4.556 ±0.432	4.0-5.4	S	NS	4.871 ±0.400	4.2-5.7	NS	NS	4.718 ±0.439
ML	8.456 ±0.429	7.6-9.2	S	S	8.418 ±0.488	7.6-9.5	S	NS	8.436 ±0.453
MH	3.775 ±0.259	3.4-4.1	NS	NS	3.935 ±0.226	3.6-4.2	NS	NS	3.858 ±0.252

The biometric trait values, especially the minimum limits, of the *C. suaveolens* individuals captured by us are lower than those of the *C. suaveolens* individuals from other regions of the country [4, 6]. The *C. suaveolens* individuals from the Gilău and Bonțida zones have the minimum value of body length smaller and the maximum value of condylobasal length greater than those from Central Europe [10, 11]; they have the minimum value of hindfoot length lower and the maximum values of tail and condylobasal lengths greater in comparison with the East European *C. suaveolens* populations [2, 12].

Conclusions. 1. In the studied two hilly zones (Gilău and Bonțida) in the Someşul Mic basin, six insectivore mammal species were recorded: *Sorex araneus, S. minutus, Neomys fodiens, N. anomalus, Crocidura leucodon* and *C. suaveolens*.

2. The differences between the adult and subadult age groups were found to be significant in most of the ten biometric traits determined, but the differences between the males and females were significant only in some traits, those of the males being greater. Exceptionally, none of the biometric traits of males and females was significantly different in the *Crocidura suaveolens* population from the Bonțida zone.

3. In the Gilău and Bonțida zones, the populations of *Sorex araneus* and *S. minutus* are morphologically similar to the populations from the northern part of Romania and Central Europe, while the *Crocidura leucodon* and *C. suaveolens* populations are similar to those from different regions of Romania and Central Europe.

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AN ORIGINAL DATA ACQUISITION SYSTEM FOR MONITORING THE BIOELECTRIC ACTIVITY OF FROG HEART

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SUMMARY. - An electrical signal generated by the frog heart was for the first time photographically recorded in 1883. Since then, the recording devices and methods have been continuously improved. One of the most powerful signal recording and processing technique is the computer-based data acquisition. During the last years, part of our work was focused on designing and improving a data acquisition system (DAQS) to be used for the study of the signals generated by the self-pacing myocardium. An original four-modular DAQS was built and our recordings show that its capacities in signal acquisition and processing are comparable to those of other similar tools.

The first step towards the study of certain electrical signals generated by the heart became possible in the 1880s, after the construction of a device (*i.e.* the Lippmann capillary electrometer) capable to acquire voltage fluctuations from living structures. To record the traces for further analysis, photographic methods were used [15].

In the years that followed, parallel to the evolution of the acquiring tools, the signal storage methods have also been improved.

The transposing of the traces reflecting a bioelectric process on film or photographic paper directly from the screen of the oscilloscope [7] was for many years the single recording method and it is still used [1, 11]. The construction of memory oscilloscopes made possible the storage of the traces and their reactivation for ulterior measurements [4-6].

In the last decade, due to the explosion of the computational techniques, a new generation of data acquisition methods has been developed and they are widely used today [2, 3, 9, 14, 16].

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Combining an astonishing data transferring speed with practically unlimited storage capacities of the harddisks, the computer-based data acquisition systems (DAQS) became powerful tools for the study of biological signals all over the world.

Beside these characteristics, one of the most important facilities offered by the PC is that the information, once stored on the harddisk, can be actually used any time. Moreover, many programs may also process the data by means of sophisticated analyzing software including, for example, harmonic analysis and statistical methods.

Our first attempt to acquire electrical signals generated by isolated frog hearts was performed by means of an ECG recorder. Two active electrodes and an indifferent one were properly placed into a self made perfusion chamber filled with Ringer solution. An ECG trace, similar to the human one, appeared on the screen of the apparatus.

The next step we made was to adapt the perfusion techniques to the requirements of a direct extracellular recording. To solve the problems of the electrode attachment to a moving organ, as well as to ensure a proper tensioning of the heart during the recordings, we had to design a novel perfusion device. Firstly, the signals were recorded with the ECG apparatus. We connected then its amplifier module to a self made data acquisition board, which was able to communicate with the PC by means of a proper program. The results of that work were previously reported [12, 13]. New improvements were made, both regarding the perfusion device and the acquiring software package, thus resulting our present DAQS.

We describe here the main technical characteristics of this system and the methods we used to monitor electrical signals generated by the frog heart.

Material and methods. *Animals.* The hearts can be prepared from any frog species. The size of the animals is not important because the fitting devices from the perfusion chamber are adaptable to the heart length.

The data acquisition system. Our system consists of four main modules. These are: the perfusion module - which ensures the proper conditions for the heart activity, the signal amplifier - which increases the signal amplitude to the requested value, the data acquisition (DAQ) board - which pre-processes and transmits the data to an IBM compatible PC - the fourth component of the DAQS (see, for illustration, Fig. 1).

The perfusion module consists of a perfusion tank (1) and a perfusion device. Each reservoir (R1,R2 and R3) of the perfusion tank can contain any requested solution, one of them always being the Ringer one for the reference cardiac activity. The Ringer solution can be instantly replaced by one from the other reservoirs by manipulating their taps (2).

Three plastic tubes (3) connect the reservoirs at the perfusing head (4), where the cardiac cannula (6) will also be fitted. A displacement device (5) adapts the position of the perfusing head to the heart size, thus ensuring the organ's proper tensioning. Made from steel, this cannula also plays the role of the reference electrode and it is connected at the signal amplifier by means of an electric cable.

The active electrode (7) consists of a steel spring, with an end in form of a hook and the other one straight. It has this special design because it must accomplish three tasks. Thus, the hook end stabs the tip of the ventricle and acquires the electrical activity of the tissue. The spring middle ensures a counter-force which opposes to the ventricular contraction and brings the heart to its initial length in the relaxing period. The straight end of the electrode serves for fixing it into the perfusion chamber by means of a pincers (8) connected to the amplifier.

A transparent Plexiglas lid covers the perfusion chamber (9), thus ensuring a damp atmosphere around the heart.

To amplify the electrical signals acquired by means of the devices described above, we used the amplifier of a Cardior KTD2 apparatus. To do that, both electrodes were connected to the original cables of its ECG module. The exit of the amplifier was further linked to the entrance of the DAQ board. This connection between the ECG apparatus and the DAQ board allows not only the transmission of the amplified signals to the PC, but also a visual control of the electrical activity of the heart on the ECG screen. According to the stability of the signals watched on this screen, one could establish the right moment to start the PC monitoring.

The DAQ board is an electronic device which plays two crucial roles in the acquisition process: those of a digital voltmeter and a transmission element.

The IBM compatible PC must have the minimum characteristics to be able to rule Windows 95, which is necessary for the acquiring LabView 4.1-based software package.

The heart preparation begins with the isolation procedures.

The frog is killed by pithing and the heart is discovered. The hook end of the active electrode is implanted into the ventricular mass. The tip of the cannula is introduced into the base of the venous sinus, which is then tightly bound around the metallic pipe.

After the removal, the heart is placed into the perfusion chamber. To avoid the formation of air bubbles into the heart, the flowing of the Ringer solution must be started before fitting the cannula at the perfusion head. The straight end of the electrode is then fixed into its pincers from the perfusion device.

By manipulating its displacement device, the perfusion head is positioned according to the length of the heart. Thus, at the end of these operations, the organ must stay in horizontal position, gently tensioned by the spring zone of the active electrode (see Fig.1).

The two electrodes are then connected to the cables of the amplifier module. Starting from this moment, the self-pacing bioelectrical activity of the ventricular tissue may be watched on the screen of the ECG.

After the stabilization of heart beating, the PC monitoring may start by triggering the acquisition program.

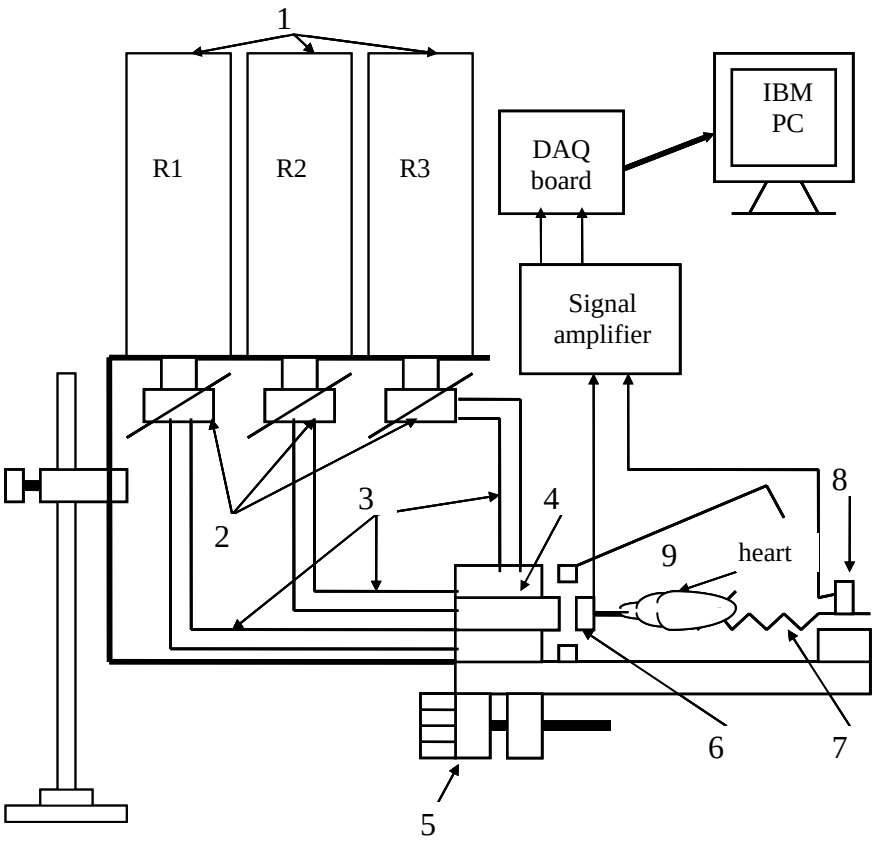

Fig. 1. *Schematic diagram of the data acquisition system.*
1 - Perfusing reservoirs. 2 – Taps. 3 - Plastic tubes. 4 - Perfusing head.
5 - Displacement device. 6 - Perfusion cannula/reference electrode.
7 - Active electrode. 8 – Pincers. 9 - Perfusion chamber.

Results and discussion. *The perfusion device.* In chronological order, the first result of our work was the realization of an original perfusion device.

In comparison to the classical Fühner model, which was the only one used in our laboratories, the new one has important improvements.

Thus, the perfusion can be successively performed from three different reservoirs which can be filled with three different solutions. As we have already mentioned, one of them must always contain the Ringer solution under normal conditions of temperature and pressure, for monitoring the reference bioelectrical cardiac functions. If the effects of any disturbing substance have to be tested, this can be solved in the Ringer solution from one of the other two reservoirs. By simply stopping the initial Ringer current and starting at the same time that containing the tested factor, the second solution will pass through the perfusing head, instantly replacing into the cannula the normal Ringer one.

This perfusing method has two main advantages.

First, the concentration of the disturbing substance is a strictly controlled factor, whereas by adding drops in the cardiac cannula the final concentration of the solution which enters the heart remains unknown.

Second, the technical characteristics of the perfusion device allow an almost instant replacement of a solution with another one from a second reservoir, with a very short transition period (see Fig. 4).

Moreover, we have thought of a third reservoir to be used for serial perfusions, such as a pretreatment with a blocker followed by a perfusion with its specific antagonist. For example, one could test the effect of epinephrine after the heart was previously perfused with its β-blocker, propranolol. Thus, the reservoir R1 (see Fig. 1) would contain normal Ringer solution, R2 - Ringer with propranolol and R3 - Ringer with epinephrine, the perfusing order being R1, R2, R3.

Another new element of our system is the mobile perfusing head. Its main role is to fit the cardiac cannula into the perfusion chamber, at the same time supplying it with the solutions which flow from the reservoirs. Taking into consideration that the hearts may have different sizes, we had to think of a technical formula in order to ensure the adaptability of this attachment device to the heart length. Thus, instead of fixing the perfusing head into the perfusion chamber, we have connected it to a thread based displacement device which ensures its foreword and backward movements, as the heart length would require.

When one has to record signals generated by a moving cell, tissue or organ, a major problem is the attachment of the electrodes so that the motion artifacts should be reduced or eliminated. Different methods have been established to solve this demand and we mention here the using of suction [8-10] or the calcium removal in order to inhibit the contractions [8].

Part of our solution to this problem is based on using a metallic cannula instead of the classical glass one. Anyhow, this must be strongly bound to the sinus wall, so that it can also serve as a reference electrode.

The other half of the problem, that of a proper and stable contact between the ventricular tissue and the active electrode, was solved by the particular design of this electrode (see the description above and Fig. 1). As we have previously presented, during the isolation procedures, the hook end of this electrode is implanted into the ventricular mass. After the isolation, the heart with the electrode hanging on its tip is attached to the perfusing head by means of the cardiac cannula. The second attachment point is represented by the pincers, where the straight end of the electrode is fixed. The next operation is to move backward the perfusing head till the spring middle of the electrode remains gentle stretched even during the relaxing period. This state of permanent tension ensures the indispensable tissue-electrode contact along the whole cardiac cycle. The elastic force accumulated by the spring during the contraction also serves for bringing the heart back to its initial length.

The DAQ board. We have mentioned before that this electronic device was designed to ensure both the digitization of the signals and their transmission to the PC.

As a digital voltmeter, the DAQ board receives the amplified signals and converts them into numerical values. This task is achieved by means of a 10-byte analog-to-digital converter, which is included into a 80C 552 Philips microcontroller.

As a transmission element, the DAQ board sends in real time the converted data to the PC by means of its serial interface. The communication protocol was by software established at 57600 kbytes/s, thus being transmitted 150 values per second.

The software package. The data acquisition and processing are performed by means of a self-designed, LabView-based software package which consists of two parts.

One of the programs receives the value strings sent by the serial interface and transforms them into analog graphic representations. Thus, the signals rule on the monitor almost in real time. Parallel to this processing task, the program also saves the data into dedicated files on the harddisk.

The software package also contains a program designed to display the recordings made by the first one. Thus, an evolutionary bioelectrical process determined, for example, by changing the Ringer solution with one containing a disturbing factor, may be seen from the beginning till the end. Due to the facilities of this program, any segment from such a recording may be zoomed and analyzed.

Recorded signals. To illustrate the performances of our DAQS, we shall further present some of the recordings obtained by using it.

As we have mentioned above, from a whole recording, which can be very condensed if the experiment lasted for a long period (minutes or even hours), one could choose and magnify only a small segment.

In Fig. 2, three steps of magnification - a, b and c - can be seen.

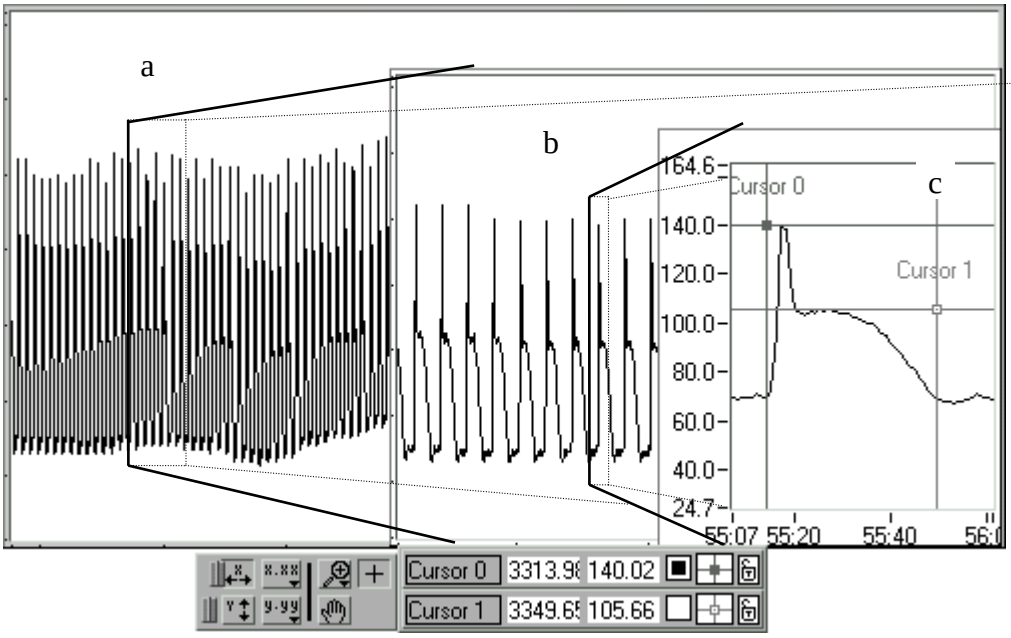

Fig. 2. *Three magnification steps of a bioelectric recording acquired from frog ventricular myocardium.*

In the cell c, a single zoomed wave is presented. Cursors 0 and 1 illustrate how the signal parameters can be measured both in amplitude and in duration. The numbers from the palette below the picture represent the units of the measured points, which are translated into absolute values of amplitude and time after the calibration.

The c cell shows a single bioelectrical wave recorded from the ventricular myocardium. According to Weidmann [15], this would represent a so called "injury action potential", which was for the first time photographically recorded in 1883.

More recently, in 1992, Neunlist and Zou [10], using an original optical method, obtained an integrated transmembrane potential recorded from frog ventricular epicardium. Although this signal, being acquired from a volume of tissue approximately 200 µm in diameter, may differ from that of individual cells [8], it expresses the main characteristics of a well known cardiac action potential. Thus, the authors describe a fast depolarization period with a rise time that can range from less then 1 ms to 20 ms, a first repolarization period followed by the depolarization plateau and, finally, the late repolarization phase. The maximal amplitude was reported to be of about 100 - 120 mV and the total duration of this integrated action potential ranged from 700 to 900 ms.

The individual signals acquired in our laboratory by means of the previously described DAQS also seem to present the characteristic steps of an action potential.

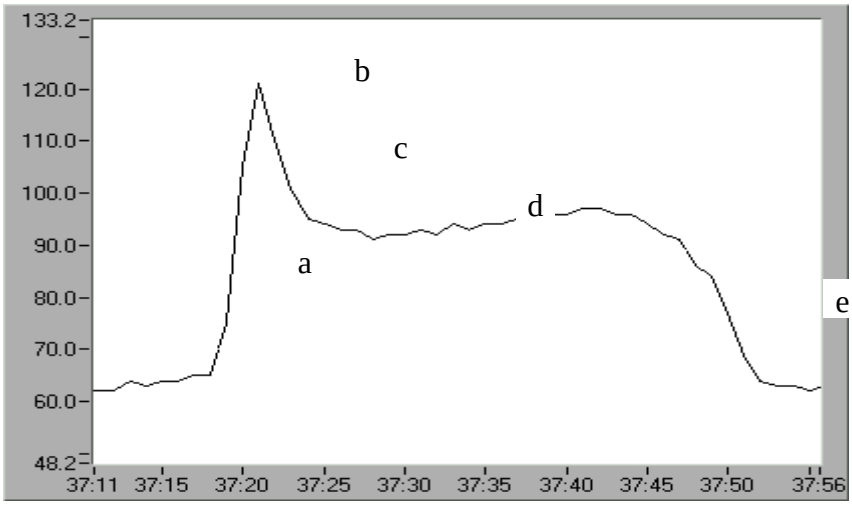

Fig. 3. *Individual signal acquired from self-pacing frog ventricular myocardium.* a; b; c; d; e - Specific phases in the development of the signal.

As can be seen in Fig. 3, the signal starts with a rapid up-stroke (phase a) that was found to last for 25 - 30 ms. The maximal spike amplitude (point b) range from 25 to 30 mV. After a rapid decrease of the amplitude (phase c), the subsequent characteristic plateau follows (phase d). Finally, the potential returns to its base-line value by means of a prolonged decreasing period (phase e).

As a characteristic of their recording devices, Neunlist and Zou [10] reported a peak-to-peak noise level of 2-7% from the action potential amplitude.

In our recordings, the maximum level of the peak-to-peak noise was 5% of the signal amplitude.

As we have already mentioned, a difficult recording problem is the fixing of the electrodes into/on the tissue when the bioelectrical signals are acquired from a moving organ.

The authors cited above have recorded their signals by means of an optical fiber which was positioned on the surface of the ventricle. When this fiber was not properly fixed by suction, a major motion artifact appeared some 50 - 100 ms after the upstroke of the action potential.

Although our signal is always followed by the ventricular contraction 90 - 100 ms after the spike, no motion artifact appears (see Fig. 3). Since the motion artifacts were totally eliminated, we may conclude that our technical solution related to the attachment of the electrodes is a valuable one.

Finally, we illustrate the capacities of our DAQS to reflect the effect of a disturbing factor on the cardiac bioelectric activity. As ca be seen in Fig. 4, a change in the signal shape, as well as in the frequency is developing next to the vertical bar. This sign marks the moment when the normal Ringer solution was replaced with the one containing the tested substance. Thus, the picture above shows the transition from a normal self-pacing ventricular activity to that determined by the administration of methanol solved in Ringer solution. Without discussing here the physiological mechanism of this effect, we only mention that our devices and methods seem to surprise well enough such a process during its development.

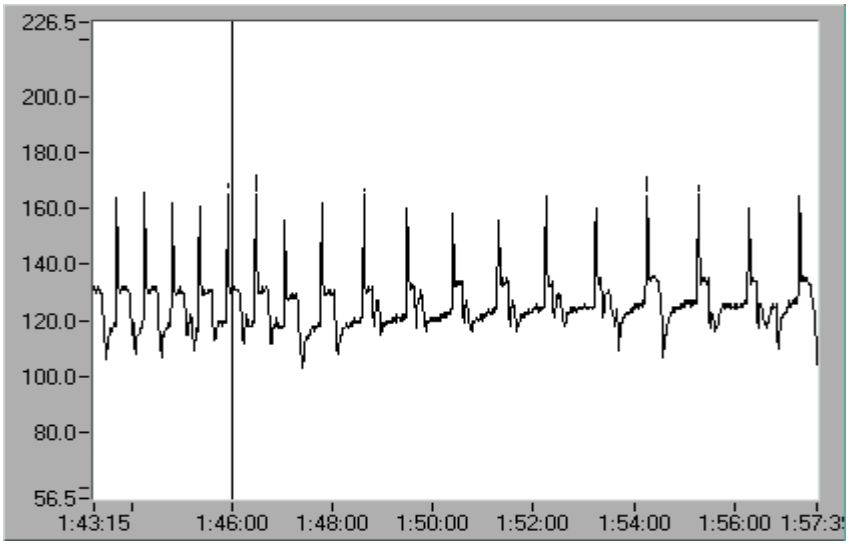

Fig. 4. *Effect of methanol on the bioelectrical activity of the frog ventricle.* The vertical bar marks the substance administration.

As a **conclusion** of the present work, we believe that our DAQS may be considered a valuable tool for the study of the frog heart bioelectrical activity either alone or in relation to other cardiac parameters.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI, BIOLOGIA, XLV, 1, 2000

CHARACTERISATION OF *NICOTIANA PLUMBAGINIFOLIA* TRANSFORMANTS, CONTAINING *METC* GENE FROM *ESCHERICHIA COLI*. I. ANALYSIS OF NUCLEIC ACIDS

GABRIELA ISPAS[*], LENUȚA RÁKOSY-TICAN[x], MICHAEL JACOBS[**] and VALÉRIE FRANKARD[xx]

SUMMARY. - *Nicotiana plumbaginifolia* plants, transformed with *metC* gene from *Escherichia coli* have been characterised at nucleic acid level. The transformed nature of the analysed plants has been proved.

Amino acid biosynthesis is an essential process for plant growth and development. The essential amino acids: lysine, threonine, methionine and isoleucine derive all from aspartate, via a branched biosynthetic pathway.The main sulphur- containing amino acids in plants and animals are methionine and cysteine.

Methionine is synthesised in plants and microorganisms *de novo*, from cysteine and O-phospho-homoserine (plants) and, respectively, from cysteine and O-succinyl-homoserine (bacteria). In contrast, in animals methionine represents the essential sulphur-containing amino acid and is the precursor of cysteine.

The steps of methionine biosynthesis in plants, downstream from cysteine are presented in Fig. 1. The precursor O-phospho-homoserine represents the crossing-point between aspartate, methionine and threonine pathway. *Cystathionine gamma synthase* (CS) may use either cysteine or sulphide as a sulphur supplier. The enzyme *cystathionine gamma synthase* (CS) catalyses the first committed step that consists in a transsulphurylation reaction between cysteine and O-phospho-homoserine to form the intermediate cystathionine and is regarded as a major regulatory point in the pathway [6]. Cystathionine cleavage by *cystathionine beta lyase* (Cbl) yields ammonia, pyruvate and homocysteine. In the next step, homocysteine is methylated by *methionine synthase* (MS) leading to methionine production. Most of the enzymes involved in methionine biosynthesis are localised in chloroplast. Only *methionine synthase* appears to be localised in the cytoplasm [4]. The localisation of *cystathionine beta lyase* enzyme is still a debate.

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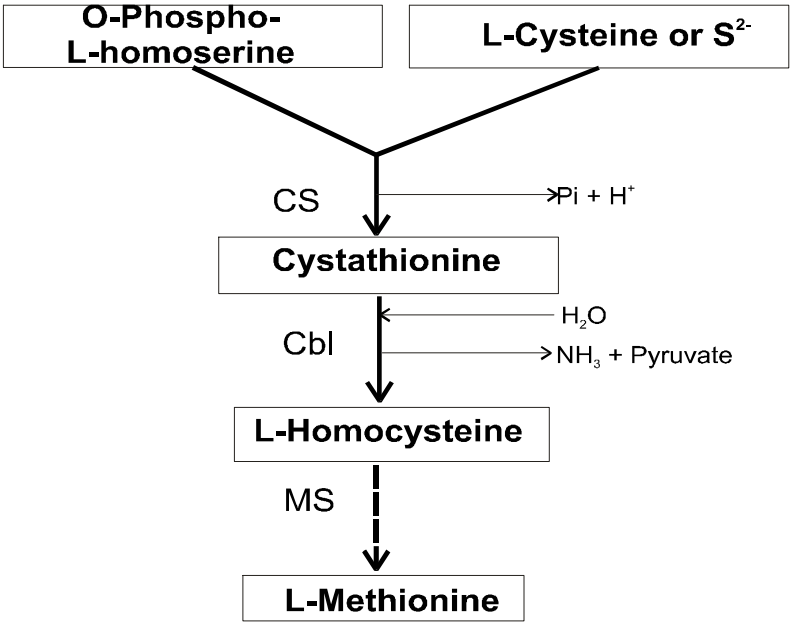

Fig. 1. *Branched pathway of methionine synthesis in plants.*

Enzyme assays performed on chloroplast-enriched fractions show that the majority of the Cbl activity, if not all, is localised in the plastid [2]. An attempt to identify the *cystathionine beta lyase* localisation in plant cells is the experiment initiated in the Laboratory of Plant Genetics (Free University Brussels).

The strategy of this work is based on the following idea: if a Met-auxotrophic mutation at the *cbl* level in plants may be complemented by a TP – *metC* construct, implying a chloroplastic localisation for MetC (cystathionine beta lyase enzyme in bacteria), the Cbl enzyme localised in chloroplast is necessary and sufficient for methionine biosynthesis.

The strategy followed in our experiments is presented in Fig. 2. The goal of our work is the characterisation of *metC* transformants, obtained in the Laboratory of Plant Genetics (Free University Brussels). In order to prove the successful complementation of the auxotrophic mutation, two transformants (T_1 and T_4) and the wild type (P_2) *Nicotiana plumbaginifolia* have been analysed at biochemical and nucleic acid level.

The work presented in this paper points on two main problems: 1. the presence of *metC* insertion(s) in analysed plants and 2. the *metC* transcription level in T_1 and T_4.

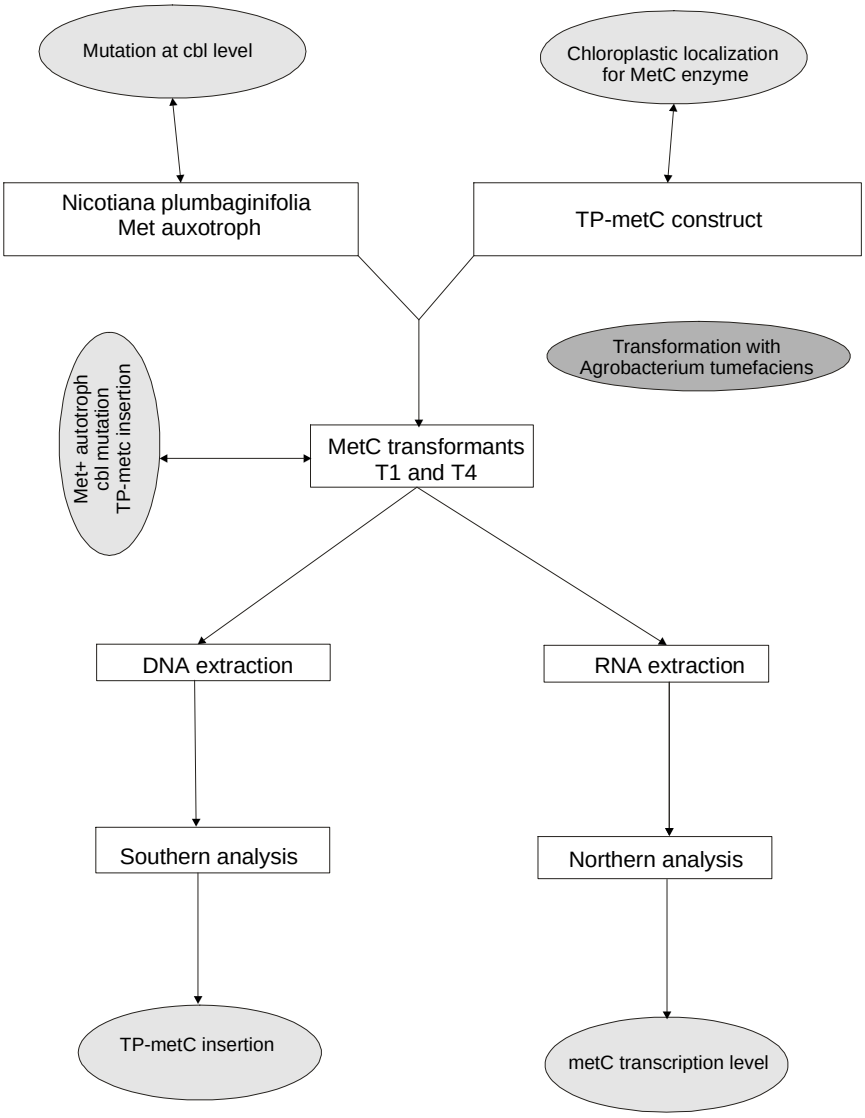

Fig. 2. Step by step strategy.

Materials and methods. *Plant material. Nicotiana plumbaginifolia* plants, auxotrophic for methionine, carrying a mutation at *cbl* level [5], have been transformed using *Agrobacterium tumefaciens* technique.

A pBIN vector has been used, containing: 1. the *metC* gene from *Escherichia coli* (coding for cystathionine beta lyase enzyme) under the control of a CaMV 35S promoter; 2. the *nptII* gene (neomycin phosphotransferase) for the selection of transformants, based on their resistance on kanamycin-containing medium; 3. the transit peptide (TP) corresponding to the spinach Rubisco small subunit, fused in frame with *metC* gene.

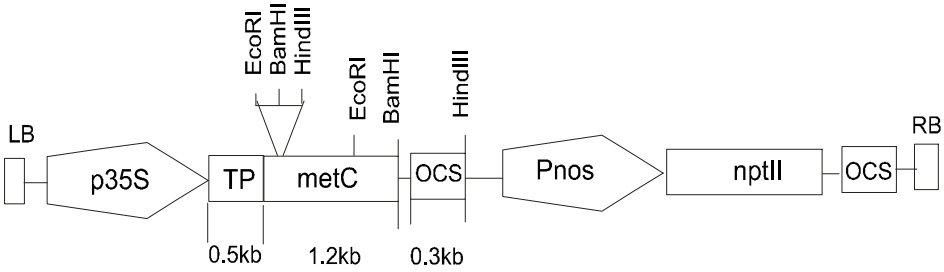

Fig.3. *The metC construct.*
LB - Left border. p35S - CaMV promoter. TP - Transit peptide. metC - Cystathionine beta lyase gene from *Escherichia coli*. OCS - Octopine synthase terminator. Pnos - Nopaline synthase promoter. nptII - Neomycin phosphotransferase gene. RB - Right border.

The recovered *metC* transformants (T_1 and T_4) are able to survive on medium without methionine. This is a direct proof that the exogenous *metC* gene complements the cbl mutation in auxotrophic *N. plumbaginifolia* mutants, due to the fact that the MetC enzyme targeted in chloroplast is sufficient to reinstall the autotrophy for methionine.

DNA and RNA analyses. *Nicotiana plumbaginifolia* genomic DNA was extracted from leaves using the technique described by Dellaporta *et al.* [1]. The DNA was digested using three different enzymes: EcoRI, BamHI and HindIII. On the agarose gel (0.8%), 10 μg DNA was loaded on each slot.The total RNA was isolated and RNA electrophoresis was performed according to Goldberg [3]. Standard Southern and Northern blot techniques have been used, with 0.4 N NaOH as a transferring agent. For DNA and RNA transfer, positively charged nylon membrane, purchased from Boehringer, has been used. The prehybridisation and hybridisation were performed in Church buffer (25% 1 M sodium phosphate buffer, 5% 5 M sodium chloride; 0.2% 0.5 M EDTA, 7% SDS).

The BamHI fragment of the *metC* gene (Fig.3) was used as a probe for Southern and Northern analyses. The BamHI fragment of the *metC* (1.2 kb) was separated on 0.8% agarose gel, electroeluted and further labelled with α-^{32}P-dCTP, 3000 Ci/mmol, using Rediprime Kit from Amersham.

Results and discussion. *Southern analysis.* For the analysed transformants (T_1 and T_4) three radioactive signals, corresponding to the three different restriction enzymes used, EcoRI, BamHI and HindIII, were obtained, at the expected locations: 1.2 kb for BamHI, 0.9 kb for EcoRI and 1.5 kb for HindIII (Fig.4).

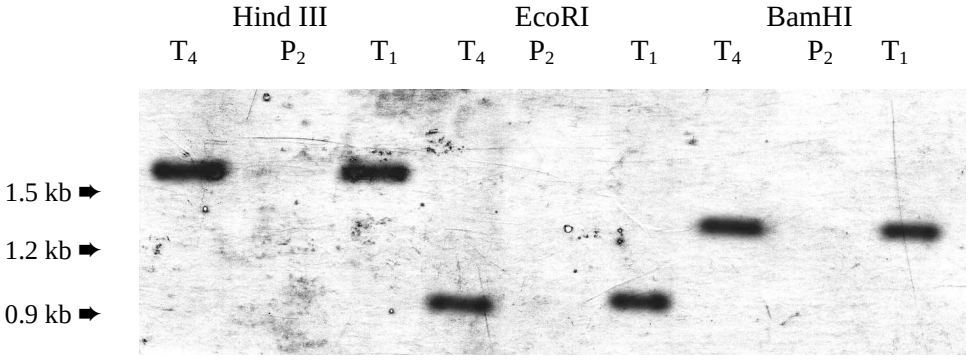

Fig. 4. *Southern analysis using* metC *probe.*

The restriction enzymes used for DNA analysis cut the BamHI fragment of the *metC* gene at the following positions:

Restriction enzyme	Restriction site in BamHI fragment (1.2 kb)
BamHI	0/1187 bp
EcoRI	891 bp
HindIII	12 bp

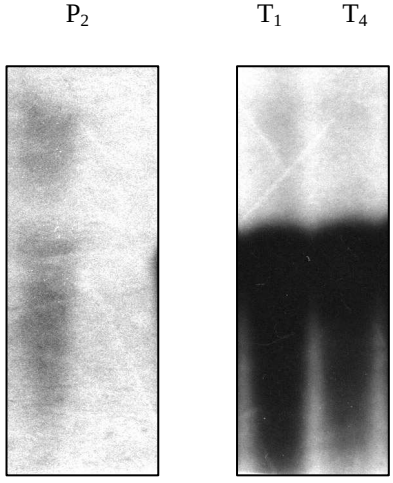

Fig. 5. *Northern analysis using* met C *probe.*

Corresponding to the cutting pattern of EcoRI and BamHI in the BamHI probe, the radioactive probe will hybridise with the two corresponding plant genomic DNA fragments : 0.9 kb for EcoRI and 1.2 kb for BamHI.

In the case of HindIII the first restriction site is located in the *metC* gene at position 12 bp, and the second restriction site is located after the *metC* terminator. The HindIII fragment will comprise the *metC* gene and *OCS* terminator, having a size of 1.5 kb. The corresponding radioactive signal is recovered after the hybridisation with BamHI probe at the expected location: 1.5 kb.

Northern analysis. A very strong signal was obtained for the analysed transformants, while no signal was present for the wild type (Fig. 5).

The transcription rate for *metC* gene in the transformants is imposed by the 35S promoter. This high inducible promoter allows a high transcription rate of the *metC* gene. As a fact, a large amount of mRNA coding for MetC protein will be produced and, consequently, the radioactive signal will be very strong in the Northern analysis, even after a short exposure time.

Due to the small homology between the *metC* gene and the *cbl* gene, the radioactive *metC* probe will not hybridise with the endogenous Cbl-mRNA from wild type. No signal for P_2 was obtained in the Northern analysis. Nevertheless, a small amount of Cbl-mRNA will be produced in wild type, under the control of the *cbl* natural promoter.

Conclusions. 1. The analysed T_1 and T_4 plants contain *metC* gene from *Escherichia coli*. The transcription rate of this gene in the transformants is very high, due to the presence of the 35S promoter.

2. The absence of hybridisation with the radioactive *metC* probe in the case of the wild type is most likely due to the fact that the homology between the *metC* bacterial gene and the endogenous *cbl* gene is not sufficient to allow the hybridisation under the established conditions.

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CERCETĂRI ENZIMOLOGICE ASUPRA NĂMOLURILOR DIN LACURILE SALINE DE LA BAZNA ŞI BLAJ

MIHAIL DRĂGAN-BULARDA[*], IOAN-CĂLIN POPTELECAN[*], VASILE MUNTEAN[**], RADU CRIŞAN[**] şi DANIELA PAŞCA[**]

SUMMARY. – **Enzymological Research on Muds from the Salt Lakes in Bazna and Blaj**. Seasonal enzymological analyses were performed on muds from two salt lakes in Bazna (Sibiu county) and Blaj (Alba county). Four enzymatic activities (phosphatase, catalase, actual and potential dehydrogenase), as well as nonenzymatic catalytic H_2O_2-splitting capacity were measured quantitatively. The studied enzymatic and nonenzymatic catalytic activities were high through all seasons, with irregular seasonal variations. The enzymatic indicators of mud quality were calculated. The mud from the salt lake in Bazna had a higher enzymatic potential than that from the salt lake in Blaj, according to the values of the enzymatic indicator of mud quality. Other four enzymatic activities (maltase, cellobiase, invertase and lactase) were determined qualitatively. The results of the qualitative analyses confirmed those of the quantitative ones in respect of the higher enzymatic potential of mud from the salt lake in Bazna.

În țara noastră s-au efectuat numeroase cercetări enzimologice asupra sedimentelor lacurilor saline, în special asupra acelora folosite în balneoterapie [1-3, 5, 6, 8, 9, 11-16]. Lucrarea de față își propune să lărgească sfera cercetărilor întreprinse de autorii citați, prin studierea unui lac deja cunoscut pentru calitățile terapeutice ale nămolului său (Bazna) şi a unuia încă neintrat în circuitul balnear (Balta Sărată de la Blaj).

Sedimentul lacului salin de la Bazna este folosit de multă vreme ca nămol terapeutic, pentru tratarea unor afecțiuni reumatismale degenerative, inflamatorii, post-traumatice, neurologice periferice, ginecologice etc. Nămolul lacului Balta Sărată de lângă Blaj nu este exploatat într-un mod organizat în balneoterapie, dar calitățile lui curative sunt atestate de localnicii care în sezonul cald practică împachetări cu nămol, cu efecte benefice asupra stării lor de sănătate.

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Material şi metode. Asupra nămolurilor din lacurile de la Bazna (jud. Sibiu) şi Blaj (jud. Alba) au fost efectuate cercetări enzimologice cantitative şi calitative sezoniere în perioada 1996-1997. Din lacul de la Bazna au fost prelevate şi analizate probe de nămol din trei puncte: nord, centru (de unde se extrage şi nămolul pentru tratament) şi sud. Din lacul Balta Sărată de la Blaj eşantioanele au fost prelevate din două puncte: nord şi sud.

Au fost determinate cantitativ următoarele activităţi enzimatice şi catalitice neenzimatice: activitatea fosfatazică (metoda Krámer şi Erdei [10]); scindarea enzimatică (activitatea catalazică) şi neenzimatică a H_2O_2 (metoda Kappen [7]); reducerea clorurii de 2,3,5-trifeniltetrazoliu (TTC) în probe neautoclavate fără adaos de glucoză (activitatea dehidrogenazică actuală) sau cu adaos de glucoză (activitatea dehidrogenazică potenţială) (metoda Casida şi colab. [4]). Activitatea fosfatazică se exprimă în mg fenol/2,5 g nămol substanţă uscată; capacitatea de scindare a H_2O_2 se exprimă în mg H_2O_2 descompusă/1,5 g nămol substanţă uscată; activitatea dehidrogenazică se exprimă în mg formazan/0,5 mg nămol substanţă uscată.

Analizele enzimologice calitatitve au cuprins activităţile maltazică, celobiazică, zaharazică şi lactazică. Determinarea acestor activităţi enzimatice s-a efectuat prin cromatografie pe hârtie, tehnica circulară (Wright şi colab. [17]). Intensitatea activităţilor enzimatice stabilită pe baza spoturilor de culoare date de monozaharidele formate în urma hidrolizei substraturilor enzimatice a fost marcată cu semne de +.

Rezultate. Toate cele cinci activităţi determinate cantitativ au avut valori decelabile în toate sezoanele şi la toate punctele studiate. Excepţie face activitatea dehidrogenazică actuală, care nu a putut fi evidenţiată în punctele nordice de prelevare din ambele lacuri, în sezoanele de primăvară, toamnă şi iarnă, respectiv activitatea dehidrogenazică potenţială, fără valori decelabile în partea de nord a lacului Balta Sărată de la Blaj, primăvara şi toamna (Tabelul 1). Valoarea maximă a activităţii dehidrogenazice actuale este consemnată vara în partea de sud a lacului Balta Sărată (8,822 mg formazan/0,5 g nămol substanţă uscată). Valoarea maximă a activităţii dehidrogenazice potenţiale este înregistrată primăvara, în centrul lacului de la Bazna. Se poate afirma, deci, că în sedimentele ambelor lacuri studiate există o activitatea dehidrogenazică a cărei intensitate depinde mai puţin de adaosul unor surse suplimentare de carbon, probabil datorită prezenţei în aceste nămoluri a unei cantităţi suficiente de substanţe organice, care asigură o dezvoltare bună a microorganismelor a căror activitate respiratorie este măsurată prin testul reducerii TTC.

Activitatea fosfatazică înregistrează atât valoarea maximă (4,068 mg fenol/ 2,5 g nămol substanţă uscată – toamna) cât şi cea minină (0,111 mg fenol/ 2,5 g nămol substanţă uscată – iarna) în partea de sud a lacului de la Bazna. Desigur, valoarea maximă înregistrată toamna este datorată acumulării în sediment a resturilor organice, la sfârşitul perioadei de vegetaţie.

Tabel 1

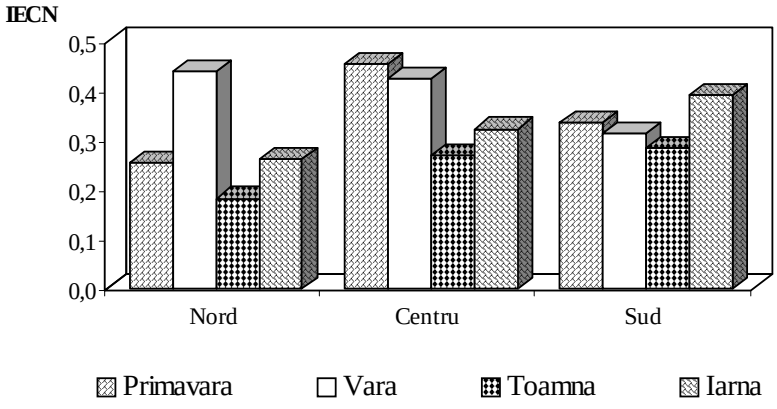
Rezultatele analizelor activităților enzimatice și catalitice neenzimatice cantitative

Lacul	Sezonul	Locul prelevării	Activitatea fosfatazică (mg fenol/2,5 g nămol s.u.)	Activitatea catalitică (mg H_2O_2 descompusă/1,5 g nămol s.u.)		Activitatea dehidrogenazică (mg formazan/0,5 g nămol s.u.)	
				Enzimatică	Neenzimatică	Actuală	Potențială
Bazna	Primăvara	Nord	1,535	28,591	30,306	1,000	1,954
		Centru	3,916	24,341	38,338	6,098	7,871
		Sud	1,430	34,190	23,393	3,455	5,290
	Vara	Nord	0,511	21,195	70,653	4,064	4,550
		Centru	0,186	12,792	66,654	4,951	5,597
		Sud	1,102	26,392	38,573	2,774	3,098
	Toamna	Nord	1,776	21,611	27,013	0	0,239
		Centru	2,750	28,970	37,662	0,226	1,174
		Sud	4,068	27,932	29,641	1,745	1,846
	Iarna	Nord	0,429	24,289	52,725	0,000	0,042
		Centru	3,098	18,964	45,304	2,245	3,068
		Sud	0,111	43,01	42,258	3,009	3,981
Blaj	Primăvara	Nord	2,295	28,188	39,796	0	0
		Sud	1,609	8,093	20,232	8,301	4,597
	Vara	Nord	3,386	26,889	57,853	4,747	6,165
		Sud	3,119	35,718	44,43	8,822	6,717
	Toamna	Nord	0,256	36,686	40,51	0	0
		Sud	1,213	25,694	30,721	4,622	3,373
	Iarna	Nord	2,617	17,134	33,163	0	0,196
		Sud	1,081	19,037	33,459	7,655	2,008

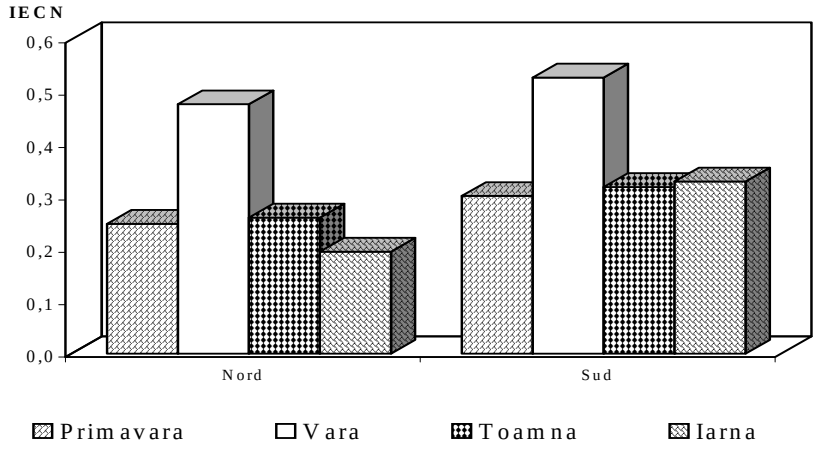
Intensitatea minimă a capacității de scindare a H_2O_2 este consemnată primăvara, în partea de sud a lacului Balta Sărată de la Blaj (8,093 mg H_2O_2 descompusă/1,5 g nămol substanță uscată – activitatea catalazică, respectiv 20,232 mg H_2O_2 descompusă/1,5 g nămol substanță uscată – scindarea neenzimatică). Valorile maxime ale acestor activități sunt înregistrate iarna în partea de sud a lacului de la Bazna (43,010 mg H_2O_2 descompusă/1,5 g nămol substanță uscată – activitatea catalazică), respectiv vara, în partea de nord a aceluiaşi lac (70,653 mg H_2O_2 descompusă/1,5 g nămol substanță uscată – scindarea neenzimatică). Aşadar, pe lângă o activitatea catalazică remarcabilă, notăm o şi mai intensă activitate de scindare neenzimatică a H_2O_2.

Pe baza valorilor absolute ale activităților enzimatice şi catalitice neenzimatice s-a calculat indicatorul enzimatic al calității nămolului (IECN), care rezultă din împărțirea la numărul activităților studiate a sumei raporturilor dintre valorile reale şi cele teoretice maxime [12].

În Fig. 1 şi 2 sunt reprezentate grafic valorile indicatorilor enzimatici sezonieri ai calității nămolurilor din fiecare punct de prelevare din cele două lacuri. Se poate observa că valoarea cea mai ridicată a potențialului enzimatic înregistrat sezonier în cele trei puncte de prelevare din lacul Bazna se întâlneşte primăvara, în zona centrală a lacului (IECN = 0,453). Valoarea cea mai scăzută a acestui potențial este consemnată toamna, în partea de nord a lacului (IECN = 0,182). Surprinzătoare este evoluția potențialului enzimatic în partea de sud a lacului, cu valori descrescătoare ale IECN dinspre primăvară spre toamnă, cu un maxim consemnat iarna.



F i g. 1. *Indicatorii enzimatici sezonieri ai calit¾ßii n¾molului din cele trei puncte de prelevare din lacul de la Bazna*.

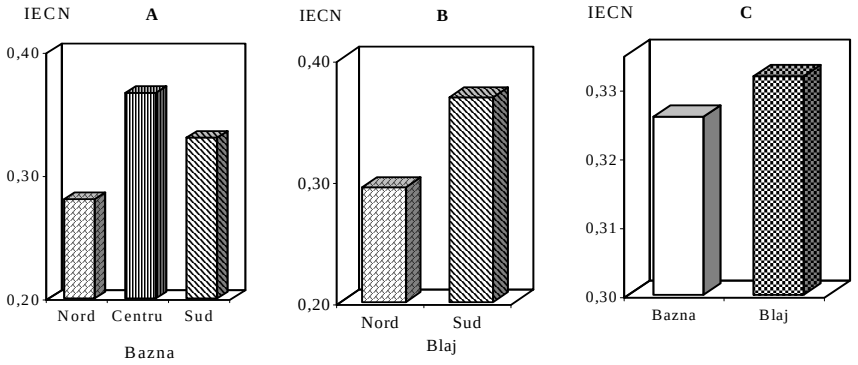


F i g. 2. *Indicatorii enzimatici sezonieri ai calit¾ßii n¾molului din cele dou¾ puncte de prelevare din lacul de la Blaj*.

În cazul lacului Balta Sărată de la Blaj, nămolul din partea de sud are un potențial enzimatic superior celui al nămolului din partea de nord. Valorile maxime ale IECN sunt înregistrate vara (0,527 – sud, respectiv 0,476 – nord). În celelalte sezoane indicatorii enzimatici ai calității nămolului depăşesc valoarea de 0,300 în sud, iar în nord se situează în jurul valorii de 0,200.

În Fig. 3 sunt reprezentate valorile indicatorilor enzimatici anuali ai calității nămolurilor din fiecare punct de prelevare, respectiv valorile indicatorilor enzimatici globali pentru cele două lacuri studiate. În lacul de la Bazna potențialul enzimatic cel mai remarcabil este consemnat în zona centrală (IECN = 0,367), de unde se şi extrage nămolul folosit pentru tratamente în stațiune. Potențialul enzimatic cel mai scăzut se înregistrează în partea de nord a lacului (IECN = 0,281), fapt explicabil şi prin impactul semnificativ al factorului antropic, în această parte a lacului deversându-se intermitent nămolul recuperat de pe pacienți, în urma tratamentelor aplicate în stațiune. Sedimentul lacului Balta Sărată de la Blaj are valori ale IECN mai apropiate (0,369 în sud, respectiv 0,295 în nord).

Calculați pe baza valorilor medii globale ale activităților enzimatice şi catalitice neenzimatice determinate în fiecare punct de prelevare din cele două lacuri, în fiecare sezon, indicatorii enzimatici globali ai calității nămolurilor din cele două lacuri (Fig. 3, C) caracterizează la modul cel mai cuprinzător potențialul enzimatic al sedimentelor lacurilor studiate. Pe această bază se poate afirma că potențialul enzimatic al nămolului din lacul Balta Sărată de la Blaj (IECN = 0,332) este superior celui al nămolului din lacul de la Bazna (IECN = 0,326), dar diferența este destul de mică.



F i g. 3. *Indicatorii enzimatici ai calit¾ßii n¾molului din cele dou¾ lacuri de la Bazna ¿i Blaj studiate*.
A ¿i B – IECN anuali pentru fiecare punct de prelevare. C – IECN globali.

Într-o lucrare citată anterior [12] s-a făcut o clasificare a 56 de lacuri saline din România, pe baza valorilor indicatorilor enzimatici ai calității sedimentelor lor. Deşi clasificarea s-a făcut în urma determinării a 7 activități enzimatice şi catalitice neenzimatice (față de cele 5 studiate în cazul de față s-a mai analizat capacitatea de reducere neenzimatică a TTC în amestecuri de reacție cu sau fără adaos de glucoză), modalitatea de calculare a indicatorilor permite raportarea la această clasificare. În acest sistem, lacul Balta Sărată de la Blaj s-ar situa pe locul 34, având aceeaşi valoare a IECN cu lacul Balta Albă, iar lacul de la Bazna pe poziția imediat următoare, înaintea lacului Nou Format de la Ocna Sibiului.

În ceea ce priveşte activitatea enzimelor determinate calitativ, rezultatele obținute sunt prezentate în Tabelul 2. Se poate constata că toate cele 4 oligaze determinate au putut fi evidențiate în nămolul celor două lacuri. Şi de această dată nămolul lacului Balta Sărată de la Blaj este mai activ pe plan enzimologic, comparativ cu cel din lacul de la Bazna, trei din cele patru activități enzimatice evidențiate fiind mai intense în primul.

Tabel 2

Rezultatele analizelor enzimatice calitative

Lacul	Locul prelevării	Activitatea enzimatică determinată			
		Maltazică	Celobiazică	Zaharazică	Lactazică
Bazna	Centru	++	+	+	+
Blaj	Sud	+++	++	++	+

Concluzii. 1. Este consemnată existența unor activități enzimatice destul de intense în sedimentele celor două lacuri studiate, care variază în funcție de anotimp și de locul de prelevare. De asemenea, se înregistrează un nivel ridicat al activității catalitice neenzimatice de scindare a H_2O_2.

2. Pe baza valorilor indicatorilor enzimatici globali ai calității nămolurilor din cele două lacuri studiate se poate afirma că nămolul din lacul Balta Sărată de la Blaj (IECN = 0,332) este superior celui din lacul Bazna (IECN = 0,326). Observația are o importanță deosebită, luând în considerare faptul că nămolul din lacul de la Blaj nu este exploatat științific în balneoterapie, ci numai empiric, de către localnicii care îi atribuie calități terapeutice.

3. Analiza calitativă a celor patru oligaze (maltază, celobiază, zaharază și lactază) crește complexitatea aprecierii potențialului enzimatic general al nămolurilor din cele două lacuri studiate, rezultatele analizelor calitative confirmându-le pe cele ale analizelor cantitative.

4. Cercetările efectuate ilustrează importanța sistemului de clasificare a lacurilor saline pe baza valorilor indicatorilor enzimatici ai calității nămolurilor, care permite identificarea și recomandarea folosirii în balneoterapie a nămolurilor mai active din punct de vedere enzimatic și care sunt terapeutic mai eficiente, fapt dedus empiric, în urma unei îndelungate tradiții. În acest sens se impune atenției lacul Balta Sărată de la Blaj.

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INDUCȚIA ENZIMATICĂ ÎN SOL CA TEST ECOTOXICOLOGIC PENTRU POLUANȚI ANORGANICI ŞI ORGANICI

ALIONA POPA*

SUMMARY. - Enzymatic Induction in Soil as Ecotoxicological Test for Inorganic and Organic Pollutants. In a laboratory experiment modelling soil pollution, salts of bivalent heavy metals (Hg^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, Co^{2+} and Cu^{2+}) and organic substances (the detergents "Rex" and "Ariel", the herbicide 2,4-D as well as fuel oil and phenol), as potential pollutants, were added to samples of an alluvial soil for studying their effect on the induction of microbial synthesis of levansucrase. Sucrose, as specific substrate of levansucrase, served as inductor. Rates of the additions (per 100 g air-dry soil) were the following: 0.001, 0.01 and 0.1 g heavy metal; 0.1, 1 and 2 g detergent; 0.001, 0.01 and 0.1 g 2,4-D; 0.1, 1 and 5 ml fuel oil; 0.001, 0.01 and 0.1 ml phenol; and 10 g sucrose. Soil samples, to which no pollutant or no sucrose was added, were the controls. All samples, representing 20 and 17 experimental variants (treated with heavy metal ions and potential organic pollutants, respectively) were moistened to 60% of water-holding capacity and incubated at room temperature for 18 days, then analysed to determine their levansucrase activity by means of circular paper chromatography.

The results have shown that the heavy metal ions inhibited activity and synthesis of levansucrase in the order: Hg^{2+}> Cd^{2+}> Co^{2+}> Zn^{2+}> Pb^{2+}>Cu^{2+}. Both detergents enhanced activity and synthesis of levansucrase, the enhancing effect of the detergent "Ariel" (manufactured with addition of enzymes) was more pronounced than that of the detergent "Rex" (containing no enzymes). 2,4-D strongly inhibited, while fuel oil and phenol slightly inhibited the activity and synthesis of levansucrase. The effect of heavy metal ions, detergents and 2,4-D increased with increasing rate of additions, but the effect of fuel oil and phenol did not change in dependence of the rate of additions.

The conclusion has been drawn that induction of microbial levansucrase synthesis in soil may be used as an ecotoxicological test for both inorganic and organic pollutants.

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Ideea inducției secvențiale a sintezei enzimelor de către microorganismele din sol a fost formulată pe baza unor cercetări, în care probe de sol au fost compostate cu melasă. Zaharoza din melasă, acționând ca substrat enzimatic, a indus sinteza microbiană a levansucrazei şi dextransucrazei care au catalizat apoi sinteza polizaharidelor levan şi dextran. La rândul lor, aceste polizaharide au indus sinteza microbiană a levanazei şi dextranazei, polizaharidaze care catalizează hidroliza levanului şi, respectiv, a dextranului. Dat fiind că levanul şi dextranul contribuie la agregarea particulelor de sol, la formarea structurii favorabile a solului, levansucraza şi dextransucraza joacă un rol pozitiv, iar levanaza şi dextranaza au un rol negativ din punctul de vedere al fertilității solului [1]. La aceste cercetări se fac referiri şi într-o lucrare de sinteză asupra activității şi importanței agricole a polizaharidazelor din sol [2].

În prezenta lucrare s-a studiat posibilitatea folosirii inducției sintezei microbiene a levansucrazei în sol ca test ecotoxicologic pentru poluanți anorganici şi organici.

Material şi metodă. S-a efectuat un experiment de laborator pentru modelarea poluării solului. S-a lucrat cu acelaşi sol aluvial (probe de 100 g, uscate la aer) şi cu aceiaşi poluanți anorganici şi organici, care s-au folosit şi pentru studierea activității dehidrogenazice [3].

Ca inductor a servit substratul specific al levansucrazei, zaharoza, care s-a adăugat sub formă de pulbere în cantitate de 10 g/100 g sol uscat la aer.

Pentru studierea poluanților anorganici şi organici s-au realizat 20 şi, respectiv, 17 variante experimentale. Schema lor este prezentată în Tabelele 1 şi 2.

Solul fiecărei variante experimentale a fost umezit la 60% din capacitatea de reținere a apei. Compostarea a avut loc la temperatura camerei şi a durat 18 zile.

După compostare, probele de sol au fost lăsate să se usuce la aer, apoi li s-a determinat activitatea levansucrazică. În acest scop, s-au preparat amestecuri de reacție din câte 3 g sol + 2 ml toluen (antiseptic) + 10 ml soluție apoasă de substrat (zaharoză 10%, greutate/volum). Drept martori au servit amestecuri de reacție la care, în locul soluției de zaharoză, s-a adăugat apă distilată (10 ml); s-a preparat şi un amestec de reacție martor fără sol (constând numai din soluție de zaharoză şi toluen). Schema amestecurilor de reacție este redată în Tabelul 3.

Tabel 1

Schema variantelor experimentale realizate în vederea urmăririi inducției enzimatice în sol ca test ecotoxicologic pentru poluanți anorganici

Varianta	Sol (g)	Zaharoză (g)	Ionul metalic	Concentrația finală a ionului metalic (g/100 g sol)
V1	100	10	Hg^{2+}	0,001
V2	100	10		0,01
V3	100	10		0,1
V4	100	10	Cd^{2+}	0,001
V5	100	10		0,01
V6	100	10		0,1
V7	100	10	Zn^{2+}	0,001
V8	100	10		0,01
V9	100	10		0,1
V10	100	10	Pb^{2+}	0,001
V11	100	10		0,01
V12	100	10		0,1
V13	100	10	Co^{2+}	0,001
V14	100	10		0,01
V15	100	10		0,1
V16	100	10	Cu^{2+}	0,001
V17	100	10		0,01
V18	100	10		0,1
V19	100	10	Sol martor compostat cu zaharoză	-
V20	100	-	Sol martor necompostat cu zaharoză	-

Tabel 2

Schema variantelor experimentale realizate în vederea urmăririi inducției enzimatice în sol ca test ecotoxicologic pentru poluanți organici

Varianta	Sol (g)	Zaharoză (g)	Substanța organică	Concentrația finală a substanței organice (g sau ml/100 g sol)
V1	100	10	Detergent "Rex" (fără enzime)	0,1 g
V2	100	10		1 g
V3	100	10		2 g
V4	100	10	Detergent "Ariel" (cu enzime)	0,1 g
V5	100	10		1 g
V6	100	10		2 g

Tabel 2 (continuare)

Varianta	Sol (g)	Zaharoză (g)	Substanța organică	Concentrația finală a substanței organice (g sau ml/100 g sol)
V7	100	10	2.4-D	0,001 g
V8	100	10		0,01 g
V9	100	10		0,1 g
V10	100	10	Motorină	0,1 ml
V11	100	10		1 ml
V12	100	10		5 ml
V13	100	10	Fenol	0,001 ml
V14	100	10		0,01 ml
V15	100	10		0,1 ml
V16	100	10	Sol martor compostat cu zaharoză	-
V17	100	-	Sol martor necompostat cu zaharoză	-

Tabel 3

Schema amestecurilor de reacție pentru determinarea activității levansucrazice în solul variantelor experimentale realizate în vederea urmăririi inducției enzimatice

Numărul amestecului de reacție	Concentrația poluantului în variantele experimentale	Sol (g)	Toluen (ml)	Zaharoză, soluție 10% (ml)	Apă distilată (ml)
1	Minimă*	3	2	10	-
2		3	2	-	10
3	Mijlocie*	3	2	10	-
4		3	2	-	10
5	Maximă*	3	2	10	-
6		3	2	-	10
7	Sol martor compostat cu	3	2	10	-
8	zaharoză	3	2	-	10
9	Sol martor necompostat	3	2	10	-
10	cu zaharoză	3	2	-	10
11	Martor fără sol	-	2	10	-

* Vezi Tabelul 1 pentru concentrația ionilor metalici şi Tabelul 2 pentru concentrația substanțelor organice.

Amestecurile de reacție au fost incubate la 37^0C timp de 10 zile, apoi analizate pentru evidențierea levanului. Prezența levanului indică activitatea levansucrazică, deoarece levanul s-a format sub acțiunea levansucrazei. Analiza a fost efectuată prin metoda cromatografiei pe hârtie, tehnica circulară. S-a folosit hârtie Whatman 1. Din faza apoasă a amestecurilor de reacție s-au aplicat câte 14 µl (în câte două reprize a 7 µl) pe punctele de start ale hârtiilor cromatografice circulare. După uscare, cromatogramele au fost developate într-un sistem de dizolvanți alcătuit din *n*-propanol, acetat de etil şi apă distilată în proporție de 6:1:3 (volum/volum/volum). Developarea a avut loc la temperatura camerei şi a durat 2 ore. Final, cromatogramele au fost pulverizate cu un reactiv conținând uree şi acid *o*-fosforic; acest reactiv evidențiază, în mod specific, cetozele libere şi combinate, inclusiv levanul [4].

Rezultate. Pe cromatogramele redate în Fig. 1-6, levanul este uşor de identificat, fiind spotul observabil la punctul de start (R_f=0).

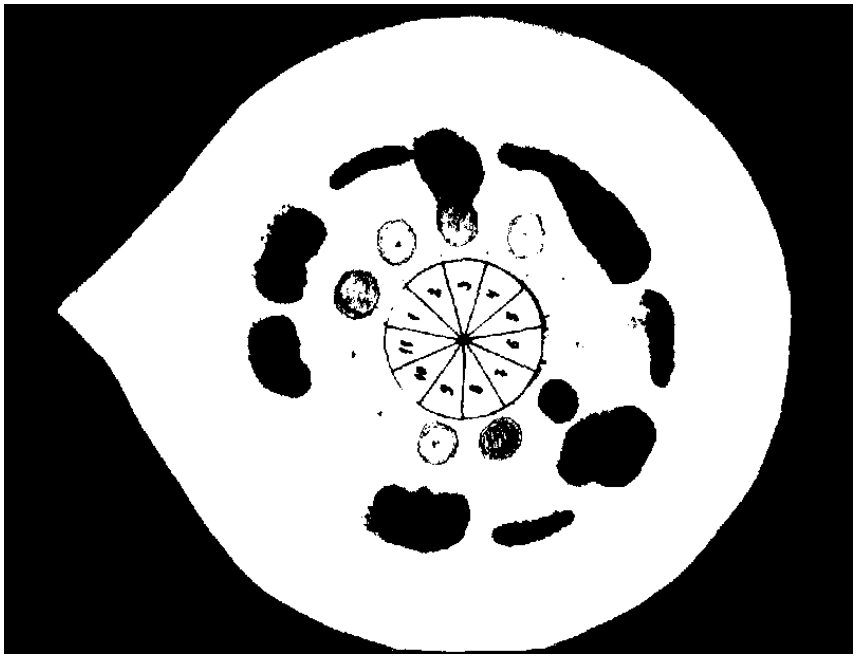

Fig. 1. *Activitatea levansucrazică în solul variantelor experimentale tratate cu* Hg^{2+}. Amestecurile de reacție 1-11: vezi Tabelul 3.

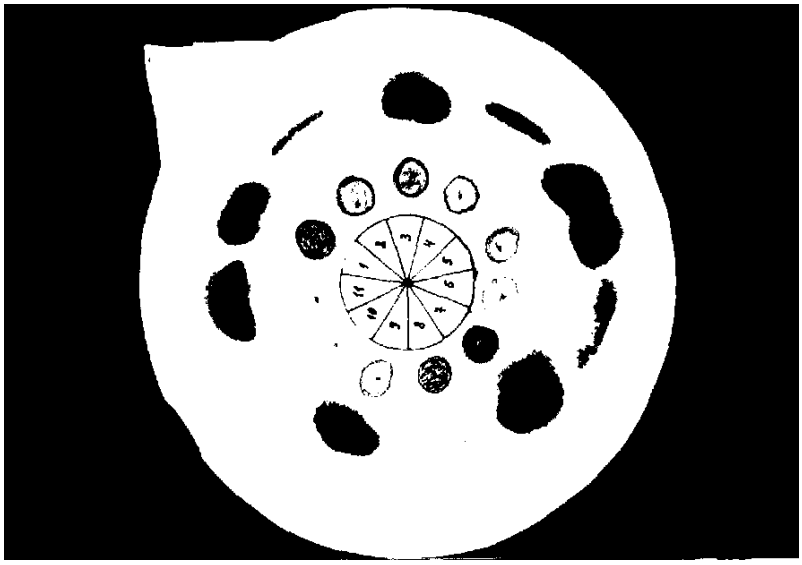

Fig. 2. *Activitatea levansucrazică în solul variantelor experimentale tratate cu* Cu^{2+}.
Amestecurile de reacție 1-11: vezi Tabelul 3.

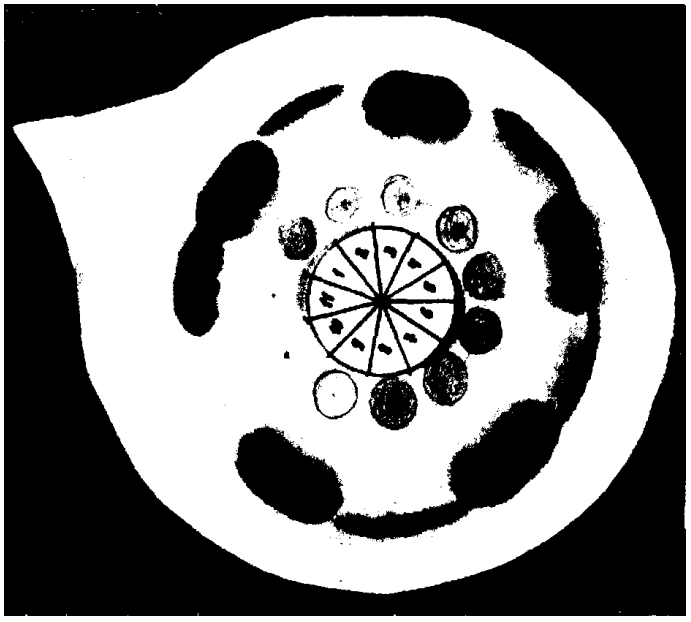

Fig. 3. *Activitatea levansucrazică în solul variantelor experimentale tratate cu detergentul "Rex"* (fabricat fără adaos de enzime).
Amestecurile de reacție 1-11: vezi Tabelul 3.

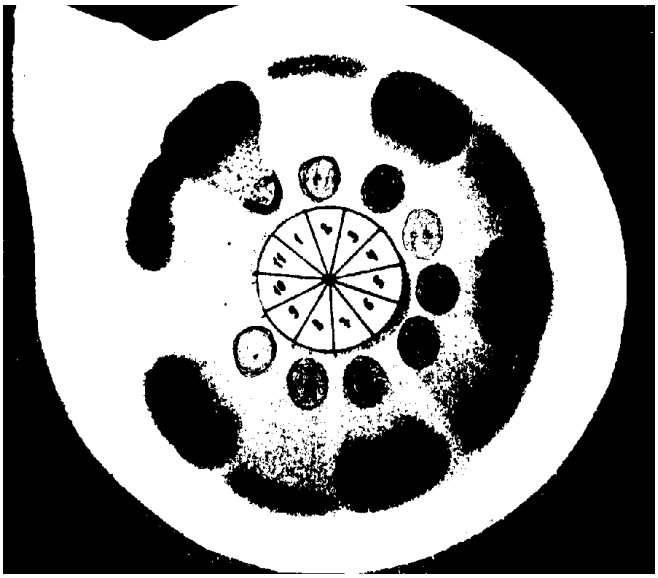

Fig. 4. *Activitatea levansucrazică în solul variantelor experimentale tratate cu detergentul "Ariel"* (fabricat cu adaos de enzime).
Amestecurile de reacție 1-11: vezi Tabelul 3.

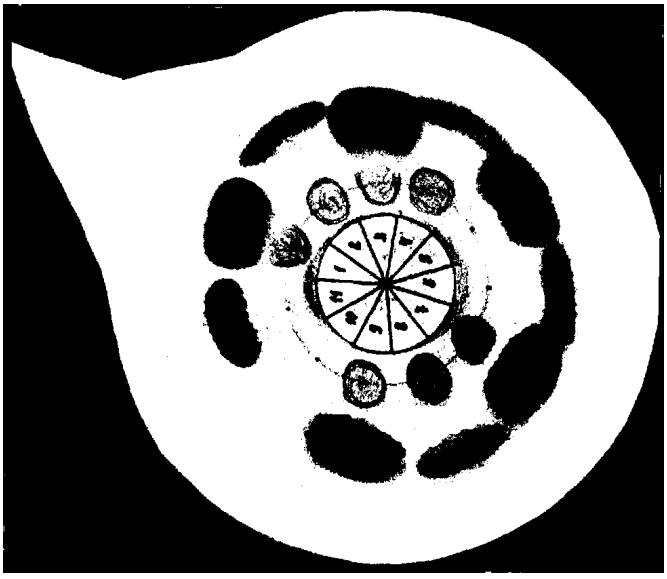

Fig. 5. *Activitatea levansucrazică în solul variantelor experimentale tratate cu ierbicidul hormonal 2,4-D.*
Amestecurile de reacție 1-11: vezi Tabelul 3.

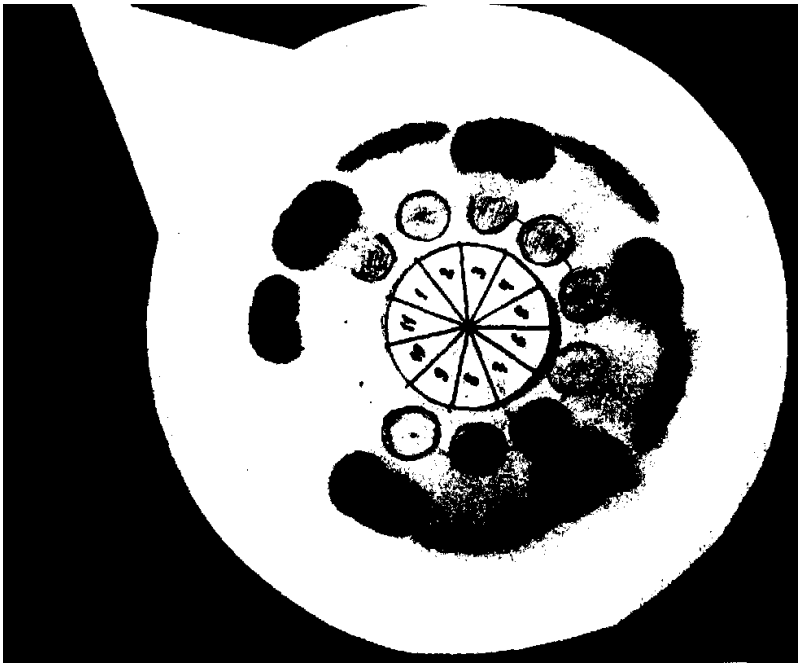

Fig. 6. *Activitatea levansucrazică în solul variantelor experimentale tratate cu motorină.* Amestecurile de reacție 1-11: vezi Tabelul 3.

Rezultatele s-au stabilit pe baza comparării spoturilor de levan la diferitele amestecuri de reacție.

A. Compararea amestecurilor de reacție cu sol martor 7-10 arată că spotul de levan este cel mai intens la amestecul de reacție 7, în care solul a fost compostat şi incubat cu zaharoză, ceea ce înseamnă că în timpul compostării a avut loc inducția sintezei microbiene a levansucrazei care şi-a păstrat activitatea şi după terminarea compostării. La amestecul de reacția 8, conținând sol compostat cu zaharoză, dar incubat fără zaharoză, se observă un spot mai slab de levan care s-a format în timpul compostării. Amestecul reacție 9, preparat din sol necompostat, dar incubat cu zaharoză, de asemenea arată prezența unui spot mai slab de levan care s-a format sub acțiunea levansucrazei preexistente (prezentă în sol înainte de compostare).În schimb, amestecul de reacție 10, în care solul nu a fost nici compostat şi nici incubat cu zaharoză, arată absența spotului de levan. Adăugăm că levanul este absent şi în amestecul de reacție 11 (martor fără sol), ceea ce dovedește că zaharoza folosită a fost un preparat pur, lipsit de levan.

B. Compararea amestecurilor de reacție 1, 3 şi 5 (conținând sol tratat cu cele trei concentrații de poluant, apoi compostat şi incubat cu zaharoză) cu amestecul de reacție 7 face posibilă stabilirea efectului poluantului asupra activității şi inducției sintezei microbiene a levansucrazei. Compararea amestecurilor de reacție 2, 4 şi 6 (în care solul a fost tratat cu poluant şi compostat cu zaharoză, dar incubat fără zaharoză) cu amestecul de reacție 10 de asemenea permite sesizarea efectului poluantului asupra activității şi sintezei levansucrazei.

Toți ionii de metale grele au inhibat activitatea şi sinteza levansucrazei, în următoarea ordine: Hg^{2+}> Cd^{2+}> Co^{2+}> Zn^{2+}> Pb^{2+}> Cu^{2+}. În Fig. 1 şi 2 redăm, pentru exemplificare, cromatogramele ilustrând activitatea levansucrazică în variantele experimentale tratate cu Hg^{2+} (ionul metalic cel mai inhibitor) şi în cele tratate cu Cu^{2+} (ionul metalic cel mai puțin inhibitor).

Efectuând comparările A şi B, putem constata că efectul inhibitor al Hg^{2+} este, în mod foarte evident, mult mai puternic decât cel al Cu^{2+}. Totodată, efectul inhibitor creşte cu creşterea concentrației de Hg^{2+} şi Zn^{2+}. Astfel, din amestecurile de reacție 5 şi 6, în care solul a fost tratat cu concentrația maximă de Hg^{2+} (0,1 g/100 g sol), levan nu s-a evidențiat (vezi Fig. 1), ceea ce dovedeşte că Hg^{2+} în această concentrație nu numai că a oprit inducerea sintezei microbiene a levansucrazei, dar a inactivat şi levansucraza preexistentă în sol.

Se poate vedea din cromatogramele redate în Fig. 3 şi 4 că detergenții "Rex" şi "Ariel" au stimulat activitatea şi sinteza levansucrazei. Efectul detergentului "Ariel" (fabricat cu adaos de enzime) a fost mai pronunțat decât cel al detergentului "Rex" (fabricat fără adaos de enzime). Efectul a crescut cu creşterea concentrației, fiind cel mai evident la 2 g detergent/100 g sol.

Ierbicidul hormonal 2,4-D (acid 2,4-diclorfenoxiacetic) a inhibat activitatea şi sinteza levansucrazei (Fig. 5). Gradul de inhibiție a crescut cu mărirea concentrației de 2,4-D. Astfel, amestecurile de reacție 5 şi 6, în care solul a fost tratat cu 2,4-D în concentrația maximă (0,1 g/100 g sol), arată absența levanului, ceea ce înseamnă că 2,4-D în această concentrație a inactivat levansucraza preexistentă şi a oprit inducția sintezei microbiene a levansucrazei.

Din Fig. 6 se poate deduce că motorina a cauzat o slabă inhibiție a activității şi sintezei levansucrazei, iar gradul de inhibiție nu s-a schimbat, în mod evident, în funcție de concentrația motorinei.

Efectul fenolului a fost similar cu cel al motorinei.

Concluzii. 1. Ionii metalelor grele au inhibat activitatea şi sinteza levansucrazei, în ordinea: Hg^{2+}> Cd^{2+}> Co^{2+}> Zn^{2+}> Pb^{2+}> Cu^{2+}.

2. Detergenții "Rex" şi "Ariel" au stimulat activitatea şi sinteza levansucrazei, efectul detergentului "Ariel" fiind mai pronunțat decât cel al detergentului "Rex".

3. 2,4-D a inhibat puternic, iar motorina şi fenolul au inhibat într-o măsură mică activitatea şi sinteza levansucrazei.

4. Efectul ionilor metalelor grele, al detergenţilor şi 2,4-D a crescut cu mărirea concentraţiei lor, în timp ce efectul motorinei şi fenolului nu s-a schimbat în funcţie de concentraţie.

5. Inducţia sintezei microbiene a levansucrazei în sol poate fi folosită ca test ecotoxicologic atât pentru poluanţi anorganici cât şi pentru cei organici.

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RECENZII

Constantin Toma, Rodica Ruginã, **Anatomia plantelor medicinale. Atlas** (*Anatomy of Medicinal Plants. Atlas*), Editura Academiei Române, Bucureşti, 1998, 320 pages including 155 plates.

This book, the Authors of which are well-known and reknown plant anatomists, is the first treatise on anatomy of medicinal plants in the Romanian literature.

As the Authors emphasise in the Preface, the histo-anatomic analyses, by means of macroscopical and microscopical methods (the microscopical methods using sometimes microchemical reactions, too), play a key role in determination of the medicinal plant species, of their organs, fragments and powders, and present a major theoretical and practical importance for many human activities related *e.g.* to pharmaceutics, food industry, agronomy, silviculture, forensic science.

The book consists of two parts (the general and the special parts).

The General Part (pages 23 - 54) is entitled "General Histology and Anatomy" and comprises 12 chapters and 3 addenda. The chapter titles are the following: 1. Meristems and definitive primary tissues; 2. Meristems and definitive secondary tissues; 3. Primary structure of the root; 4. Transition from the structure of root to that of the stem; 5. Secondary structure of the root; 6. Anomalies in the structure of root; 7. Primary structure of the stem; 8. Secondary structure of the stem; 9. Anomalies in the structure of stem; 10. Structure of the leaf; 11. Structure of the seed; and 12. Structure of the fruit. One of the addenda deals with the chemical composition of the plant cell, whereas the other two are keys, namely for the determination of dried fragments of plants and plant organs and for the determination of strongly fragmented plants (plant powders).

The Special Part (pages 55 - 306) contains the description of 128 plant species, of which 111 are native and 17 are exotic. The structure of vegetative organs (root, rhizome, stem, leaf) and, for some plants, the structure of reproducing organs (flower, fruit) are described in the alphabetical order of the families, genera and species, starting with Pteridophytes (2 families, 2 genera, 2 species) and continuing with Gymnosperms (4 families, 4 genera, 4 species) and Angiosperms (44 families, 102 genera, 122 species). The descriptions are accompanied by 1154 original drawings and 285 drawings reproduced from papers of other authors. The drawings are grouped in 155 plates as already mentioned. Due to the rich illustrations, the book is really an atlas, beside being a treatise, too.

The bibliographical list comprises 78 titles of books and articles published in Romania or in many other countries.

The book ends with a 2-page abstract in English; Alphabetical index of the common names of the species cited in the text, with mention of the family; Alphabetical index of the genera and species, with mention of the family; and Etymological index of the scientific terms used.

Based on the excellent quality of this comprehensive and updated book, I warmly recommend its publication in world-wide spoken languages.

Anatomia plantelor medicinale is a valuable source of information for a broad circle of readers: students and researchers in biology, pharmacology, agronomy, silviculture, ecology, biochemistry, medicine; highschool teachers; experts in valorification of medicinal plants, in toxicology and legal medicine; and also for other readers interested in the medicinal plants.

CORNELIA DELIU

Cristina Dobrotă, Masamichi Yamashita, **Creşterea şi dezvoltarea plantelor** (*Growth and Development of Plants*), Casa de editură Gloria, Cluj, 1999, 223 pages with 41 figures and 5 tables in the text.

The book is structured into five parts and 17 Chapters which cover the topic of plant growth and development at four levels of organisation: cell, tissue, individual and community levels.

Part I, comprising Chapters 1-3, presents the processes expressed at cell level concerning growth and development such as: the unequal division, the cell polarity, the role of cytoskeleton, the physiological mechanism of cell expansion, the cell wall structure, the genic control and the influence of growth regulators on cell division and on cell expansion.

In Part II, Chapters 4-7 deal with aspects related to embryogenesis, meristem organisation and functioning, differentiation of vascular tissues, and the competence and determination processes in shoots, roots, leaf and flower development.

Part III, with Chapters 8-10, describes plant growth and development at individual level presenting: iterative growth through modules, the formation of branches, leaves and floral organs and the functional integration of plant organs.

Part IV, containing Chapters 11-15, concerns the environmental influences on plant growth and development. Photomorphogenesis, phototropism and photoperiodism are presented as growth and developmental responses to th1e light perception and transduction. The influence of low and high temperature, flooding and oxygen shortage, salinity and gravitation are also discussed.

In Part V, Chapter 16 deals with growth and development at community level in relation to the growth rate and the habitat productivity. The primary plant strategies, the morphological plasticity, the cellular acclimation and the stored growth are presented. Chapter 17 describes techniques for measuring plant growth such as: direct sampling techniques, relativised measures of growth, indirect sampling techniques, plastochron index, physiological measurement, remote sensing and modelling techniques.

The book written by Lecturer Cristina Dobrotă and Professor Masamichi Yamashita is remarkable through the integrating conception and the clarity of explanation of such complex processes as plant growth and development are. Each chapter ends with conclusions which are very useful for a better understanding of intimate mechanisms and processes. This comprehensive book, based on a rich and up-to-date bibliography comprising 239 titles, and also on personal investigations of the authors, is a very valuable source of information for students in biology, agronomy and forestry and experts working in different fields of plant sciences.

CORNELIA DELIU

L. Rákosy-Tican, **Utilizarea tehnicilor de electrofuziune în hibridarea somatică a plantelor** (*Electrofusion Techniques and Their Use in Plant Somatic Hybridization*), Presa Universitară Clujeană, Cluj-Napoca, 1998, 187 pages with 57 figures and 14 tables in the text and with 8 tables enclosed.

This comprehensive book reviews the state of the art of electrofusion techniques and their use in plant somatic hybridization. The book is highly original representing the Ph. D. thesis of the author (Babeş-Bolyai University, 1994). Its publication was made possible under a TEMPUS-PHARE project entitled "Masters' Degree Course in Plant Genetic Manipulation" (S-JEP-09697-95), funded by EU.

The book is structured into two parts.

Part I, "Electrofusion of higher plant protoplasts", presents: recent advances in plant protoplast electromanipulation (dielectrophoresis, electrorotation, electrofusion, electroporation and electrostimulation of plant protoplasts); basic studies on cereal mesophyll protoplast electrofusion and ultrastructural studies of electrofused plant protoplasts (transmission electron microscopy).

Part II, "Plant somatic hybridization through electrofusion techniques", deals with: theoretical aspects of higher plant somatic hybridization and its perspective in plant breeding; studies on plant regeneration from isolated protoplasts (potato mesophyll protoplasts, *Nicotiana africana* cell suspension-derived protoplasts); studies on asymmetric somatic hybridization of *Nicotiana africana* and *Solanum tuberosum* cv. Désirée, through mass electrofusion; studies on asymmetric somatic hybridization of *N. africana* and *N. tabacum* KY14; and technique used for electrofusion of preselected protoplast pairs to produce intraspecific somatic hybrids in *Nicotiana*.

Associate Professor Lenuța Rákosy-Tican's book, summarizing more than 11 years of research in plant protoplast electrofusion, is addressed to students and scientists in biology, biotechnology and agronomy, interested in the achievements of a modern and spectacular branch of plant genetic engineering.

The book may be characterised by a series of qualities: the novelty of the topic both for Romanian and international biological literature, basic and up-to-date information supported by 387 references, originality of research, logical division into parts, chapters and subchapters and clarity of the descriptions, simple, concise and attractive style, richness and originality of illustrations and a very beautiful cover.

The book has a table of contents in English. But taking into consideration its excellent qualities mentioned above, I strongly recommend its translation into world-wide spoken languages.

DORINA CACHIȚĂ-COSMA

Lenuţa Rákosy-Tican (Editor), **Plant Genetic Engineering - Lab Manual. Inginerie genetică vegetală - caiet de lucrări de laborator,** Presa Universitară Clujeană, Cluj-Napoca, 1998, 167 pages with 27 figures and 22 tables in the text.

The lab manual edited by Associate Professor Lenuţa Rákosy-Tican is a collection of methods described to be used for practical work in plant genetic engineering laboratories. It was published under the TEMPUS-PHARE project S-JEP-09697-95: Masters' Degree Course in Plant Genetic Manipulation. This manual is the result of a very successful co-operation of well-known specialists in plant genetic engineering and their Romanian colleagues, who joined their efforts in European spirit in order to set up a teaching laboratory in this modern field of biology.

The manual comprises four chapters. First, I present their authors:

Chapter I: Lenuţa Rákosy-Tican (Universitatea "Babeş-Bolyai", Cluj); Paul Anthony (University of Nottingham, UK) and Ioana Dinu (Institutul de Cercetare şi Producţie pentru Cultura Cartofului, Braşov);

Chapter II: Dr. Michael R. Davey, Paul Anthony and Nigel Blackhall (University of Nottingham, UK) and Călin Andraş (Ph.D. student, Universitatea "Babeş-Bolyai", Cluj);

Chapter III: Dr. Günther Hahne and Jean Molinier (CNRS, Strasbourg, France); and

Chapter IV: Professor Michel Jacobs and Fanny Frulleux (Vrije Universiteit, Brussel, Belgium).

The contents of the chapters (and subchapters) are specified below:

Chapter I: Plant tissue and protoplast culture (Plant genetic engineering laboratory design; Sterilization techniques; Inoculation of plant explants - organ culture; The use of hemocytometer to determine total cell counts in a cell suspension; The use of fluorescein diacetate (FDA) to determine cell viability; Protocol for sunflower hypocotyl protoplast isolation and culture; Isolation and culture of protoplasts from cell suspension of *Passiflora giberti*; Isolation and culture of protoplasts from seedling leaves of *Passiflora edulis*; Electrofusion of plant protoplasts - experimental protocol; Protocol for cereal protoplast electrofusion);

Chapter II: Mutagenesis - Transformation - Cryopreservation - Analysis of chromosomes and transgenes (Screening for mutation in *Arabidopsis thaliana*; Screening of *Arabidopsis thaliana* for barbiturate mutants; Leaf disk transformation of lettuce; Histochemical staining of tissues for GUS activity; Protocol for protoplast isolation and fusion in rice; Cryopreservation of rice cell suspension cultures; Examination of the chromosomes of cultured cells; Examination of the meiotic chromosomes of *Tradescantia* sp.; PCR analysis of transgenes);

Chapter III: Bacterial transformation and DNA analysis (Introduction of a plasmid into *Agrobacterium tumefaciens* and test in plants; Small-scale preparations of a plasmid DNA; How to write a Lab Report?); and

Chapter IV: Analysis of mutants (Callus initiation in *Daucus carota*; Influence of hormones on growth of cell suspensions; Protoplast isolation, transformation and fusion; Characterisation of biochemical mutants; Characterisation of resistant mutants of *Nicotiana sylvestris*; Segregation of kanamycin resistance in *Arabidopsis thaliana* transformants; Expression of endogenous and exogenous *Adh* genes in *Nicotiana plumbaginifolia* transformed with *Arabidopsis Adh*).

This manual in A4 format is combbound and its chapters are printed on different coloured papers. It was especially

designed to be used in the laboratory and to allow an easy way to find and follow practical information and also to stimulate the students' practical work and skill development.

Finally, I consider that the laboratory manual edited by Associate Professor Lenuța Rákosy-Tican is the best book of its kind in Romania. Therefore, I warmly recommend it to all laboratories of plant biotehnology for teaching and research work.

DORINA CACHIȚĂ-COSMA

Enzymes in the Environment: Activity, Ecology and Applications, Edited by Richard P. Dick, Oregon State University, Corvallis, OR, USA, 1999, 164 pages.

This volume comprises the Preface written by the Editor, and the abstracts of papers presented at the International Conference on Enzymes in the Environment, held in Granada (Spain), July 12-15, 1999. The abstracts are preceded by the Conference schedule.

As Professor R.P. Dick emphasises in the Preface "The core of the conference was on enzymes or enzyme-mediated processes as biological catalysts in soil, sediments and aquatic ecosystems. The conference had two broad themes: microbial ecology and environmental enzymology".

Ten symposia were organised. Their titles (and numbers of oral and poster presentations) are specified below: I. Enzymes in soil systems (3 and 32); II. Limnic systems (3 and 18); III. Plants and soil enzymes (3 and 15); IV. Enzymes in solid/liquid phases (4 and 7); V. Nutrient oyeling and organic matter decomposition (4 and 30); VI. Enzymic methodologies (3 and 12); VII. Bioremediation and extracellular enzymes (5 and 23); VIII. Bioremediation - genetically designed organisms and enzymes (3 and 1); IX. Marine ecosystems (4 and 4) and X. Enzymes as environmental sensors (4 and 30).

The past and present of environmental enzymology are outlined in the inaugural lecture "Biochemical context of enzymes in the environment" (J.N. Ladd) and its future is discussed in the closing lecture "Environmental enzymology in the 21th century" (R. G. Burns).

Two lists are enclosed to the volume. The first is a list with the names and addresses of the corresponding authors (166 persons from 32 countries, including Romania). The second list is the author index (457 authors).

This volume is a valuable source of information for all experts working in different fields of environmental science and technology.

ȘTEFAN KISS and ELENA MANOLACHE

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI, BIOLOGIA, XLVI, 1, 2001

ADVANCES IN SOIL ENZYMOLOGY (PARTS I-III)

STEFAN KISS[*]

SUMMARY. - Under the general heading "Advances in Soil Enzymology", a series of five review articles, based on recent literature, are elaborated. They deal with: I. Enzymology of oil-contaminated soils; II. Enzymology of soils affected by industrial emissions (with addenda on soil enzymological effects of military waste disposal operations, enzymology of urban soils and enzymology of roadside soils); III. Enzymology of technogenic soils; IV. Enzymology of soils inoculated with microorganisms; and V. Soil enzyme activities as influenced by earthworms.

The present article comprises Parts I-III in the series of five review articles and adds new data to the reviews published in 1998 as Parts I-III in "Enzymology of Disturbed Soils" [35].

Introduction. After publication of our reviews on five soil enzymological topics [33-35], a great number of papers dealing with the same topics have appeared in the world literature. With the aim to review the investigations described in these recent papers, a series of five review articles are elaborated. A smaller number of papers, which appeared before publication of our reviews [33-35], but became available to us only recently, are also considered. Corresponding to the five topics, the series of five review articles taken as a whole consists of the following five parts: I. Enzymology of oil-contaminated soils; II. Enzymology of soils affected by industrial emissions; III. Enzymology of technogenic soils; IV. Enzymology of soils inoculated with microorganisms; and V. Soil enzyme activities as influenced by earthworms.

The present article consists of Parts I-III and adds new data to the reviews published in 1998 as Parts I-III in [35]. Parts I and II are structured into the same 3+3 chapters (Chapters 1-6) as Parts I and II in [35], but Parts III comprises only six chapters (Chapters 7-12) and not 22 chapters as in [35].

Part I. ENZYMOLOGY OF OIL-CONTAMINATED SOILS

Chapter 1. **Soil enzyme activities as affected by accidental oil contamination**

Contamination with crude oil

Enzymological research in the Russian Federation. In a review of studies on pollution and recultivation of the oil fields of the enterprise "Tatneft'" (Tataria), Sattarov *et al*. [65] point out that oil pollution of soil causes inhibition of proteolysis and decrease in dehydrogenase activity.

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Enzymological research in Azerbaijan. Akhundova and Maslovetskaya [1] carried out soil enzymological studies in the seashore areas of the Zykh and Shikhovo districts located on the Apsheron Peninsula. The soils in both areas are oil-polluted which is related to oil extraction on the Caspian Sea. Soil was sampled at distances of 250 m (sample A), 500 m (sample B) and 1000 m (sample C) from the seashore. The oil content in soil samples A, B and C was much higher in the Shikhovo soil (1400, 450 and 100 mg/100 g soil, respectively) than in the Zykh soil (400, 75 and 12 mg/100 g soil, respectively). In concordance with the pollution degree, catalase and dehydrogenase activities were lower in the Shikhovo than in the Zykh soil, and invertase activity was not detectable at all in the Shikhovo soil. At the same time, only traces of protease activity and the same level of urease activity were recorded in both soils. In the Shikhovo soil, catalase and urease activities were highest in sample C, while dehydrogenase activity gave the highest value in sample B. In the Zykh soil, catalase and dehydrogenase activities were highest in sample C, and invertase and urease activities in sample A.

Contamination with crude oil and oil products

Enzymological research in the Russian Federation. Gabbasova *et al.*[19] have studied typical chernozems polluted with crude oil and oil products on the territories of the Tuimazy, Shkapovo and Arlan oil fields (Bashkiria). One of the studied plots is located in the zone of an oil well and other three plots were polluted due to rupture of pipelines. Adjacent, unpolluted plots were the controls. Soil was sampled from different depths. The enzymological analyses showed that at each depth urease activity was higher and invertase activity was lower in the polluted than in the unpolluted soils.

Contamination with oil products

Enzymological research in Spain. According to a short report, Trasar-Cepeda *et al.* [76] have found that, in Galician soils exposed to various degrees of pollution by fuel oil, alterations of enzyme activities by themselves showed no pattern allowing precise qualification of soil disturbance. They concluded that for qualification of soil disturbance enzyme activities generally need to be supplimented by information on other biochemical soil properties.

It should be mentioned that in the same volume, in which this short report of Trasar-Cepeda *et al.* [76] has appeared, Schinner *et al.* [66] have drawn a diametrically opposite conclusion (see page 11 in the present article).

Enzymological research in France. Brohon and Gourdon [9] have determined dehydrogenase activity in surface (0-25 cm) soil samples from two areas of the same contaminated industrial site in Central France. Soil l is mostly contaminated with hydrocarbons (386 mg/kg dry soil) and some heavy metals (*e.g.* 75 mg Cu/kg dry soil), whereas soil 2 contains less hydrocarbons (81 mg/kg dry soil) and more heavy metals (*e.g.* 335 mg Cu/kg dry soil). Both contaminated soils are sandy loam alluvial soils. Uncontaminated soil was not used. For measurement of soil toxicity the Lumistox bioassay (which, based on reduction of light emission by

Vibrio fisheri, is similar to the Microtox test) and the MetPlate bioassay (based on the inhibition of biosynthesis of β-galactosidase in *Escherichia coli*) were also applied. Dehydrogenase activity was found to be higher in soil l than in soil 2, which means that the hydrocarbons inhibited this activity to a lesser extent than did the heavy metals. Another finding was that in evaluation of the results of toxicity bioassays great care must be given to soil microbial activity (estimated by measurement of dehydrogenase activity).

Contamination with oil field wastewaters

Enzymological research in the Russian Federation. Gabbasova *et al.*[18] have determined dehydrogenase and invertase activities in polluted and unpolluted plots of a humid meadow soil in the Krasnokams district (Bashkiria). The pollution was the result of the rupture of a pipeline with saline wastewaters containing 30-35% crude oil. The soil samples were examined 1.5 months after the pollution. The oil remained in the surface (10 cm) layer of soil. The concentration of salts was also highest in this layer, but it was high even at depths of 50-80 cm. Dehydrogenase activity increased in the oil-polluted layer and decreased in the deeper, salinised layers. Invertase activity decreased in all layers.

In another study, Gabbasova *et al*. [19] have determined urease and invertase activities in nine plots installed on chernozems and grey forest soils contaminated with both oil and saline wastewaters, in a plot on meadow chernozem-like soil polluted only with saline wastewaters and, comparatively, in adjacent, unpolluted plots, all being located on the territories of the Tuimazy, Shkapovo and Arlan oil fields (Bashkiria). The pollution was caused by rupture of pipelines. The enzyme activities were measured in soil samples taken from different depths. Pollution with oil and wasterwaters led to increased urease activity. This activity significantly correlated with soil C content enriched by the polluting oil, which was attributed to the oil-enhanced growth of the urease-producing microorganisms. But pollution only with wastewaters caused a decrease in urease activity. Invertase activity was inhibited by pollution with both oil and wastewaters only. During aging of the polluted soils and biodegradation of oil, values of the enzyme activities, especially those of the invertase activity, manifested a trend to approach the values measured in the control soils.

Chapter 2. **Enzymological evaluation of the biological effects of oil contamination of soils in experimental models**

Field experiments

Contamination with crude oil

Enzymological research in the Russian Federation. Kireeva *et al.*[32] have dealt with enzymes participating in the C cycle in a grey forest soil, on which microplots (1.3 x 1.3 m) were installed and contaminated with 0, 8, 16 and 25 l crude oil/m^2. The results obtained in this experiment concerning activities of enzymes participating in the P and N cycles were published in 1997 and reviewed on pages 18-19 in [35].

Of the enzymes participating in the C cycle, invertase, cellulase, amylase and xylanase were studied. Soil was sampled from the 0-20-cm layer and from deeper layers of the microplots 3 days, 1, 6 and 12 months after contamination, in the first year, and then three times in the vegetation period during 10 years. Each activity measured in the 0-20-cm layer decreased in parallel with the rate of contamination and time in the first year. But after 10 years, invertase activity exhibited higher values in the contaminated than uncontaminated soil and the increase showed a parallelism with the rate of contamination. In contrast with these results obtained in the grey forest soil, invertase activity in a dark-grey forest soil was not significantly affected by any rate of contamination during the first 3 days, but 1 year after the contamination invertase activity gave increased values at 8 and 16 l crude oil/m^2 and decreased values at 25 l crude oil/m^2. At the same time, the other carbohydrase activities in the dark-grey forest soil behaved like in the grey forest soil.

Enzymological research in Venezuela. In a short report, López-Hernández *et al.* [45] presented some data on a field experiment, in which a savanna soil in Eastern Llanos was contaminated with oil. Following contamination, urease and phosphatase activities were systematically determined during a 60-day period. Oil contamination increased both activities to a maximum value at day 20; the activities decreased between days 20 and 30 and showed a slight increase in the period of 30-60 days.

Laboratory experiments

Contamination with crude oil

Enzymological research in Azerbaijan. Akhundova and Maslovetskaya [1] treated samples of an unpolluted soil with 0, 10, 100 and 200 mg crude oil/100 g soil. Their enzymatic activities, determined after 1, 10, 20, 30 and 60 days of incubation, showed that, in the first period of incubation, the crude oil, depending on its rate, decreased catalase, dehydrogenase and invertase activities, which later recovered at each crude oil rate. The recovery took place in 30 days (catalase), in 20 days (dehydrogenase) or in 60 days (invertase). Urease activity was not affected by either crude oil rate or incubation time.

Enzymological research in the Russian Federation. Kireeva *et al.* [32] contaminated surface (0-20 cm) samples of a grey forest soil with crude oil at rates ranging from 0.5 to 25% (volume/weight) and moistened them to 60% of WHC. Uncontaminated samples served for comparison. Carbohydrase activities in the samples were determined 3 and 15 days and 1 and 3 months after contamination (invertase and cellulase) and 3 days and 1, 6 and 12 months after contamination (amylase and xylanase). Each activity decreased with rate of contamination and incubation time. The decrease was most pronounced in the invertase activity.

Enzymological research in Venezuela. In the laboratory experiment briefly described by López-Hernández *et al.* [45], the soil, from which samples were collected, was the same as in the field experiment (see above). The oil contamination, incubation time and the results concerning urease and phosphatase activities were also the same as in the field experiment.

Contamination with oil products

Enzymological research in Austria. Bauer *et al.* [4] presented a synthesis of their investigations reviewed on pages 25-26 in [35].

Enzymological research in the Russian Federation. In continuation of the investigations, the first results of which were published in 1995 and 1997 and reviewed on pages 26-27 in [35], Kireeva *et al.* [32] treated surface (0-20 cm) samples of a grey forest soil with *n*-hexadecane, cyclohexane, aromatic oil fraction and partial oxidative degradation products of oil hydrocarbons (1-hexadecanol, palmitic, benzoic and salicylic acids) at rates of 0, 0.5, 1.0 or 2.0% (on soil weight basis). Moisture content of soil was brought to 60% of WHC. After 3 days and 1, 3, 6, 12 and 25 months of incubation, the samples were analysed for determination of their carbohydrase (invertase, cellulase, amylase and xylanase) activities. The analytical data presented in the paper show that, 3 months after addition of hydrocarbons at 2% rate, each activity was slightly increased by *n*-hexadecane and cyclohexane and strongly decreased by the aromatic oil fraction. One can deduce from Table 1 that the partial oxidative products of hydrocarbons also inhibited each activity; the strongest inbibitor was salicylic acid.

Table 1

Effect of partial oxidative products of hydrocarbons, applied at a rate of 2%, on carbohydrase activities in a grey forest soil [32]

Partial oxidative product	Carbohydrase activities			
	Invertase (mg glucose)	Cellulase (mg glucose)	Amylase (mg maltose)	Xylanase (mg xylose)
1-Hexadecanol	16.6	0.41	0.38	0.305
Palmitic acid	12.4	0.28	0.24	0.218
Benzoic acid	8.3	0.10	0.18	0.116
Salicylic acid	1.4	0.06	0.12	0.076

Enzymological research in Germany. The investigations on the effect of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), applied alone or in combination with heavy metals, on dehydrogenase activity in two regosols located in the Berlin area were described in a paper published by Koch and Wilke in 1996 and reviewed on pages 27-28[*] in [35]. These investigations were referred to by Wilke [80] in a review work on the soil microbiological effects of PAHs, PCBs and surfactants. The communication presented by Wilke and Koch [81] at the 16th World Congress of Soil Science (Montpellier, 1998) also dealt with these investigations.

According to Wilke [80] and Wilke and Koch [81], the PAHs fluoranthene and benzo[a]pyrene and the PCB-52 (2,2', 5,5'-tetrachlorobiphenyl) at a rate of 10 μg/g soil can cause up to 20% inhibition in soil dehydrogenase activity.

[*] There is a printing error on this page: in line 19, <0.2 should be corrected into <0.01.

Enzymological research in Romania. Popa [63, 64] carried out two experiments for studying the effect of fuel oil on enzyme activities and microbial enzyme synthesis in an alluvial soil (pH 7.5). Soil was sampled from the 5-15-cm layer. Air-dried samples (100 g each) were contaminated with 0, 0.1, 1 and 5 ml fuel oil dissolved in 10 ml acetone.

In the first experiment, after evaporation of acetone, the soil samples were moistened with 10 ml water and incubated at laboratory temperature for 24 hours, then analysed for determination of their actual and potential dehydrogenase activities. The analytical data showed that the fuel oil at each rate inhibited both activities and the degree of inhibition increased with increasing rate of fuel oil addition.

In the second experiment, after evaporation on acetone, the soil samples were amended with 0 or 10 g sucrose (inductor of the microbial synthesis of levansucrase), then moistened to 60% of WCH and incubated at laboratory temperature for 18 days. Following incubation, the samples were air-dried and, then, analysed for estimation of their levansucrase activity. It was found that the fuel oil slightly inhibited activity and biosynthesis of levansucrase, but the inhibitory effect did not change in dependence of the rate of fuel oil addition.

Enzymological research in Spain. Peña *et al.* [62] contaminated samples of a native Galician soil with diesel oil at different rates, up to 40 ml/100 g soil. The oil contamination caused a considerable decrease in urease activity; the degree of decrease was dependent on the rate of oil contamination. β-Glucosidase and phosphomonoesterase activities underwent only slight diminutions which were not dependent on oil rate. Although the microbial biomass strongly diminished oil rate-dependently, the soil respiration showed a strong, oil rate-dependent increase, which was attributed to survival and metabolic activity of the hydrocarbon-oxidising microorganisms. N mineralisation increased oil rate-dependently due to increase in ammonification, while nitrification remained unchanged.

Chapter 3. **Enzymological evaluation of the biological effects of the remediation of oil-contaminated soils in experimental models**

Field experiments

Contamination with crude oil

Enzymological research in the Russian Federation. Khaziev [30] described a 2-year experiment, in which two commercial biopreparations (Bacispecin and Devoroil), containing hydrocarbon-oxidising microorganisms, were used for remediation of an oil-contaminated leached chernozem in Bashkiria. Bacispecin is prepared from *Bacillus* sp. strain 739, whereas Devoroil is a mixture of several species of hydrocarbon-oxidising microorganisms. In each year, after mineral fertilisation of the contaminated soil, the plots were treated with 10 g of Bacispecin or Devoroil/m^2 or remained untreated (controls). At the end of the experiment, the degree of oil degradation was found to be 71 and 60% (in the Bacispecin- and Devoroil-treated soils, respectively), which is significantly higher than the 20% oil

degradation registered in the control soil. In comparison with dehydrogenase and invertase activities of the control soil, dehydrogenase activity exhibited 34 and 9% increases in the soils treated with the two biopreparations, while invertase activity showed a 17% decrease in the Bacispecin-treated soil and no changes in the Devoroil-treated one.

Kireeva *et al.* [31] have described a 3-year experiment on a grey forest soil contaminated accidentally with crude oil in Bashkiria. For remediation, the soil was amended with mineral (NPK) fertilisers and farmyard manure. The experimental variants and the results obtained in the first year, during which five soil enzyme activities were measured, had been published in 1986 and referred to on page 29 in [35].

After the first year, the experimental plots were divided into two halves. In the second and third years, one half was not further amended, whereas the other half received the same amendment at the same rate as in the first year.

Results of the determination of dehydrogenase and catalase activities have indicated that the amendment with mineral fertilisers and farmyard manure was the best measure for increasing the enzyme activities. Yearly administration of mineral fertilisers and farmyard manure was, in general, more efficient than a single administration. Dehydrogenase activity became, even in the first year, higher in the amended contaminated soil than in the uncontaminated soil, but the level of the catalase activity in the uncontaminated soil was not attained by any amended contaminated soil in any year.

Kiyamova [36] contaminated experimental plots, installed on the territory of the "Dzhalil'neft'" oil field (Tataria), with 12 l crude oil/m^2 and inoculated them with hydrocarbon-oxidising microorganisms from the culture collection of the Kazan State University and with microbial strains isolated from soil sampled in the zone of an oil well (Butulma city). The inoculations led to 30-80% increases in protease activity and even to higher (1.5-2-fold) increases in cellulolytic activity of the soil.

Laboratory experiments

Contamination with oil products

Enzymological research in the United States of America. As the refined oil products, including diesel oil, inhibited urease activity in the three Californian soils studied, urea was not recommended as a nitrogen fertiliser for remediation of soils contaminated with refined oil products (for details see pages 49-50 in [35]).

But - as Frankenberger [17] proved -, urea peroxide providing (due to soil catalase and urease activities) O_2 (aeration) and nutrient (NH_4^+) necessary for bioremediation of oil-contaminated soils, is a recommendable compound. He studied a Californian sandy loam soil (pH 7.4) contaminated with diesel oil. The surface (0-0.15 cm) layer, containing on average 2200 mg total petroleum hydrocarbons/kg soil, was sampled.

Experiments were carried out for studying the effects of pH of buffer solution, temperature and duration of incubation and concentration of urea peroxide on the release of NH_4^+ and formation of NO_3^- from urea peroxide added to samples of the contaminated soil. In another experiment, the thermal stability of urease in the contaminated soil was studied. The results of these experiments have shown that the release of NH_4^+ from urea peroxide was greatest at pH 7.5 to 8.5 and at 40°C, increased linearly during the first 24 hours of incubation and was approaching steady state at 0.25% concentration of urea peroxide. Urease was stable from 40 to 60°C, but it was irreversibly inactivated at >60°C.

In a final experiment, 25-g samples of the contaminated soil were subject to the following treatments:

a) sterilisation (0.16 Mrad γ-irradiation from a ^{60}Co source with 8 hours of exposure);

b) application of water to adjust the moisture level to 10% (weight/weight) (-33 kPa); and

c) application of urea peroxide (200 mg/kg soil) to field-moist soil.

The treated samples were incubated at 23°C for 2, 4, 6 and 8 weeks, then analysed for total petroleum hydrocarbons (TPH). Fig. 1 shows that, after 8 weeks of incubation, TPH declined from 2100 to 1680 mg/kg soil in the sterilised sample, indicating that abiotic factors contributed slightly to decrease in TPH over time. The moist treatment, in which only water was added, promoted a 50% decrease in TPH. The application of urea peroxide resulted in the greatest decline in TPH (from 2180 to 170 mg/kg soil, *i.e.* the decline was 92%).

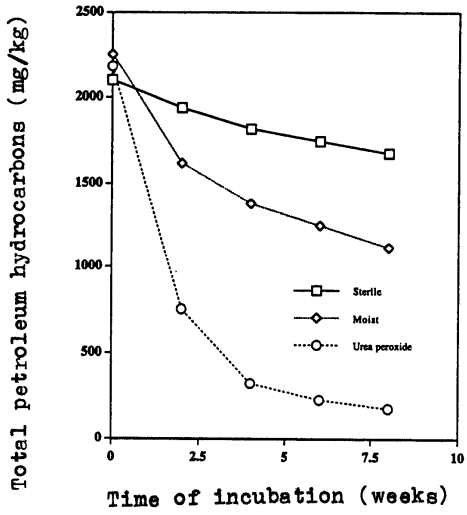


Fig. 1. *Decline in total petroleum hydrocarbons in diesel oil-contaminated soil, upon sterilisation, the addition of moisture and urea peroxide* [17].

Enzymological research in Austria. As the alpine environment is also exposed to oil pollution, Margesin and Schinner [47] have initiated an oil remediation experiment with alpine soils, at low temperature (10°C). Soil samples were taken from the uncontaminated C horizons in two typical areas (meadows) of the Tyrolean Alps. Soil A (collected at Kühtai) is a carbonate-free loamy sand (pH 6.3), soil B (collected at Hahntennjoch) is a carbonate-rich sandy loam (pH 7.2). The samples were contaminated by addition of 4000 mg diesel oil/kg soil (dry matter). The experiment comprised three variants:

a) soil NPK-fertilised to adjust C:N ratio to 10:1, N:P to 5:1 and P:K to 0.5:1 and, thus, to enhance the growth of the indigenous oil-degrading microorganisms;

b) soil NPK-fertilised as in *a* and inoculated with the culture of a diesel oil-degrading, cold-adapted nocardioform actinomycete (isolate RM7/11) (rate of inoculation being $5x10^5$ cells/g dry soil), to assess the effect of inoculum on oil degradation; and

c) soil poisoned with 0.3% $AgNO_3$ solution, to assess the abiotic oil degradation.

All variants were incubated in the dark at 10°C for 155 days. During incubation (at time 0 and at intervals of 7-14 days), the soil (the water content of which was maintained at 60% of WHC) was analysed for determination of the residual content of hydrocarbons; several physicochemical parameters as well as biological ones (including INT-dehydrogenase activity and basal respiration) were also determined.

The results have shown that the oil degradation process was similar in both soils. The biotic oil degradation in variant *a* reached the maximum during the first 33 days of incubation, resulting in a 50 and 60% oil degradation in soils A and B, respectively. During this time, the inoculum (variant *b*) showed a little higher biodegradation activity in both soils, but with further incubation no difference in oil degradation was detected between variants *a* and *b*. After 155 days of incubation, the residual content of hydrocarbons was 400 mg/kg in soil A and 380 mg/kg in soil B, *i.e.* the oil degradation was 90 and 95%, respectively. The abiotic oil degradation was about 30% in both soils. INT-dehydrogenase activity and basal respiration corresponded to the course of biodegradation activities in the soils.

In another experiment performed by Schinner *et al*. [66], samples of an agricultural soil were contaminated with diesel oil (5000 mg/kg soil). A part of the samples were NPK-fertilised, then all samples were incubated at 20°C for 120 days. At the end of incubation, the residual content of hydrocarbons was 1150 and 538 mg/kg soil in the unfertilised and fertilised samples, respectively. Urease, dehydrogenase and catalase activities and soil respiration were characterised by stress reactions: the activities increased immediately after oil contamination, then continuously decreased and increased again, which was attributed to succession of soil microbial populations. Urease activity was more sensitive to oil contamination than were dehydrogenase and catalase activities. Another finding was that the

contaminating oil induced the microbial synthesis of lipase. The conclusion was drawn that the soil enzymes give important information on the physiological and toxicological conditions in soil and are, thus, useful tools to monitor the impact of soil contamination and to evaluate the success of bioremediation. This conclusion is in good agreement with the opinion of Balba *et al.*[3], who consider that the assay of dehydrogenase activity is a useful, simple method for feasibility assessment and evaluation of the bioremediation of oil-contaminated soils.

Enzymological research in The Netherlands. Using soils from different sites contaminated with mineral oil and uncontaminated soils, van der Waarde *et al.*[78] have compared, in closed batch experiments, dehydrogenase activity (TTC reduction), FDA-hydrolysing activity, CO_2 production and bacterial numbers as indicators for oil biodegradation. The oil content was also determined. Dehydrogenase activity was found to be the parameter that had the best correlation with oil removal and, in several soils, also with CO_2 production. Biodegradation of oil in a peat-like soil did not take place and could not be monitored with CO_2 production, FDA hydrolysis or bacterial numbers, but the absence of bioremediation was confirmed with TTC reduction.

Part II. ENZYMOLOGY OF SOILS AFFECTED BY INDUSTRIAL EMISSIONS

Chapter 4. **Studies of the soil enzymological effects of the components of industrial emissions, through experiments modelled in the laboratory or in** in situ **artificial microcosms**

As these studies were summarised in excellent reviews, they were not dealt with in [35], in which only some of these excellent reviews were cited. Now we add to these reviews the papers by Dick *et al.* [13] and Dick [12] on soil enzyme activities as indicators of soil quality and soil health, respectively, and the paper by Kuperman and Edwards [42] on the soil biological, including soil enzymological, effects of acidic deposition.

Chapter 5. **Studies of the soil enzymological effects of industrial emissions originating from a point source (an industrial plant)**

Nonferrous metallurgical plants

Enzymological research in the United States of America. For complex studies, including determination of dehydrogenase activity, Kelly and Tate [29] have collected soil samples from several sites located at different distances (at 0.5-0.6 up to 6.5 km) from a zinc smelter that has been in operation since 1898 in a Mid-Atlantic state. All sites are downwind from the smelter. The top 15-cm soil

layer was sampled from four nonremediated sites (sampling period: autumn 1995) and from three nonremediated sites and four sites remediated in 1986, 1991, 1993 and 1995, respectively (sampling period: summer 1996). For remediation, a 2:1 (wet weight) mixture of municipal sewage sludge and power plant fly ash was surface-applied at a single rate of ~448 t/ha. For sites treated before 1992, limestone was also added to the mixture in an amount equivalent to 22 t/ha.

It has been found that in the nonremediated soils both total (soluble and insoluble) heavy metal (Zn, Cd, Cr, Cu, Ni and Pb) contents and soluble Zn content as well as decreased dehydrogenase activity reflected proximity to the smelter. Thus, the soil collected from the site, which is located at l.6 km from the smelter and is most contaminated with heavy metals, had, in autumn 1995 and in summer 1996, about 7 and 4 times lower dehydrogenase activity, respectively, than the soil collected from the less contaminated, most distant site (at 6.5 km from the smelter). Contrarily to dehydrogenase activity, the soil microbial biomass did not vary significantly in dependence of the distance from the smelter and, thus, in dependence of heavy metal contents. Similarly, no parallelism was recorded between dehydrogenase activity and total number (colony-forming units, CFUs) of bacteria and number (CFUs) of Zn-resistant bacteria.

Remediation of soils resulted in increase of pH (from 4.5-6 to 6.2-6.9), reduction of soluble heavy metal content and increase in dehydrogenase activity. The activity showed again no parallelism with the microbial biomass, but it changed in parallel with the changes in total number of bacteria and in the number of Zn-resistant bacteria.

The results obtained in these field studies were compared by Kelly *et al.* [28] with those found in a laboratory experiment, in which 20-kg air-dry samples of a loamy sand were treated with $ZnSO_4$ (at a rate of 6000 mg Zn/kg dry soil), brought to 33.3 kPa moisture content and incubated at room temperature for 420 days. No $ZnSO_4$ was added to the control samples. All samples were analysed after 15, 45, 90, 200 and 420 days of incubation.

The mean values of the soluble Zn content did not change significantly during the incubation; they were 4660 and 1.08 mg/kg soil in the treated and control samples, respectively. Dehydrogenase activity was lower in the treated than in the control samples and tended to decrease during the incubation; at day 420, dehydrogenase activity of the treated samples was 93% lower than the controls. In the treated soil as compared to the control soil, microbial biomass was lower for 200 days, whereas total number of bacteria gave lower values only at days 15 and 45, and the number of Zn-resistant bacteria tended to increase between days 45 and 420. In other words, dehydrogenase activity proved, under both field and laboratory conditions, to be a more sensitive indicator of the presence of high amounts of soluble heavy metals in soil than are microbial biomass and total number of bacteria.

Enzymological research in Poland. The first results of the soil chemical, enzymological and microbiological investigations in the areas surrounding the zinc smelter in Miasteczko Śląskie were published in 1975 and 1984 and reviewed on pages 77-78 in [35].

In continuation of these investigations, Olszowska [57] used four experimental plots set up at 11.0, 8.0, 5.5 and 0.5 km from this smelter. The plots correspond to the pollution zones I, II, III and IV, respectively. Zones I and II are located within the Świerklaniec Forest District, whereas zones III and IV belong to the Koszecin Forest District. In all zones, the soil is podzolic. In zones I-III, the vegetation is dominated by pine (*Pinus sylvestris*), but zone IV is covered by *Deschampsia flexuosa* grassland. Samples were taken from the organic Oh and humus A layers twice a year (in spring and autumn) during four years (1988-1989 and 1991-1992) and submitted to chemical and enzymological analyses. The air was also analysed.

As expected, the SO_2 and NO_2 (expressing NO_x) contents in air and the heavy metal contents in the dustfall increased from zone I toward zone IV. For exemplification, we quote the data obtained for zones I and IV, respectively, in 1992: 13.12 and 38.53 mg SO_2/m^2/month; 0.16 and 0.61 mg NO_2/m^2/month; 0.97 and 12.70 kg Pb/ha/year; 0.29 and 6.80 kg Zn/ha/year; and 0.012 and 0.096 kg Cd/ha/year. But the total dustfall was higher in zone I than in zone IV (440 and 219 kg/ha/year, respectively).

Excepting invertase and β-glucosidase activities which did not change significantly depending on the degree of soil pollution, the other activities measured (urease, asparaginase, acid phosphatase and dehydrogenase) decreased with increasing heavy metal content in soil. Each activity correlated significantly with the organic C content in soil. The negative correlation of each activity with the Pb, Zn or Cd content had higher (significant or insignificant) coefficients in the humus A layer than in the organic Oh layer.

Based on the results of these investigations, Olszowska [57] has drawn the conclusion that urease, asparaginase and dehydrogenase activities may be used as sensitive and early indicators of the stress caused by chemical pollution of the soil.

The effect of the emissions from the zinc smelter in Miasteczko Śląskie on enzyme activities in podzolic soils under *Pinus sylvestris*-dominated forest stands was studied by Januszek [25], too. Samples were collected from the humus and mineral-humus horizons in heavily and medium-polluted sites at Brynica and Pniowiec, respectively (Świerklaniec Forest District) and in unpolluted sites in Herby (Herby Forest District). As expected, the heavy metal contents in the humus and mineral-humus horizons increased with increasing degree of pollution. For example, the contents of Cu and Pb (mg/kg soil) were 96, 653 and 1875, and 77.6, 92.5 and 584.0 in the unpolluted, medium- and heavily polluted humus horizons, respectively.

The enzyme activities in the humus and mineral-humus horizons behaved rather irregularly in dependence of the degree of heavy metal pollution. Thus, dehydrogenase activity was highest in the medium-polluted or unpolluted soil. Invertase activity gave the highest value in the heavily polluted soil, whereas urease was most active in the medium-polluted soil. Only the phosphatase activity was highest in the unpolluted soil.

In the medium-polluted Pniowiec soil, the enzyme activities were determined separately in the mineral-humus horizon under four tree species. Dehydrogenase, urease and phosphatase activities were highest under *Quercus robur* and lowest under *Larix decidua*. Invertase was most active under *Larix decidua* and least active under *Pinus sylvestris*. The mineral-humus horizon under *Betula verrucosa* gave an intermediary value for each of the four enzyme activities determined.

The soil enzymological and microbiological effects of the emissions from a zinc smelter located in the Upper Silesian Industrial District and from a copper plant located in the Legnica-Glogów Copper District were studied by Zwoliński *et al.* [82]. An unpolluted area in the Sieradz province was the control. All areas are characterised by podzolic soil and *Pinus sylvestris*-dominated plant cover. The experimental plots (0.5 ha each) were set up at 5.5, 8.0 and 11.0 km from the zinc smelter, and at 0.8, 2.0 and 3.5 km from the copper plant. The amounts of dustfall decreased with increasing distance of the plots from the pollution source. Thus, it was about 86, 83 and 65 t/km^2/year emitted by the zinc smelter, and 118, 87 and 72 t/km^2/year emitted by the copper plant. It was only 32 t/km^2/year in the control area. The dusts contained a considerable amount of heavy metals (Cu, Zn, Pb, Cd).

Invertase, β-glucosidase, urease, asparaginase and dehydrogenase activities decreased with increasing degree of soil pollution, but phosphatase activity was not affected by pollution. Soil respiration determined by measurements of CO_2 production and O_2 uptake distinctly declined with the increase of pollution. As the CO_2 production decreased to a larger extent than did the O_2 uptake, the respiratory coefficient ($Q=CO_2/O_2$) decreased very markedly. Cellulose decomposition was also strongly diminished by pollution. Nitrification was more strongly decreased than ammonification.

Significant negative correlations were found between invertase activity, cellulose decomposition, total dustfall and heavy metals (Cu, Pb, Cd). As regards β-glucosidase activity, such dependence referred only to heavy metals (Zn, Pb, Cd).

Numbers of bacteria and microfungi decreased, but the number of actinomycetes increased with increasing degree of pollution. The percentage share of pleomorphic rods (*Arthrobacter, Mycobacterium, Nocardia, Streptomyces*) and pigment-producing Gram-negative rods (*Pseudomonas, Flavobacterium*) in the total number of bacteria and actinomycetes increased with increase of pollution, but in the case of spore-forming rods (*Bacillus*) an opposite tendency was evident.

The effect of the emissions from the Boleslaw zinc smelter on dehydrogenase and phosphatase activities in the mor humus of soils under *Pinus sylvestris*-dominated forests located at 10-12 km from the smelter, in the Olkusz

Forest District, and at about 65 km from the smelter, in the Jedrzejów Forest District, was studied by Januszek [25]. The heavy metal contents (mg/kg soil) in the mor humus of the more polluted Olkusz and the less polluted Jedrzejów stands were the following: 368.1 and 108.6 (Zn); 470.9 and 107.3 (Pb); 11.6 and 6.8 (Cu); 6.0 and 0.74 (Cd); 145.7 and 144.4 (Mn); and 1437.8 and 695.2 (Fe), respectively. Mean dehydrogenase activity (mg TPF/100 g soil/24 hours) was significantly ($p<0.05$) lower in the more polluted humus (2.24) than in the less polluted one (4.92), but phosphatase activity (mg phenol/5 g soil/2 hours) in the more and the less polluted humus (20.31 and 22.52, respectively) was not significantly different.

Dusts collected from electrofilters operating in zinc and copper smelters and in other industrial plants were added to experimental plots installed in the Niepolomice Forest. These field experiments and the results obtained in the chemical, enzymological and microbiological analyses of the soil in dust-treated and untreated (control) plots were described in papers published in the 1979-1989 period and reviewed on pages 78-82 in [35].

Of the 240-m^2 plots installed in a mixed pine-oak forest stand in the Niepolomice Forest in 1980 (see page 79 in [35]), Olszowska [58] has selected, for chemical and enzymological analyses, the plots treated with zinc dust and those treated with cadmium dust at rates of 0, 100, 500, 1000, 2000 and 5000 t/ha/year. She took samples from the Oh and A horizons of the plots in springs and autumns of four years (1988-1989 and 1991-1992) and used the mixed (Oh + A) samples for determination of organic C content, pH and six enzyme activities.

The mean values of the analytical data were calculated for the whole, 4-year experimental period. They have shown that the organic C content tended to decrease and the pH tended to increase in dependence of the rate of dusts. The Zn dust, in comparison with the Cd dust, led to a less pronounced decrease in organic C content and to a more pronounced increase in pH. Thus, at the 0 and the highest rate of dusts, organic C contents were 11.89 and 9.46% (Zn dust) and 7.54 % (Cd dust), whereas pHs in H_2O were 3.98 and 5.33 (Zn dust) and 4.06 (Cd dust), respectively.

All enzyme activities were negatively affected by the dusts. Invertase, β-glucosidase, asparaginase and acid phosphatase activities did not show, whereas urease and dehydrogenase activities did show a clear trend to decrease with increasing rate of dusts. At the highest rate, the Zn dust, in comparison with the Cd dust, was less inhibitory on β-glucosidase and asparaginase activities and more inhibitory on urease, acid phosphatase and dehydrogenase activities; there was no difference between the two dusts in respect of their inhibitory effect on invertase activity. Dehydrogenase activity was the most sensitive enzyme activity: it suffered significant ($p<0.001$) decreases, which were 89.1 and 92.6% at the highest rate of Zn and Cd dust, respectively.

In the same 4-year period (1988-1989 and 1991-1992), Olszowska [59] has also studied the effect of liming and fertilisation on the same chemical parameters and enzyme activities in plots treated with Zn and Cd dusts at the same

rate as those mentioned above. In the spring of 1987 a part of the plots were amended with lime (2 t/ha) and mineral fertilisers (200 kg NPK/ha). In the spring of 1989, the NPK fertilisation was repeated at a half rate.

Comparison of the mean values in the control and dust-treated plots has indicated that liming and fertilisation did not result in significant changes in soil organic C content, but raised soil pH insignificantly in the Zn dust-treated plots and significantly ($p<0.05$) in those treated with Cd dust.

Under the influence of liming and fertilisation, invertase, asparaginase and dehydrogenase activities increased significantly ($p<0.05$) in the Zn dust-treated plots, whereas β-glucosidase activity decreased significantly and dehydrogenase activity increased significantly in the Cd dust-treated plots; urease and acid phosphatase activites did not undergo significant changes in either Zn- or Cd-treated plots. It was drawn again the conclusion that dehydrogenase activity is the most sensitive indicator of soil pollution with heavy metals and also of the effcts of technologies applied for remediation on such soils.

Galiulin *et al.* [20] have carried out laboratory experiments using soil samples taken in an agricultural area located at 1-2 km from the metallurgical enterprise "Orzel Bialy" (Katowice voivodship). This enterprise had been founded in the middle of the 19th century, but since 1989 it is specialised in recovery of lead from broken accumulators for producing antimonous lead.

Due to the industrial emissions, the studied soil (a loamy sand; pH in H_2O 6.40) was polluted with heavy metals, the contents of which (in mg/kg soil) were: 1850 (Zn), 492.1 (Pb), 476.6 (Mn), 26.32 (Cu), 14.96 (Cd), 10.73 (Cr), 10.58 (Co), 0.497 (Mo).

The experiments aimed to study the removal of heavy metals by chelating compounds and the effect of these compounds on soil catalase and dehydrogenase activities and microbial decomposition of cellulose.

Fifty-g soil samples were treated with *a*) 1, 5, 10 or 20 mM EDTA (potassium ethylenediamine tetraacetate)/kg soil; *b*) 5 mM EDTA + 10 mM citric acid or 10 mM acetic acid or 10 or 50 mM nitric acid/kg soil. The aqueous solutions of the compounds were added in such volumes that brought the moisture content of soil to 70% of WHC. Some soil samples were treated with a 1:1 (volume:volume) mixture of sediment of liquid dung and water up to 70% of WHC. Soil samples to which only water was added served as controls. All samples were incubated at 30^0C for 63 days. The contents of soluble heavy metals (Zn, Pb and Cd), extractable with water (at 1:5 soil:water ratio), were determined after 3 and 63 days of incubation, whereas catalase and dehydrogenase activities and cellulose decomposition were measured several times during the incubation period.

After 3 days of incubation, it was found that the amounts of soluble heavy metals increased with increasing rate of EDTA, but the effect of 5 mM EDTA + citric or acetic or nitric acid was not better than that of 5 mM EDTA alone. Further incubation to 63 days did not improve extractability of heavy metals, excepting a single case: 5 mM EDTA + 50 mM nitric acid led to solubilisation of more heavy metals than did 5 mM EDTA alone.

After 14 days of incubation, the samples treated only with EDTA exhibited increased catalase activity in the following order of the EDTA concentrations: 20 mM ≈ 10 mM > 5 mM > 1 mM; dehydrogenase activity increased in the order 10 mM > 20 mM, and showed no changes at 5 and 1 mM. Catalase activity increased in the EDTA + citric acid treatment and decreased in the other treatments (EDTA + acetic acid, EDTA + nitric acid, sediment of liquid dung); dehydrogenase activity increased under the influence of two treatments (EDTA + citric acid; sediment of liquid dung) and decreased under the influence of the other treatments. The negative effect of the EDTA + nitric acid treatment was stronger on dehydrogenase than on catalase activity.

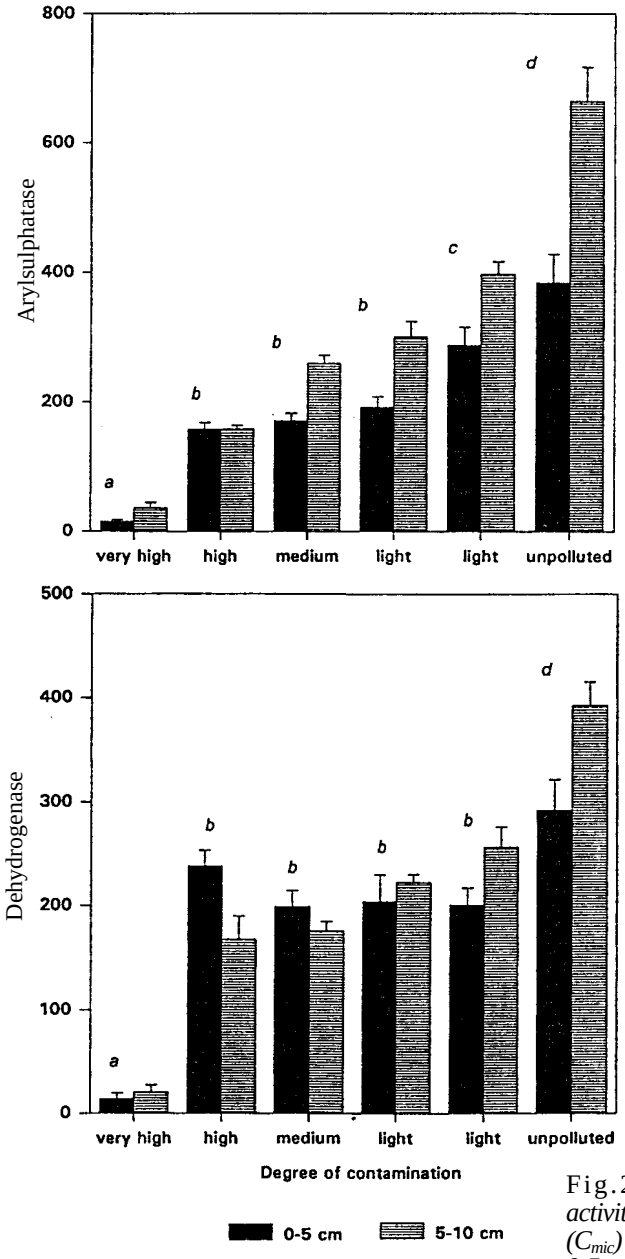
After 63 days of incubation, catalase activity remained increased in the samples treated with EDTA only, but in these samples dehydrogenase activity at 1 and 5 mM EDTA was not different from the activity measured in the control samples and decreases occurred in the activity at the 10 and 50 mM EDTA. The decreasing effect of the EDTA + nitric acid treatment on both activities was again evident and stronger (85.8%) on dehydrogenase activity than on catalase activity (54.5%). Contrarily, microbial decomposition of cellulose was most intense in the EDTA + nitric acid treatment during the whole (63-day) incubation period.

The conclusion was drawn that much attention should be paid to the concentration of chelating agents used for remediation of heavy metal-polluted soils in order to avoid soil pollution by the chelating agents themselves.

Enzymological research in the Russian Federation. The first results of the soil enzymological research in the Sredne-Uralsk area were reviewed on page 90 in [35]. Kaigorodova and Vorobeichik [27] and Kaigorodova [26] have also described investigations carried out in forests located around the Cu smelter in Sredne-Uralsk. For example, they have determined systematically the rate of cellulose decomposition and urease activity in surface samples collected at 1-km distance from the smelter (in the "technogenic desert") and at 30 km from the smelter (in an unpolluted area). In the technogenic desert, the forest litter was completely destroyed and replaced by a 2.5-5-cm thick peat-like layer consisting of partly decomposed mass of the moss *Pohlia nutans*, whereas in the unpolluted area the surface was covered by pine litter.

Cellulose decomposition rate was 200-2000 times lower and urease activity was 2.3-2.5 times lower in the peat-like layer on the technogenic desert than in the forest litter on the unpolluted area.

Mukatanov and Shigapov [52] have determined invertase and dehydrogenase activities in plots located at different distances from the Cu smelter in Karabash (Chelyabinsk region). Invertase activity (expressed in mg glucose/g soil) was 0.30, 0.60, 0.65 and 2.36, and dehydrogenase activity (expressed in mg TPF/g soil) was 0.026, 0.030, 0.030 and 0.450 in samples taken at 1.9, 2, 6 and 18 km from the smelter, respectively. These data mean that the emissions from the smelter caused decreases in both soil enzyme activities.



Enzymological research in Austria. Tscherko and Kandeler [77] have studied the effect of the emissions from the aluminium smelter near Ranshofen, Upper Austria on soil microbial biomass, arylsulphatase and dehydrogenase activities. The electrolysis process of the smelter operated between 1939 and 1992. The smelter emitted about 170 t F/year (1982), mainly gaseous HF (about 80%) and aerosols of cryolite, NaF and $A1F_3$. In May and October 1993, soil was sampled from six grassland sites located at distances of 0.5, 0.5, 1.0, 3.5, 4.2 and 15.0 km from the smelter. Sampling depths were 0-5 and 5-10 cm. The water-extractable F content decreased from the average value of 124 mg/kg soil (in the very highly contaminated soil at 0.5 km from the smelter) to 10 mg/kg soil (in the unpolluted soil at 15 km from the smelter).

Fig. 2 shows that arylsulphatase activity as related to microbial biomass C increased in parallel with diminution of contamination degree.

Fig.2. *Arylsulphatase and dehydrogenase activities as related to microbial biomass C (C_{mic}) in F-contaminated soil sampled from 0-5- and 5-10- cm depths in May 1993* [77].

Expression of enzyme activities: arylsulphatase in µg *p*-nitrophenol/g C_{mic}/hour, and dehydrogenase in µg TPF/mg C_{mic}/16 hours. Bar shows arithmetic mean of five replicates and whisker indicates standard error. Degree of contamination corresponds to different distances from the smelter: very high (0.5 km), high (0.5 km), medium (1 km), light (3.5 and 4.2 km), unpolluted (15 km). Bars not sharing the same letter are significantly different at $p < 0.05$.

As expected, the difference was greatest between the unpolluted and the very highly contaminated soil. Values of dehydrogenase activity as related to microbial biomass C in highly, medium- and lightly contaminated soil were not significantly different, but the greatest difference in this activity, too, appeared between the unpolluted and the very highly contaminated soil.

The linear correlation coefficients between the water-extractable F concentrations and the microbial biomass, arylsulphatase and dehydrogenase activities were r=-0.80, -0.84 and-0.86, respectively.

As microbial biomass and dehydrogenase activity decreased substantially where the concentration of F exceeded 100 mg/kg soil, whereas arylsulphatase activity was already inhibited at 20 mg F/kg soil, the conclusion was drawn that the ratio of arylsulphatase to microbial biomass C can be used as a sensitive index for evaluating environmental stress such as F contamination.

Enzymological research in Uzbekistan. The serozems around the nonferrous metallurgical plant in Almalyk were studied by Fedorov [16]. He found that the Cu content in these soils was highest at 0.5 km from the plant, whereas the largest amount of Zn was recorded in soils at 1-3 km from the plant, and even at 23 km the Cu, Zn and Pb contents exceeded 1.5-2.0 times the heavy metal contents of unpolluted soils. In irrigated serozems the heavy metal contents were high in the whole arable layer, while in the non-irrigated serozems the heavy metals accumulated in the top 7 cm.

In the highly polluted serozems urease activity as well as nitrification and N_2-fixing capacities suffered 1.4-2.4-fold decreases. The effects of heavy metals were more pronounced in the non-irrigated than in the irrigated serozems.

Enzymological research in France. For chemical and enzymological study of the soils in the industrial fallow of Mortagne du Nord (Nord-Pas de Calais), Lattaud *et al*. [44] have selected five sites accordong to a heavy metal gradient. Sites I-III are under herbaceous vegetation and sites IV-V are located in a poplar plantation. Two unpolluted sites (in a grassland and a poplar plantation, respectively) served as controls. At all sites, soil was sampled from the surface layer and from a deeper one for determination of pH, heavy metal (Zn, Cu, Pb and Cd) contents, alkaline and acid phosphatase activities. For determination of these activities the surface and deeper soil samples were taken from the 0-15- and 15-25-cm depths, respectively.

The soils in both layers were nearly neutral at sites I-III, alkaline at sites IV-V and acid at the control sites. The amount of each heavy metal was higher in the surface than in the deeper soil layer. The contents of heavy metals in the surface layer increased in the order: Zn > Pb > Cu > Cd (sites I, III-V) or Zn > Cu> Pb >Cd (site II). Contents (μg/g soil) of the most abundant Zn and of the least abundant Cd in the surface layer varied between 26363 (site II) and 2812 (site V), and between 149 (site II) and 21 (site V), respectively.

At each site, both alkaline and acid phosphatase activities were detectable in both soil layers, but they were higher in the surface than in the deeper layer. Alkaline phosphatase activity gave the highest values in the alkaline soils of sites

IV and V, while acid phosphatase was most active in the acid soils of the control sites. But the finding, that acid phosphatase activity was higher in the alkaline soils of the less polluted sites IV and V than in the nearly neutral soils of the more polluted sites I-III, indicates that this enzyme activity was influenced not only by soil pH, but also by the polluting heavy metals. However, the statistical analyses, which confirmed the correlation of acid phosphatase activity with soil pH, revealed no correlation of this activity with the Zn content.

Ironworks

Enzymological research in Poland. Zwoliński *et al.* [82] have dealt with the soil enzymological and microbiological effects of the emissions (containing SO_2 and dust bearing heavy metals) from a metallurgical complex (ironworks and steel mill) located in the southern part of the Niepolomice Forest. An experimental plot (0.5 ha) on podzolic soil under pine (*Pinus sylvestris*)-dominated plant cover was installed at 22 km from the pollution source. A plot with similar soil and vegetation characteristics in an unpolluted area (Sieradz province) served for comparison. For analyses, nonrhizospheric samples were taken from the 0-5-cm layer (horizon A_1) of the plots in springs and autumns of the 1982-1984 period.

Comparison of the polluted and unpolluted plots by using the mean values of analytical data recorded during the three years of the experiment led to a series of results, of which some will be specified below.

Invertase, β-glucosidase and urease activities, cellulose decomposition, ammonification and number of bacteria were not significantly affected, while asparaginase activity was slightly increased, phosphatase activity was slightly decreased and dehydrogenase activity, respiration, nitrification and numbers of actinomycetes and microfungi were strongly decreased by the pollution.

The conclusion could be drawn that the emissions had a polluting effect even on the area located at a great distance (22 km) from the metallurgical complex, but the degree of pollution should be considered low.

Ore enrichment works

Enzymological research in Romania. The soils in the area of a nonferrous heavy metal mine located in the vicinity of the village of Turț (Satu Mare county) were studied, from chemical, microbiological and enzymological viewpoints, by Kolosváry [37]. Sulphide ores (sphalerite, galena, calcopyrite, pyrite) are mined and processed here for enrichment. The area is affected by atmospheric pollution deposits. Along a transect, soil samples were taken periodically (during 1995 and 1996) from the 0-10-cm depth and analysed for determination of pH, heavy metal (Zn, Pb, Cu, Co, Cr, Ni, Cd, Mn) contents, total number of the aerobic heterotrophic bacteria, number of the colony-forming units (CFUs) of *Azotobacter*, respiration, actual and potential dehydrogenase, catalase and phosphatase activities.

The number of *Azotobacter* CFUs was found to be a more synthetic indicator of pollution than the content of any of the eight metals analysed. Actual dehydrogenase and catalase activities, like respiration and pH, significantly correlated (at least at $p < 0.05$) with the number of *Azotobacter* CFUs in 1996, but not in 1995, whereas potential dehydrogenase and phosphatase activities, like total number of aerobic heterotrophic bacteria, never gave significant correlations with the number of *Azotobacter* CFUs. The lack of correlations in 1995 and appearance of some correlations in 1996 may be related to some diminution of atmospheric pollution deposits in 1996.

Pulp and paper mills

Enzymological research in the Russian Federation. Enzyme activities in alluvial soils around a mill manufacturing cellulose and cardboard were studied by Antonenko [2] during 1992 and 1993. This mill operates near the bank of the Selenga River, a tributary of the Baikal Lake. For analysis of atmospheric pollution deposits snow samples were collected along 3-5-km distances from the mill. The samples contained 80 times more Na^+, 10-15 times more K^+ and 5-10 times more NH_4^+ than the snow on unpolluted areas.

Due to the pollution, the soil enzyme (invertase, urease, protease, polyphenol oxidase) activities showed decreased values; the productivity of agrocoenoses and natural phytocoenoses also decreased.

Other chemical factories

Enzymological research in the Russian Federation. Results of the investigations, in which the effect of the emissions (polluting the atmospehere with fluoride and S-containing compounds) from the cryolite factory located near the town of Polevskoi on the catalase activity in litter (01 and 02 horizons) and soil (A_1 horizon) of experimental plots set up in pine plantations at 1, 1.5, 4, 7 and 26 km from the factory, were published by Kovalenko *et al.* in 1996 and reviewed on pages 105-106 in [35]. The investigations were continued and the new results were published by Kovalenko *et al.* [38] and Shebalova and Babushkina [69].

Kovalenko *et al.* [38] have studied only the permanent plots installed at 1.5, 7 and 26 km from the factory, *i.e.* a strongly polluted, a medium-polluted and an unpolluted plot, but have determined, beside catalase activity, other enzyme activities, too.

In all plots, the enzyme activities in the litter exhibited broad seasonal and annual variations and were not always dependent on the degree of pollution. At the same time, most of the activities (catalase, dehydrogenase, peroxidase, polyphenol oxidase, cellulase) in the soil (A_1 horizon) decreased with increasing degree of pollution.

There was a direct relationship between sulphate reductase activity and sulphate content in litter and soil. As the increased sulphate content originated from the emissions of the cryolite factory, sulphate reductase activity indicates the degree of pollution with sulphate and participation of this enzyme in the removal of the pollutant.

The investigations of Shebalova and Babushkina [69] made it possible to compare enzyme activities in the litter (01 and 02-03 horizons) and soil (A_1 horizon) of a strongly and a weakly polluted plot. In both plots, invertase, cellulase, protease, catalase, peroxidase and polyphenol oxidase activities were higher in the litter than in the soil. Dehydrogenase activity was lower in the 01 horizon, but higher in the 02-03 horizon than in the A_1 horizon. Sums of the invertase, cellulase, dehydrogenase and polyphenol oxidase activities of the three horizons were higher values in the weakly than in the strongly polluted plot, whereas the reverse was true for the sums of catalase and peroxidase activities. The sums of protease activity were similar in the two plots.

Enzymological research in Poland. Bielińska *et al.* [7] have determined enzyme activities in mostly sandy soils under several degraded forest stands located in the vicinity of the nitrogen fertiliser factory in Pulawy. As Table 2 shows the enzyme activities tended to decrease with decreasing distance from the factory. In the soils of the three closest stands (1P-3P) dehydrogenase activity was not detectable at all, but phosphatase activity was highest in the soil of stand 3P. All activities, excepting phosphatase activity, were highest in the soil of the most distant stand (5Ż).

The finding on the depth dependence of the enzyme activities should also be emphasised. Dehydrogenase activity (which results from the activity of living, proliferating microbial cells) is lower in the upper (5-10-cm) soil layer than in the deeper (10-20-cm) layer, whereas urease, protease and phosphatase activities (which are the result of the activities of accumulated enzymes) give higher values in the upper than in the deeper soil layer. As the enzymes in the proliferating microbial cells are more sensitive to inactivating factors than are the accumulated soil enzymes, the finding emphasised above indicates that the upper soil layer was more strongly affected by the emissions from the factory than the deeper soil layer.

Enzymological research in Germany. The soil chemical, microbiological and enzymological properties in an area affected by alkaline dust deposits which originated from the emissions of a phosphate fertiliser factory have been studied by Langer *et al.* [43] 8 years after the closing down of the phosphate-manufacturing operations. High total concentrations of the major dust constituents (P, Na, F, Cd) and increased pH values were still found in the affected soils.

In 2-year field experiments, highly and medium-polluted and unpolluted quackgrass (*Agropyron repens*) stands along a gradient of decreasing dust deposition were compared. Humus accumulation and microbial activities were highest in the most polluted soil and lowest in the unpolluted one. Pot experiments confirmed a positive correlation between the input of alkaline dust and microbial activities (basal respiration, C mineralisation, litter decomposition) and enzyme (INT-dehydrogenase, alkaline phosphatase and arylsulphatase) activities. Microbial biomass was, however, lowest in the highly polluted soil.

Table 2

Chemical properties and enzyme activities of soils in degraded forest stands located in the vicinity of the nitrogen fertiliser factory in Pulawy [7]

Region	Forest station	Stand number	Stand surface (ha)	Dominant plants	Distance from the factory (km)	Soil depth (cm)	pH in KCl	Total C (%)	Total N (%)	C:N	Enzyme activities*			
											Dehydro-genase	Urease	Protease	Phos-phatase
Pulawy	Wronów	1 P	3.39	Birch, bird cherry	0.5	5-10	3.1	1.50	0.63	2.4	0	177.21	9.52	10.76
						10-20	3.5	0.82	0.24	3.4	0	171.54	6.65	4.39
		2 P	11.25	Birch, bird cherry	0.8	5-10	3.1	2.84	1.02	2.8	0	189.72	11.17	12.72
						10-20	3.4	1.60	0.45	3.6	0	154.21	7.78	7.12
		3 P	9.77	Pine, oak, alder	2.0	5-10	3.5	1.45	0.49	3.0	0	233.55	12.36	22.06
						10-20	3.6	0.74	0.27	2.7	0	179.04	9.02	10.17
Żyrzyn	Zagórki	1 Ż	5.60	Pine, birch, spruce, alder	7.0	5-10	3.4	12.01	4.56	2.6	0.57	249.16	18.27	17.20
						10-20	3.7	2.01	0.64	3.1	0.63	218.86	11.17	3.26
		2 Ż	3.63	Pine, alder	8.0	5-10	2.8	7.69	2.83	2.7	0	198.67	9.22	18.68
						10-20	3.4	2.04	0.63	3.2	0	182.41	5.53	5.49
	Kozi Bór	3 Ż	7.42	Pine, hornbeam, aspen	15.0	5-10	2.8	2.60	1.30	2.0	0.35	265.76	18.76	12.29
						10-20	3.1	1.65	0.74	2.2	0.52	221.30	10.78	5.33
	Wola Osińska	4 Ż	3.90	Oak, maple, larch, spruce, alder	15.5	5-10	3.4	2.65	1.23	2.2	0.31	245.32	11.21	18.46
						10-20	3.5	1.17	0.53	2.2	0.46	185.47	6.44	8.17
		5 Ż	9.78	Pine, oak, alder	16.0	5-10	3.7	3.10	1.75	1.8	0.57	345.39	21.87	20.67
						10-20	3.9	1.40	0.74	1.9	0.63	216.58	8.96	9.72

* Expression of enzyme activities: dehydrogenase in μg TPF/g dry soil/24 hours; urease in μg NH_4^+ - N/g dry soil/24 hours; protease in μg tyrosine/g dry soil/hour; and phosphatase in μg *p*-nitrophenol/g dry soil/hour.

Coal-fired power plants

Enzymological research in the Russian Federation. Results of the investigations on the soil enzymological effects of the emissions from the power plants operating in the Nazarovo Basin were described by Nikitina *et al.* in 1988 and Naprasnikova in 1993 and reviewed on page 111 in [35]. These power plants belong to the Kansk-Achinsk Fuel-Energetic Complex (Siberia) and use, for electricity generation, the brown coal stripmined in this basin.

Sorokin and Gukasyan [74] and Sorokin [73] have dealt with the effects of the power plant emissions in the Nazarovo Basin on microorganisms in soils, on enzymes and microorganisms in litters and also on microorganisms living on the surface of leaves (phyllosphere).

The forests selected for the investigations included the polluted Dorokhov and Pioner spruce forests located at 5-7 km from the power plant, the unpolluted Zakharin spruce forest located at 70-80 km from the power plant and the unpolluted Adadym birch forest.

Soils, litters and leaves were sampled and analysed 30 times during the vegetation periods in four years (1982-1985). Mean values of the analytical data showed:

- no significant difference between the microfloras of polluted and unpolluted soils;
- decreased enzyme (dehydrogenase, catalase, invertase, urease and phosphatase) activities; decreased respiration rate; increased gelatinolytic capacity; increased number of bacteria and decreased numbers of actinomycetes and microfungi in the polluted litters as compared to the unpolluted ones;
- higher number and biomass of the phyllosphere microorganisms, but weaker physiological activities of the isolated strains in the polluted than in the unpolluted forests.

ADDENDUM

Military waste disposal operations

Enzymological research in the United States of America. The soil chemical, enzymological and microbiological investigations on an area in the U.S. Army's Proving Ground at Aberdeen, Maryland were described in detail by Kuperman and Carreiro in 1997 and reviewed on pages 112-114 in [35]. Now we mention that these investigations were referred to by Kuperman in a book entitled "Bioindicator Systems for Soil Pollution" and published in 1996 [41].

Chapter 6. **Studies of the soil enzymological effects of industrial emissions originating from multiple sources (many industrial plants manufacturing different products, but situated in the same industrial area)**

Enzymological research in Poland. In 3-year experiments, Januszek [23, 24] has determined, several times, four enzyme activities and cellulose decomposition rate in mor humus horizons of podzolised soils in spruce (*Picea abies*) forest stands

of two areas affected by different industrial emissions. One area is located in the western Sudetes (Izary and Karkonosze Mountains belonging to the Szrenica Massif, Szklarska Poreba Forest District). The other area is located in the Tatra Mountains (Czuba Roztocka Mountain, Tatra National Park). In each area, five permanent plots (~ 0.25 ha each) on 40-120-year-old stands (Sudetes area) and on 80-year-old stands (Tatra area) were selected for the studies. The industrial emissions affecting the Sudetes area contained more SO_2, NO_x and fluoride than those affecting the Tatra area, but nearly the same amount of dustfall affected both areas. The heavy metal content in raw humus was different in the two areas. The mean contents of Cu and Pb were 2.5 and 1.7 times, respectively, higher, whereas the mean contents of Zn, Mn and Cd were 3.9, 2.2 and 1.5 times, respectively, lower in the Sudetes than in the Tatra area.

Phosphatase activity was, on average, 1.5 times lower, but dehydrogenase, invertase and urease activities and cellulose decomposition rate were, on average, 1.5, 1.3, 1.3 and 1.5 times, respectively, higher in the Sudetes than in the Tatra area.

In another experiment, carried out by Dahm *et al*. [11], dehydrogenase activity and several microbiological parameters were deterimed in the surface (0-15 cm) layer of a podzolised soil (pH 6.1) under a 22-year-old pine (*Pinus sylvestris*) forest at Brynica (Świerklaniec Forest District), this forest being polluted by heavy metals from industrial emissions and of another podzolised soil (pH 4.5) under a 27-year-old *Pinus sylvestris*-dominated mixed (*Betula verrucosa, Quercus sessilis, Larix europaea*) forest at Herby (Herby Forest District), this forest not being affected by industrial amissions. The heavy metal contents (mg/kg soil) were 149 (Pb), 130 (Zn), 4.7 (Cu) and 2.4 (Cd) in the polluted (Brynica) soil and 60.5 (Pb), 16 (Zn), 4.8 (Cu) and 0.5 (Cd) in the unpolluted (Herby) soil.

Potential dehydrogenase activity (measured in reaction mixtures amended with a respiratory substrate: glucose, Na acetate, casein hydrolysate, Na pyruvate or soluble starch) was, as expected, higher than the actual dehydrogenase activity (measured without added substrate) in the unpolluted soil. But there was no significant ($p > 0.05$) difference between the activities in the polluted soil. This, unexpected finding, may be considered as a convincing evidence of the heavy metal toxicity on the soil microbiota. Correspondingly, respiration (CO_2 production) was significantly ($p < 0.05$) lower in the polluted than in the unpolluted soil. Total numbers of bacteria, actinomycetes and microfungi, the numbers of ammonifying and amylolytic microorganisms had also significantly lower values in the polluted than unpolluted soil. Exceptionally, the reverse was true for the numbers of denitrifying microorganisms, and the numbers of cellulolytic microorganisms in the two soils were not significantly different.

Enzymological research in Finland. Ohtonen and co-workers performed complex soil biological investigations, including enzyme analyses, in polluted pine forests in the surroundings of the industrialised city of Oulu. Results of the investigations published between 1989 and 1994 were reviewed on pages 127-128 in [35]. Now we mention that some data on biological activity, including dehydrogenase

activity of humus layer in these forests were published already in 1988 (Markkola and Ohtonen [48]) and these investigations were also the topic of a synthesis work by Ohtonen [56].

Enzymological research in Ukraine. The area studied by Kaigorodova and Vorobeichik [27] is affected by both emissions (containing heavy metals and SO_2) from a copper smelter and alkaline dust from a coal-fired power plant (Kirovograd). An unpolluted site served for comparison. Due to the pollution, litter decomposition was strongly inhibited, but practically no changes occurred in the mineral soil horizons. Thickness of litter was 7-9 cm in the polluted area and only 4-5 cm on the unpolluted site, which is related to a 5-fold decrease in the rate of cellulose decomposition in the polluted litter. But urease activity in the polluted litter remained at the same level as in the unpolluted litter.

ADDENDA

Urban soils

Enzymological research in Germany. In a complex studyof soils in the city of Dorsten (Northwest-Germany), Broll and Keplin [10] have submitted 100-m^2 permanent plots set up in 1988 at four sites of urban lawn (including the site Dorsten-Hardt) to the following extensive management practices:

- mulching several (9-10) times a year (April-October);
- mulching thrice a year (June, August, September);
- mulching twice a year (June, September); and
- mowing twice a year (June, September).

Besides some soil chemical parameters, phytomass, abundance and biomass of earthworms, soil urease activity was also determined. During 1994, this activity was measured between 30 May and 19 September at 6-week intervals. Mulching, in comparison with mowing, was more efficient in increasing urease activity. Aiming also at cost effectiveness, the management practice "mulching thrice a year" is recommended.

Machulla [46] has studied urban soils in Northern, Central and Southern Germany (Kiel, Rostock, Eckernförde, Halle/Saale and Stuttgart). At 33 sites located in parks, restoration areas and industrial fallow land, soil was sampled in October 1994 and 1995 and analysed from physical, chemical, microbiological and enzymological viewpoints. According to the nature of the anthropogenic parent materials (substrates), the studied urban soils were grouped into soils developed on 1. rubbish; 2. spoil banks; 3. industrial ashes; and 4. mud or sewage sludge. As expected, the soil properties varied between and also within the groups. Thus, minimum and maximum values of dehydrogenase activity (μg TPF/g soil/24 hours) were the following: 73 and 175 (soils on rubbish); 14 and 47 (soils on spoil banks); 19 and 39 (soils on ashes); and 18 and 237 (soils on mud or sludge), respectively. The correlations between dehydrogenase activity and different soil microbiological and chemical properties (Table 3) also varied depending on the nature of the parent

materials. The data of this table show that only the correlation between dehydrogenase activity and number of bacteria was positive and significant in each of the four urban soil groups studied, whereas dehydrogenase activity correlated positively and significantly with number of fungi, total organic C content or P content, and negatively and significantly with pH only in one of the groups.

Table 3

Correlation coefficients between soil dehydrogenase activity and different soil properties [46]

Soil property	Parent material and number of analysed samples			
	Rubbish n=60	Spoil banks n=84	Ashes n=84	Mud or sludge n=60
Number of bacteria	0.72*	0.62*	0.46*	0.84*
Number of fungi	0.54*	0.28	0.35	0.26
Microbial biomass C content	0.43	0.89*	0.71*	0.81*
Total organic C content	0.55*	0.02	-0.04	0.33
Total N content	0.67*	0.46*	0.17	0.66*
pH	-0.44	0.18	-0.40*	0.33
Clay content	0.54*	0.15	-0.12	0.69*
P content	0.29	0.42*	-0.15	-0.24
K content	0.51*	0.40*	0.24	0.05

* The asterisk indicates significance at $p < 0.05$.

Enzymological research in the Russian Federation. Sidorenko *et al.* [71] have determined FDA-hydrolysis activity and several microbiological parameters (including the number of FDA-positive bacteria[*]) in soils of the town of Serpukhov located in the south of the Moscow region. Permanent plots were set up *a)* in the town centre close to a bus station; *b)* in the zone of an enterprise, the emissions from which contain dichlorophenols; *c)* in the vicinity of a fuel oil storage base; *d)* on the territory of a building-constructing enterprise emitting cement dust; *e)* in the vicinity of a plant producing meat preparations and also bone meal; the emissions from this plant contain bone meal. The soil in an unpolluted mixed forest stand at 2-km distance from the centre of the city of Pushchino-na-Oke (Pushchino-on-Oka, also located in the south of the Moscow region) served for comparison.

The soils were sampled and analysed seasonally in the 1995-1996 period. All results have indicated the following order of the FDA-hydrolysis activity: oil-polluted soil > dichlorophenol-polluted soil > unpolluted soil > soil in the centre of the town > bone meal-polluted soil > cement dust-polluted soil. Similarly, the FDA-positive bacteria were most numerous in the oil-polluted soil and least numerous in the cement dust-polluted soil. FDA-hydrolysing activity in the urban

[*] Viable bacteria reacting positively to vital staining with FDA.

soils significantly correlated with their C content. It was also found that the ratio between bacterial and fungal biomasses was much higher in the urban soils (2-20%) than in the unpolluted soil (0.6%).

Studying the soils in Rostov-on-Don, the large industrial centre in Southern Russia, Bezuglova *et al.* [5, 6] have determined catalase and invertase activities in park soils in the recreational zone of the city. Buried soil layers and soils sealed up, *i.e.* covered by solid materials (asphalt, concrete) were also analysed; their samples were collected in building foundation pits. Catalase activity (expressed in ml O_2/g soil/minute) varied between 6.3 and 8.5 in the upper horizon of park soils, but in virgin soils of the same type catalase activity averaged 9.2. Catalase activity was lowest in buried soil layers which had been covered by other soils 20-40 years before. Invertase activity was also low in park soils and buried soil layers especially when the parent rocks had a high carbonate content.

Enzymological research in the Czech Republic. Tesařová *et al.* [75] have determined dehydrogenase activity and a series of microbiological and chemical properties in soils of park lawns in city centres of Brno and Podolí. In Brno, soil samples were taken from two parks during 1996, whereas in Podolí soil was sampled from one park during 1995 and 1996. Total number of samplings was 3 (Brno) and 10 (Podolí). The chemical analyses proved that these soils are polluted with heavy metals and organic compounds, including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and DDT. Soils from four submontane grasslands belonging to a landscape reserve in the Bohemian-Moravian Uplands were used as unpolluted controls. These soils were sampled 10 times during 1995 and 1996.

Mean values of dehydrogenase activity (expressed in μg TPF/g soil/hour) were 3.25 and 3.96 in the two park soils in Brno and 4.2 in the park soil in Podolí, and higher (ranging from 5.40 to 7.07) in the four unpolluted grassland soils. Ammonification and nitrification capacities were also lower in urban soils than in the unpolluted ones. Soil respiration (CO_2 production) was indicator of pollution only in such soil samples which were amended with organic substrate (alfalfa meal) and incubated under laboratory conditions for 30 days.

Enzymological research in Ukraine. Maryskevych and Shpakivska [49] have selected three experimental plots in the city of Lvov (Lemberg) for soil chemical, microbiological and enzymological studies. Plot 1 is located in a suburban beech-hornbeam forest; plot 2 was installed in the city park in which planted chestnut, maple and ash are the dominant trees; plot 3 is a lawn of sown perennial grasses.

Soil was sampled from the 0-10-cm layer in June 1997. Soil pH in the three plots was 4.7, 7.2 and 7.9, respectively, and the mobile Pb content (extractable at pH 4.8) was 1.84, 7.42 and 6.60 ppm, respectively. The mobile Cu, Zn and Cd contents were under the permissible level.

Soil invertase, urease and catalase activities were several times lower, while peroxidase and polyphenol oxidase activities were several times higher in plot 1 than in plots 2 and 3. The microbial biomass C presented the order: plot 1 > plot 2 > plot 3.

Roadside soils

Enzymological research in the Czech Republic. During 1995 and 1996 Tesařová *et al.* [75] took 10 times soil samples from a secondary grassland at 5 m from the Brno-Olomouc highway. This roadside soil, strongly affected by motor vehicle exhausts, was similar to the three urban soils studied by the same authors (see above): it was also polluted with heavy metals and organic compounds including PCBs, PAHs, DDT; its dehydrogenase activity, ammonification and nitrification capacities were lower than those of unpolluted grassland soils.

Part III. ENZYMOLOGY OF TECHNOGENIC SOILS

Chapter 7. **Technogenic soils from coal mine spoils**

Enzymological research in the Russian Federation. The spoil heaps at the brown coal mines located in the forest-steppe zone in the Nazarovo Basin (which belongs to the Kansk-Achinsk Fuel-Energetic Complex, Siberia) were studied enzymologically by two research groups, headed by Naprasnikova (Institute of Geography, Siberian Branch, Russian Academy of Sciences, Irkutsk) and Shugalei (Institute of Forestry, Siberian Branch, Russian Academy of Sciences, Krasnoyarsk), respectively. Their studies published between 1982 and 1993 were reviewed on pages 181-183 in [35].

In a communication prepared for the international conference on "Problems of Anthropogenic Soil Formation", held in Moscow in 1997, Naprasnikova [55] presented a short summary of the investigations carried out by her research group. She emphasised that complex (botanical, chemical, microbiological and enzymological) comparison of 5- and 15-year-old, spontaneously revegetated spoil heaps proved the intensity of the soil formation processes which assure the regeneration of the natural ecosystems. Another emphasis is related to the pioneer vegetation on spoil heaps. The hydrolase (invertase and phosphatase) activities are very high in the rhizosphere of plants belonging to the families *Cruciferae* and *Compositae*.

In 100-m^2 plots of spoil heaps under 10-11-year-old Scots pine plantations, Shugalei [70] determined urease and proteolytic activities at different depths. Before plantation of young (2-3-year) pines, these spoil heaps were not covered with stored topsoil. The results have indicated that both activities were highest in the top layer (1.5-cm thick humus layer), whereas in the 1.5-20- and 20-50-cm layers the activities were 3-5 times lower.

Enzymological research in Germany. The first enzymological study of spoil heaps that resulted from strip mining of brown coal in the Halle-Leipzig zone, namely at Espenhain, was published by Machulla and Hickisch in 1988 and reviewed on pages 191-192 in [35].

Recently, Schneider *et al.* [67] and Hübl [22] have carried out investigations, including enzymological ones, on recultivated mine soils in the Halle-Leipzig brown coal strip mining area.

Schneider *et al.* [67] have studied the mine soils at Luckenau (Saxony) which represents an area located between Leipzig and Zeitz-Altenburg. These soils were submitted to sylvi- or agricultural recultivation about 30 years before.

The results of the determination of some chemical parameters, respiration and dimethylsulphoxide (DMSO) reduction in the studied mine soils (Table 4) indicate differences between the forest and arable soils and plant- and pH-dependent differences in the forest soils. Thus, organic C and total N contents and DMSO reduction were higher in the forest soils than in the arable soil. Organic C and total N contents, basal respiration, substrate-induced respiration (SIR) and DMSO reduction were higher in the 0-15-cm than in the 15-30-cm layer of forest soils. In the 0-15-cm layer, organic C and total N contents, basal respiration and SIR are highest in the poplar soil, whereas DMSO reduction is highest in the larch soil. It should also be mentioned that the abundance of earthworms was highest in the 15-30-cm layer of the alder soil.

Table 4

Chemical and microbiological properties of about 30-year-old recultivated forest and arable soils at Luckenau (Saxony) [67]

Soil	Dominant tree	Depth (cm)	pH in $CaCl_2$	Organic C (%)	Total N (%)	C:N	Respiration*		DMSO reduction*
							Basal	Substrate-induced	
Forest	Poplar	0-15	7.51	3.34	0.27	13	3.2	58.5	7234
		15-30	7.52	1.20	0.10	12	0.9	15.1	4048
	Alder	0-15	7.44	2.35	0.24	10	2.0	37.6	6176
		15-30	7.43	1.28	0.13	10	0.5	13.6	5538
	Larch	0-15	7.39	2.73	0.21	13	2.1	39.6	9801
		15-30	7.52	1.05	0.11	10	0.6	12.5	3198
Arable	-	0-30	7.60	1.05	0.11	10	1.3	15.0	756

* Respiration is expressed in μg CO_2/g soil/hour, and DMSO reduction in ng dimethylsulphide (DMS)/g soil/hour.

For chemical, microbiological and enzymological studies, Hübl [22] has selected four pairs of mine soils representing four textures (loam, calcareous loam, calcareous silt and composite loam) and natural soils, all located within the Westelbe Brown Coal District. In each pair, there was a young (< 15 years) and an old (> 15 years) mine soil. The natural soils (brown earths, parabrown earths and

black earths) were under long-term agricultural use. All soils were sampled and analysed in the springs of 1996, 1997 and 1998. Means of the values registered in the three years were compared.

Microbial biomass and alkaline phosphatase activity were higher in the mine soils than in the natural soils. Within each pair of mine soils, the old soil contained more biomass and was more phosphatase-active than the young soil (excepting the old loam mine soil which was less phosphatase-active than the young soil). Contrarily, invertase activity was higher in the natural soils than in the mine soils, and the young mine soils were more invertase-active than the old ones (excepting the young composite loam mine soil which was less invertase-active than the old soil).

The high microbial biomass in mine soils is attributed to the dominance of zymogenous microbial populations (r-strategists).

The first enzymological data on spoils in the Niederlausitz (Lower Lusatia) brown coal strip mining area (Cottbus region) were published by Katzur and Haubold-Rosar and by Kolk and Hüttl in 1996 and reviewed on pages 192-193 in [35].

Mine soils in Lusatia were studied enzymologically also by Emmerling *et al.* [14, 15], in mesocosm and lysimeter trials. Their studies are related to amelioration of young mine soils by application of organic waste materials.

Four young mine soils were studied; they derived from tertiary carboniferous sand (TS) and loamy sand (TlS), quaternary sand (QS) and loamy sand (QlS). Excepting QS, they were submitted to a lime treatment: base-rich brown coal ash was incorporated into TS and TlS and lime into QlS.

Six organic waste materials were tested:

SS - municipal sewage sludge sterilised with burnt lime;

BS - brown coal sludge, the waste from cleaning process in power plants;

CSS II - composted sewage sludge, fresh compost, obtained from 30% SS and 70% green waste (degradation degree: II);

CSS V - composted sewage sludge, mature compost, obtained from 30% SS and 70% green waste (degradation degree: V);

C 1 - mature biocompost, obtained mainly from household biowaste;

C 2 - mature compost, obtained mainly from green waste.

The first activity studied was dimethylsulphoxide (DMSO)-reductase activity, in mesocosm trial [14]. In August 1995, large pots (having a surface area of ~ 1 m^2 and ~ 1 m depth) were filled with T1S-derived young mine soil treated with base-rich ash (at a rate equivalent to 97 t CaO/ha), then amended with organic waste materials in the following variants and amounts (t/ha): 0 (control); 10 SS; 25 SS + 100 BS; 25 CSS V; 50 C 2 and 500 C 2. The ash and the organic waste materials were incorporated to a depth of 30 cm. The control was fertilised with mineral NPK (100, 80 and 100 kg/ha). For comparison, an about 30-year-old mine soil derived from tertiary deposits (locality: Koyne), an about 30-year-old mine soil derived from quaternary deposits (Sedlitz) and a podzolic brown earth (Klitten) were used.

Mine soil samples taken from the 0-30-cm depth in spring 1996 showed the following increasing order of the DMSO-reductase activity in the variants: control < 50 C 2 < 10 SS < 25 SS + 100 BS < 25 CSS V < 500 C 2. In other words, DMSO-reductase activity increased in each variant amended with organic waste materials. The highest increase occurred in the variant that received 500 t C 2/ha, *i.e*. the highest amount of organic waste material. The activity in this variant exceeded the activity measured in the Sedlitz mine soil and the Klitten brown earth, but it was under the level of activity registered in the Koyne mine soil. Basal respiration and microbial biomass C content also increased in the amended variants and were highest in the 500 C 2 variant.

In this mesocosm trial, alkaline phosphatase and invertase activities as well as basal and glucose-induced respirations were also determined in samples taken from the 0-30-cm depth in August 1995 (after the lime treatment and application of organic waste materials) and in springs of 1996 and 1997 [15]. The enzyme activities and respirations showed annual variations as a result of climatic variabilities and were highest, in most variants, in 1996. Phosphatase activity in the 500 C 2 variant was significantly higher ($p < 0.05$) in each year, while invertase activity in this variant was significantly higher only in 1997 than in the other amended variants and control. Basal and glucose-induced respirations exhibited the highest values in the 500 C 2 variant.

Two lysimeter trials were carried out at the lysimeter station in Grünewalde [15]. The lysimeters used have a surface area of 1 m^2 and 3 m in depth.

In the lysimeter trial "sewage slude", the effects of sludge and composted sludge on TS and TlS were compared. TS and TlS were limed with base-rich ash at rates equivalent to 46 and 97 t CaO/ha, respectively, then amended with sludge or composted sludge in the following variants and amounts (t/ha): 0 (control); 5 SS; 10 SS; 25 SS + 50 BS; 25 SS + 100 BS; 25 CSS II and CSS V for TS, and in the same variants and amounts, without 25 SS + 50 BS and 25 CSS V, for TlS. Incorporation depth was 1 m for ash and 30 cm for sludge and composted sludge.

In the lysimeter trial "compost", the effects of mature biocompost (C 1) on TS, TlS, QS and Q1S were compared. First, the mine soils were limed: TS and T1S were treated with base-rich ash at rates equivalent to 57 and 97 t CaO/ha, respectively, whereas G1S received lime (3 t CaO/ha); QS was not limed. Then, the mine soils were amended with C 1 in the following amounts (t/ha): 0, 50, 250 and 500. Depth of incorporation was 1 m for the ash, 60 cm for the lime and 30 cm for the compost.

In both trials, the controls were fertilised with mineral NPK (60, 118 and 160 kg/ha).

At the beginning of trials (June 1995 and June 1996, respectively), soils were sampled from the 0-30-cm layer. The samples, after their moisture content was brought to 40-50% of WHC, were preincubated at 16^0C for 14 days and then submitted to chemical, enzymological and microbiological analyses.

In the trial "sewage sludge" it was found that, in comparison with the controls, phosphatase activity increased insignificantly, basal and glucose-induced respirations increased significantly ($p < 0.05$ or at least $p < 0.1$) with increasing amounts of sewage sludge and increased to a lesser extent under the influence of composted sewage sludges. Such relations were not evident in the case of invertase activity.

In the other lysimeter trial, both enzyme activities and both respirations increased significantly ($p < 0.05$) with the amounts of mature biocompost, and the increases were more pronounced in TS and T1S than in QS and QlS.

*
* *

Müller *et al.* [53] consider that a decision on the recultivability of a mine wasteland should be based on the results of the following analyses of the mine soil:

- determination of soil pH, humus and N contents;
- determination of the contents of plant-available nutrients;
- evaluation of the phytotoxicity with the *Lepidium sativum* germination and root growth test;
- determination of dehydrogenase activity;
- evaluation of the microbial diversity.

Three places should be selected on the wasteland for soil sampling. Mixed samples should be taken from the depths of 0-20, 20-40 and 40-60 cm.

Analytical data are presented for the soil of a mine wasteland, but kind and locality of the mine are not specified.

Enzymological research in the United Kingdom. The opencast coal site, on which the experiments of Scullion and Malik [68] were carried out, is located in South Wales. The site was restored by replacement of the topsoil stored for some 4 years during opencast mining of coal, and by seeding to grassland in spring 1985. The site was then amended annually with poultry manure (8 t fresh weight/ha/year). During autumn 1985 and spring 1986, earthworms were inoculated into 400-m^2 plots, the earthworm input being of almost 70 individuals/m^2. No earthworms were introduced into the control plots. For physical, chemical, microbiological and enzymological analyses, soil was sampled from two depths (0-7.5 and 7.5-15 cm) in spring and autumn 1994. Microbial biomass C, basal respiration and dehydrogenase activity were measured in the samples collected in autumn 1994.

In the 0-7.5-cm soil layer, microbial biomass C was significantly ($p < 0.01$) and dehydrogenase activity was insignificantly ($p > 0.05$) higher in earthworm input plots than in the control plot. In the 7.5-15-cm soil layer, both parameters were higher, but insignificantly, in the control plots than in the earthworm input

ones. In both soil layers, basal respiration was not significantly different in the earthworm input and control plots.

Earthworm inputs increased stable aggregation and resulted in a higher proportion of the organic matter as carbohydrates. The conclusion was drawn that the results obtained emphasise the important influence of earthworms on aggregate and organic matter stabilisation, processes which are closely linked. We consider that this conclusion would receive further support, if activities of accumulated soil enzymes were also determined, as enzyme accumulation in soil is closely linked to aggregate and organic matter stabilisation.

Enzymological research in the Czech Republic. The enzymological investigations on technogenic soils in the North Czech Brown Coal District were described by Šíša and his co-workers between 1985 and 1997 and reviewed on pages 204-206 in [35]. Some of the results were also presented at an international seminar held in Cottbus, Germany in 1997 [79].

The field experiments started in 1995 at eight localities within the North Bohemian Brown Coal District were continued by Šíša *et al*. in 1996 and 1997 and the results obtained were published in 2000 [72]. Four tehnogenic soils resulting from recultivation of overburdens, a recultivated loessoid soil and three undisturbed soils were studied. All soils were cultivated with agricultural plants. Characterisation of the soils and specification of the plants are given in Table 5. Three enzymatic (invertase, phosphatase and catalase) activities and five microbial parameters, namely basal respiration, three potential respirations (N-induced, glucose-induced and N+glucose-induced respiration) and microbial biomass C were measured in the 0-20-cm layer of each soil (Table 6). All measurements were carried out 5 times in each year. Thus, the chemical, enzymological and microbiological data in Tables 5 and 6 are means of 15 measurements.

Comparison of the eight soils based on their enzyme activities and microbial parameters reveals that one enzyme activity and four microbial parameters were highest in the second Úžín soil (technogenic soil formed during recultivation of topsoiled overburdens), whereas two enzyme activities and three microbial parameters were lowest in the Čepirohy soil (formed during recultivation of a loessoid soil).

Comparison of the first and second Úžín soils clearly shows the importance of topsoil cover for the efficient recultivation of overburdens.

Comparison of soils 5 and 6 indicates that the undisturbed vineyard soil at Rudolice is more active than the recultivated vineyard soil at Čepirohy.

The Svoboda soil occupies a medium position (and not the last position) from enzymological and microbiological viewpoints, which proves that covering of the toxic overburdens with a bentonite layer and then with topsoil was an efficient remediation technology.

Table 5

Characterisation of technogenic and undisturbed soils cultivated with agricultural plants, in 3-year experiments [72]

No.	Locality	Soil	Organic C (%)	Total N (%)	Total P (mg/kg dry soil)	C:N	pH	Plants		
								1995	1996	1997
1	Úžin	Technogenic soil: overburdens not covered with topsoil	2.80	0.26	1287.78	10.67	6.75	Winter wheat	Fallow	Rape
2	Úžin	Technogenic soil: overburdens covered with topsoil (50 cm)	2.77	0.26	1667.86	10.55	6.92	Winter wheat	Fallow	Rape
3	Svoboda	Technogenic soil: toxic overburdens covered with bentonite (20 cm) and topsoil (50 cm)	1.65	0.15	955.86	10.75	6.82	Spring barley	Legume-grass	Clover-grass
4	Braňany	Undisturbed soil	2.11	0.20	1470.76	10.37	6.11	Alfalfa	Winter wheat	Winter wheat
5	Rudolice	Undisturbed soil	1.36	0.12	1520.01	11.24	8.33	Grapevine	Grapevine	Grapevine
6	Čepirohy	Recultivated loessoid soil	1.08	0.09	630.57	12.20	8.58	Grapevine	Grapevine	Grapevine
7	Třískolupy	Technogenic soil: overburdens covered with topsoil (50 cm)	1.25	0.12	688.36	10.55	8.52	Spring barley	Rape	Winter wheat
8	Havraň	Undisturbed soil	1.52	0.17	769.11	9.21	7.19	Sunflower	Ryegrass	Winter wheat

Table 6

Enzyme activities and microbial parameters in technogenic and undisturded soils, in 3-year experiments [72]

Enzyme activity or microbial parameter*	Number of locality							
	1	2	3	4	5	6	7	8
Invertase activity	5.11	5.95	5.15	5.75	3.87	2.33	3.02	4.63
Phosphatase activity	28.57	25.06	14.90	23.86	5.83	4.58	7.04	16.02
Catalase activity	0.80	0.94	0.85	0.74	1.51	1.54	1.31	0.78
Basal respiration (BR)	0.41	0.53	0.39	0.41	0.33	0.29	0.31	0.37
N-induced respiration (NR)	0.58	0.78	0.48	0.47	0.69	0.54	0.64	0.42
Glucose-induced respiration (GR)	4.29	5.89	4.11	5.54	3.43	2.16	3.51	3.71
N+glucose-induced respiration (NGR)	13.30	22.06	17.51	13.85	13.97	5.54	6.25	10.34
NR:BR	1.53	1.47	1.43	1.32	2.30	2.15	2.27	1.38
GR:BR	10.54	11.73	11.35	14.39	10.60	7.98	11.96	10.94
NGR:BR	35.51	43.24	48.56	36.55	44.54	20.23	20.97	30.87
Microbial biomass C	439.8	644.0	388.2	655.7	459.8	403.0	493.5	421.1

*Expression of enzyme activities and microbial parameters: invertase in mg reducing sugars/g dry soil/24 hours; phosphatase in μg *p*-nitrophenol/g dry soil/hour; catalase in ml 0.1 N $KMnO_4$/g dry soil/15 minutes; respiration in mg CO_2/100 g dry soil/hour; and microbial biomass C in mg C/kg dry soil.

According to the investigations described by Mikanová *et al.* [51] and Kubát *et al.* [39, 40], unexpected enzymological results were obtained in the loamy clay mine soil at Březno (located in the area of the city of Chomutov in North-West Bohemia). The site at Březno is highly polluted: both SO_2 and flying dust concentrations in air exceeded 100 $\mu g/m^3$ in 1985 to 1989. Experimental plots were installed on the mine spoil in 1979. The plots received one of the following four treatments:

1. covering with 50-cm thick topsoil layer and addition of sewage sludge (70 t/ha/year);

2. the same as treatment 1, but without addition of sewage sludge;

3. covering with 25-cm thick topsoil layer and addition of power plant ashes (400 t/ha) + sewage sludge (70 t/ha/year);

4. the same as treatment 3, but without addition of sewage sludge.

Soil was sampled from the 0-20-cm depth of plots, 5 times during 1995 and 6 times in 1996, for chemical, microbiological and enzymological analyses.

The microbiological analyses proved that the soil in each plot contained a considerable number of bacteria, actinomycetes and filamentous fungi, among which proteolytic and spore-forming bacteria, cellulolytic bacteria and actinomycetes, free-living N_2-fixing bacteria, oligotrophic microorganisms. In the four treatments, the microbial biomass C (in mg C/g dry soil) was, on average, 208.22, 195.28, 165.70 and 122.68, respectively; cumulative CO_2 production (in mg CO_2/100 g soil) in 27

days of incubation at laboratory temperature was, on average, 56.11, 55.78, 93.31 and 92.45, respectively. But dehydrogenase activity was equal to zero in the soil of all plots at all sampling times. However, these permanently negative enzymological results, unexpected in the light of the results of microbiological analyses, need further verification, as pointed out also by the authors themselves [39].

Chapter 8. **Technogenic soils from manganese mine spoils**

Enzymological research in Spain. For botanical, chemical and enzymological studies, González *et al*. [21] have selected five 1-m^2 microplots on the wasteland of a manganese mine located in Burgos. A relation was found between plant cover percentage and the ratio of phosphatase activity (μg *p*-nitrophenol/g dry soil/hour) to available P content (ppm). Thus, in microplot 1, the 20% plant cover was accompanied by a low phosphatase activity (PA): available P (av.P) ratio (121.95:2.82). In the other microplots, the plant cover ranged from 85 to 100% and the PA: av.P ratios increased as PA varied between 565 and 767 and av.P between 10 and 18 and became similar to ratio values recorded in natural soils. Another finding was that higher proportions of leguminous plants in the plant cover led to increased PA: av.P ratios.

Chapter 9. **Technogenic soils from lead and zinc mine wastes**

Enzymological research in Romania. The results obtained in the enzymological, microbiological, botanical and zoological studies of lead and zinc mine spoils submitted to biological recultivation at the Rodna mine (Bistrița-Năsăud county) were reviewed, for the 1987-1996 period, on pages 238-243 in [35].

The newer results, registered in the last years, were published by Paşca *et al*. [60, 61] and Muntean *et al*. [54].

Fig. 3 comprises the results of the enzymological analyses carried out in 1999. Before commenting the data in this figure, it is necessary to present a brief description of the three recultivation experiments started at the Rodna mine in 1987, 1988 and 1989, respectively.

In 1987, there were nine terraces formed on the spoil dump. In this year, the oldest terrace (I) was 14 years old and the youngest terrace studied (VIII) was 2 years old.

In 1987, 14 small (7 m^2 = 2 x 3.5 m) plots were installed on the 2-year-old terrace VIII (plots 1-6), on the 7-year-old terrace V (plots 7-10) and on the 10-year-old terrace III (plots 11-14).

In 1988, two large (50 m^2 = 20 x 2.5 m) plots were installed on the 5-year-old terrace VI (plots I and II).

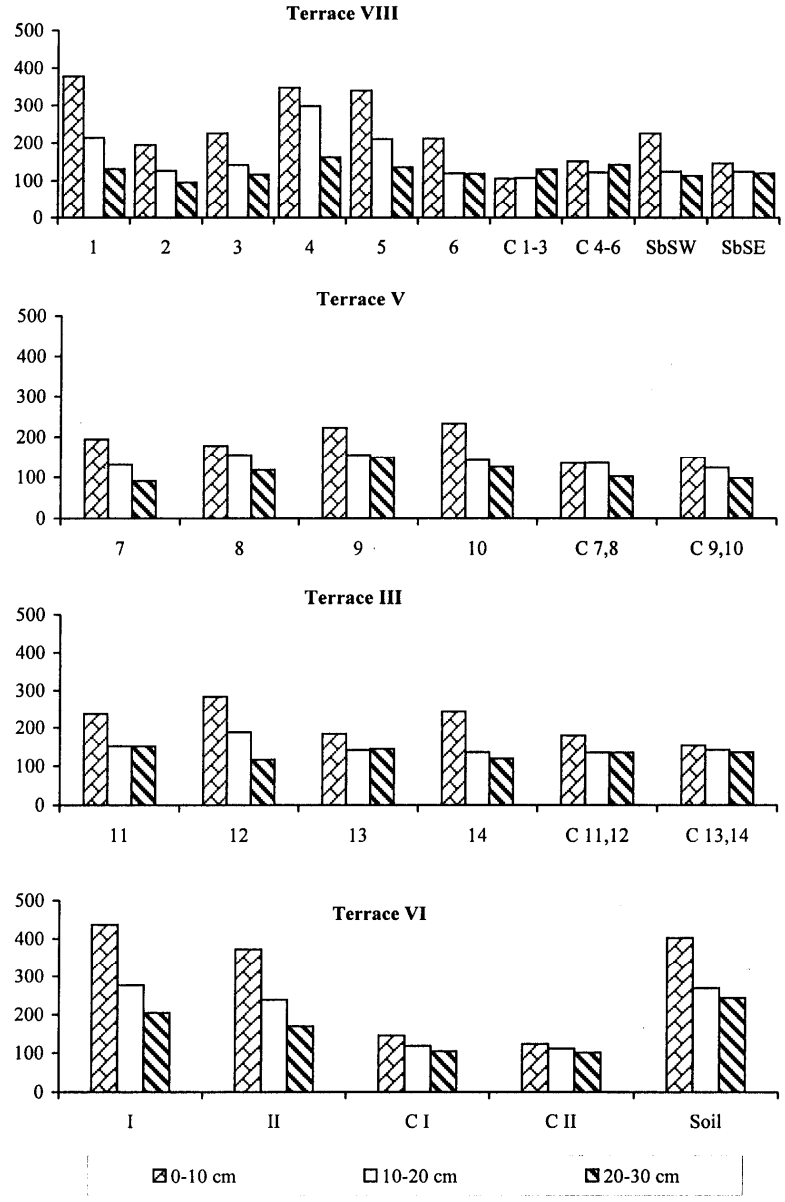


Fig. 3. *Enzymatic potential in spoils and native soil sampled from depths of 0-10, 10-20 and 20-30 cm* [60].

X axes - Plots (1-14; I-II) and their controls (C). Y axes - Enzymatic indicators of spoil (soil) quality. SbSW - Sea buckthorn, south-western aspect. SbSE - Sea buckthorn, south-eastern aspect.

The treatments applied to plots were the following: *a)* covering with soil + fertilising with farmyard manure (FYM) + NPK + sowing of Italian ryegrass (*Lolium multiflorum*) and meadow clover (*Trifolium pratense*) (RC) (plots 1 and 4); *b)* FYM + NPK + RC (plots 2 and 5); *c)* NPK + RC (plots 7, 9, 11 and 13); *d)* NPK (plots 3, 6, 8, 10, 12 and 14); *e*) covering with soil + NPK + RC (plot I); and *f)* covering with soil + NPK + adding of spontaneously revegetated 15-year-old spoil, containing seeds of plants and microorganisms adapted to the toxic environment of lead and zinc mine spoils (plot II). On each terrace, there were plots with south-western (SW) aspect (plots 1-3, 7, 8, 11, 12 and I) and plots with south-eastern (SE) aspect (plots 4-6, 9, 10, 13, 14 and II). Untreated places in the vicinity of plots were the controls. A native, soddy soil at the foot of the spoil dump also served for comparison.

In 1989, two plots (having SW and SE aspect, respectively) on the already 4-year-old terrace VIII were planted with sea buckthorn (*Hippophaë rhamnoides*) shrubs.

It should be emphasised that the treatments mentioned above were applied to the plots only in the year of their installation, *i.e.* in 1987, 1988 and 1989, respectively. In the next years, the plots received no fertilisers, and they were not sown and moistened artificially.

In the spring and autumn of 1999, samples were collected from the plots, controls and native soil. Sampling depths were 0-10, 10-20 and 20-30 cm. The samples were submitted to enzymological analyses for determination of their phosphatase, catalase, actual and potential dehydrogenase activities and nonenzymatic catalytic H_2O_2-splitting capacity.

The analytical data were used for calculation of the enzymatic indicator of spoil (soil) quality (EISQ). First, the arithmetical mean of each activity at a given depth in each plot and control as well as in native soil was calculated from the values measured in spring and autumn (*i.e*. from two values measured). Then, taking the maximum mean value of each activity as 100%, the relative activities were calculated. The sum of the relative activities is the enzymatic indicator which is considered as an index of biological quality of spoil (soil) at a given depth in a plot, control and native soil.

One can see from Fig. 3 that the EISQ values manifested a trend to decrease with sampling depth in all plots and, as expected, in the native soil. The 0-10-cm layer was more enzyme-active than the 10-20- and 20-30-cm layers in all plots and in the native soil, but the 10-20-cm layer exhibited higher enzyme activities than the 20-30-cm layer only in most of the plots and in the native soil. It should also be mentioned that the 0-10-cm layer proved to be more enzyme-active in plot I than in the native soil. The controls showed little depth-dependent changes in their EISQ values. However, a slight decreasing trend could be observed in the controls, excepting C 1-3 and C 4-6.

The results obtained in 1999 are in good agreement with those registered in the previous years and, thus, they make it possible to confirm the following conclusion: covering of lead and zinc mine spoils with an at least 10-cm-thick soil

layer is the most important measure for rapid recultivation of raw and young spoils as the favourable effect of a soil cover on the enzymatic potential of spoils proved to be long-lasting, whereas NPK fertilisation is the minimum treatment for recultivation of old spoils.

Chapter 10. **Technogenic soils from potassium salt mine wastes**

Enzymological research in Ukraine. Maryskevych *et al*. [50] set up four experimental plots on 3-15-year-old spoil dumps that resulted from strip mining of potassium salts in the area of Kalush (Ivano-Frankovsk region). The vegetation on the dumps is in different successional stages: in pioneer stage (*Salicornia europaea*), rootstock stage (*Puccinella distans*), soddy stage (*Calamagrostis epigeios, Betula pendula*) and oak forest.

Physical, chemical, enzymological and microbiological properties were examined in the 0-10-cm layer of dumps and of natural forest soils (zonal controls). It was found that succession of stages was accompanied by decrease in bulk density (from 1.35 to 1.19 g/cm^3), by increase in field moisture (from 9.2 to 40.5%) and porosity of aeration (from 16.4 to 40.6%), by decrease in pH and water-soluble K^+ and Na^+ contents and by increase in C, N and P contents.

During succession from the pioneer stage to the soddy stage, soil enzyme (invertase, urease, catalase, polyphenol oxidase, peroxidase) activities increased 3-14 times, microbial biomass thrice and basal respiration 1.5-2 times.

Chapter 11. **Technogenic soils from sulphur mine spoils**

Enzymological research in Ukraine. The spoil dumps that resulted from strip mining of sulphur in the area of Yavorov (Lvov region) were studied by Maryskevych *et al.* [50]. They installed four experimental plots on 3-15-year-old dumps under vegetation in different successional stages: in pioneer stage (*Erucastrum gallicum*), rootstock stage (*Tussilago farfara*), soddy stage (*Festuca pratensis, Poa pratensis*) and pine forest.

The 0-10-cm soil layer in the dumps was submitted to the same examinations as the soil layer of the dumps that resulted from strip mining of potassium salts in the Kalush area and similar results were obtained (see Chapter 10).

Chapter 12. **Technogenic soils on exhausted limestone quarries**

Enzymological research in Spain. For reclamation of an exhausted limestone quarry, Bonmatí *et al*. [8] used a calcareous soil amended with high amounts of sewage sludge. The soil in experimental plots was mixed with sludge at a rate of 10 or 20% dry matter or the sludge was directly applied on the soil surface. Plots with no added sludge were the controls. During 3 years, soil samples were periodically taken for chemical and enzymological analyses.

In sludge-amended soil the stable organic matter content, invertase, BAA (Nα-benzoyl-L-argininamide)-hydrolysing protease and phosphatase activities increased, but casein-hydrolysing protease activity did not increase, during the eperimental period. The evolution of organic matter and enzyme activities was slower in the soil-sludge mixtures than in the soil surface-amended with sludge. Invertase and BAA-hydrolysing activities were mostly associated with stabilised organic matter, casein-hydrolysing activity with fresh organic matter and phosphatase activity with both stabilised and fresh organic matters. Another finding was that the sludge initially added to the soil contained some type of invertase activity inhibitor.

Conclusions. The investigations reviewed in this article led to results similar to those obtained in the investigations reviewed in [35] and confirmed the following conclusions:

- the soil enzyme activities may indicate toxicity of oil contaminants on soil life and also the capacity of the soil microbiota to catalyse self-decontamination of soil;
- the multidisciplinary investigation of oil contamination of soils and their remediation should always comprise enzymological measurements, too;
- the enzyme activities are, in most situations, sensitive indicators of *a)* soil pollution caused by industrial emissions (and motor vehicle exhausts) and *b)* efficiency of the decontamination technologies applied;
- as microorganisms and enzymes participating in decontamination of polluted soils are not infallible, the prevention of pollution should remain the best way for environmental protection;
- application of enzymological methods makes it possible to indicate the degree of evolution of technogenic soils, the transformation of overburdens and other spoils and wastes into agricultural and forest soils, the efficiency of the recultivation measures applied;
-in comparison with microbiological parameters, the enzymes are more synthetic indicators of the evolution of technogenic soils because they reflect *a)* due to their accumulation in form of humus complexes, the past of technogenic soils and *b)* due to their activity, which plays a key role in nutrient cycles, the present biological status of these soils.

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INVESTIGATION OF GLYCONECTIN-GLYCONECTIN INTERACTIONS BY ATOMIC FORCE MICROSCOPY

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SUMMARY. - Cellular interactions involve many types of cell surface molecules operating via homophilic and/or heterophilic protein-protein and protein-carbohydrate binding. Our investigations using the marine sponge *Microciona prolifera* as a model system have provided direct evidence that a novel class of primordial proteoglycans, named by us glyconectins, can mediate cell adhesion via a new alternative molecular mechanism of polyvalent carbohydrate-carbohydrate binding. Biochemical characterisation of purified glyconectins revealed the presence of specific acidic glycans different from classical glycosaminoglycans. These glyconectins mediate *in vivo* cell recognition and aggregation via homophilic, species-specific, polyvalent, and calcium ion-dependent glycan-glycan interactions. The kinetic binding studies, calorimetric methods, X-ray diffraction, nuclear magnetic resonance, and other spectroscopic analyses do not supply a direct estimation of the intermolecular binding forces that are fundamental for the function of the ligand-receptor association. Recently, we have introduced atomic force microscopy to quantify the binding strength between cell adhesion proteoglycans. Measurement of binding forces intrinsic to cell adhesion molecules is necessary to assess their contribution to the maintenance of the anatomical integrity of multicellular organisms. As a model, we selected the glyconectin 1, a cell adhesion proteoglycan isolated from the marine sponge *Microciona prolifera*. Under physiological conditions, an adhesive force of up to 400 piconewtons between two cell glyconectins was measured. Such a large cohesive force as this is theoretically able to hold the weight of approximately 1600 cells in physiological solution. Thus the integrity of the multicellular sponge organism, with at least 1000 glyconectin molecules per cell, may be maintained by the multiplicity of glyconectin-glyconectin interactions. Our results suggest that the strength and polyvalency of glycan-glycan interactions are essential for cell adhesion.

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The cell surface is the "contact layer" used by cells to communicate with the outside world. Through the activities of cell surface molecules cells recognise self from non-self, send and receive physico-chemical signals and adhere to other cells. Cell recognition and adhesion are a cascade of multistep events involving the extracellular matrix glycoprotein, lectin, immunoglobulin, integrin and cadherin families, operating via homophilic and/or heterophilic protein-protein and protein-carbohydrate interactions. All living cells express surface carbohydrates that participate in cell-cell interactions. These carbohydrates in the form of glycolipids, glycoproteins, proteoglycans and mucins are principal components of many cell surfaces.

Species-specific reaggregation of dissociated marine sponge cells was the first experimental system supporting the existence of cell recognition and adhesion [33]. Later work with *Microciona prolifera* revealed that both cellular interactions are mediated by an adhesion proteoglycan molecule, however, without the quantitative and biochemical evidence about the underlying molecular mechanisms [7, 9]. Further investigations provided for the first time the direct evidence that carbohydrate-carbohydrate interactions can mediate cell adhesion [14-16]. Immunological and biochemical studies showed that the functional carbohydrate structures belong to a new class of large, fucosylated acidic glycans different from the classical glycosaminoglycans [15, 16].

Cellular interactions are not only biological phenomena because they, practically, depend upon physico-chemical phenomena. At present, many of the recent advances have occurred at the scale of the cell, the membrane, and the receptors, and have focused on the identification and characterisation of cell adhesion molecules (CAMs). In these circumstances, the accurate understanding of cell-cell and/or cell-extracellular matrix interactions at larger physical scale requires an interdisciplinary approach [21, 25, 34].

The biological relevance of CAMs has been demonstrated using different functional assays. Such investigations provide data concerning two essential aspects: the biophysical definition of adherence, and the biochemical modifications of CAMs. It is evident that the biophysical basis for any functional assay is the mechanical strength of adhesiveness. The effects of biochemical manipulations can be compared quantitatively only when a valid and accurate estimation of adhesiveness is made in a controlled experiment. Generally, either the force or the energy of the interaction can define the mechanical strength. The adhesive force can be calculated at two levels: cellular (avidity) and molecular (affinity). At the cellular level, fracture stress is defined as contact stress (force per unit area of adhesion) at the point of detachment. The surface adhesion energy density is defined as the mechanical work done to separate a unit contact area. At the molecular level, bond strength is defined as the maximum force a single molecular crossbridge can sustain. Bonding energy is defined as the energy required to break a single crossbridge [34].

Long- and short-range contacts between biological macromolecules and macromolecular superstructures are extremely important for the dynamic behaviour of biological systems. Until recently the direct measurement of force or energy of the adhesive interactions *per se* was not possible. Such interactions are usually investigated using thermodynamic and kinetic approaches. However, these methods do not supply a direct estimation of the intermolecular binding forces that are fundamental for the function of the ligand-receptor association. Distinct measurement of the force (the derivative of energy with respect to separation distance) is not possible and, as a result, the direct information concerning the distribution of interaction energy between two biological structures is incomplete.

Recently, we have introduced atomic force microscopy (AFM) to quantify the binding strength between cell adhesion proteoglycans [3, 22]. The AFM is considered a relatively new tool suitable for measuring intermolecular forces between nanometer-scale objects. It was first developed as an imaging device but, at this time, is one of the most widely used instruments for measuring intermolecular forces. The local interactions can be measured in real time with a high spatial resolution, because the AFM uses a probe with a radius of curvature typically of the order of 10-100 nm [3, 6].

Material and methods. *Isolation of glyconectin 1*. Glyconectin 1 was extracted from fresh cuts of *M. prolifera* sponge in artificial Ca^{2+}- and Mg^{2+}-free seawater (462 mM NaCl, 10.7 mM KCl, 7 mM Na_2SO_4 and 2.1 mM $NaHCO_3$) at +4°C for 16 hours. Final purification was performed as described [16].

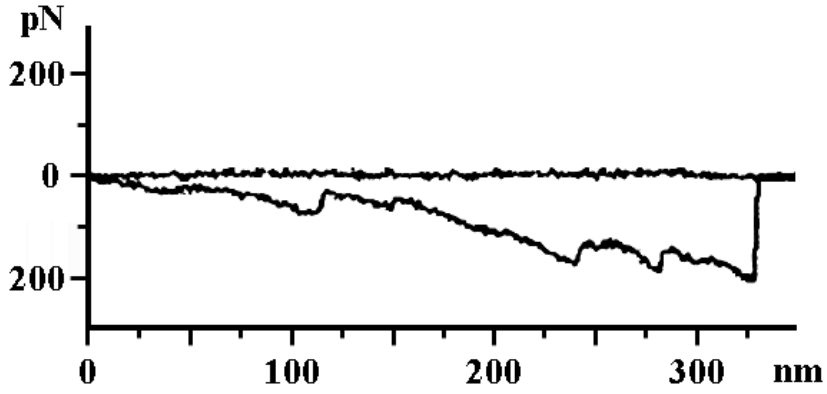
Monoclonal antibodies. Block 2 monoclonal antibodies were purified from culture supernatant of glyconectin 1-positive clone 17, by protein A-agarose chromatography. The Fab fragments were isolated by gel filtration after papain digestion of the whole antibody [15, 16].

Analytical methods. Carbohydrate analysis of glyconectin 1 glycans, colorimetric reactions for neutral hexoses, uronic acids and sulphate were conducted as previously described [1, 2, 4, 26].

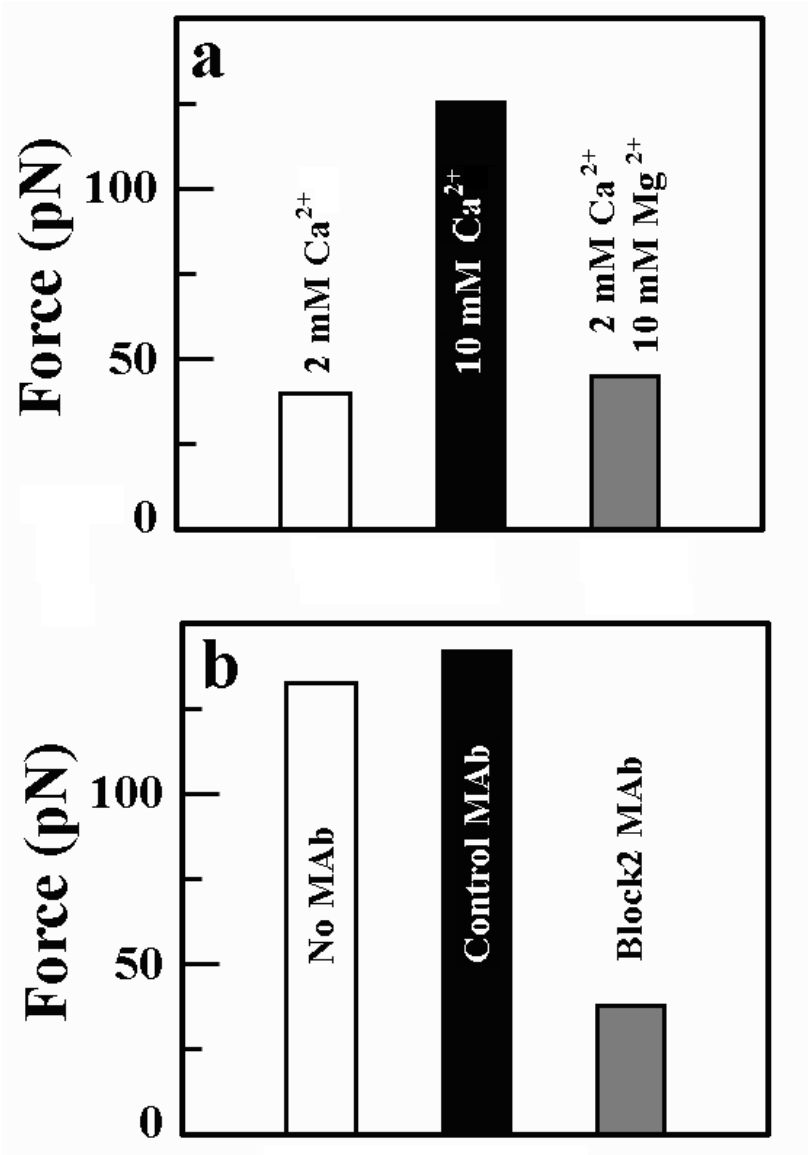
AFM experiments. Glyconectin 1 molecules were covalently attached to an AFM sensor tip and a flat surface. In order to obtain biocompatibility, 20-30 nm of gold was deposited by evaporation in high vacuum on silicon nitride cantilevers and atomically flat silicon wafers. They were then immersed in 1 mM 11,11'-dithio-bis-(undecanoic acid N-hydroxysuccinimide ester) in dry methanol, incubated overnight at 20°C, washed in dry methanol, and dried. Glyconectin 1 molecules were covalently attached by their amino groups to these self-assembled monolayers of active succinimide groups. Glyconectin solution was diluted to a final concentration of 0.2 mg/ml in 0.5 M NaCl, 2 mM $CaCl_2$, and 20 mM Hepes buffer (pH 7.4) (seawater Hepes, SWH, iso-osmotic with seawater) and incubated for 1 hour in a moist chamber at 20°C. The tip and substrate were then rinsed with SWH and mounted wet into the fluid chamber of the atomic force microscope (NanoScope III, Digital Instruments, Santa Barbara, CA, USA) [3, 6, 22].

Results and discussion. Measurements of glyconectin-glyconectin adhesion forces were performed in physiological solution, containing different concentration of Ca^{2+} and Mg^{2+}. The cantilever tip coated with glyconectin 1 was slowly moved towards the glyconectin 1 functionalised substrate surface until contact is made, followed by retraction of the tip. During such an "approach and retract" cycle the cantilever deflection was permanently monitored. The hysteresis of the cantilever is a direct measure of the adhesion force. "Approach and retract" cycles, sometimes called force distance curves or force plots, were repeated 50 times at 5 different points with a speed of 0.01-1 Hz at 20°C.

The attachment process involves exclusively the protein moiety of glyconectin. The stability of binding events during the AFM experiments indicated that very few of the glyconectin functional adhesion sites were damaged. A representative "approach and retract" curve is shown in Fig. 1. The adhesion peaks were retarded, indicating that during the surface approach there was no interaction, but on retraction the lever detected an attractive force at a distance more than 300 nm above this surface. The appearance of the "approach and retract" curves suggests the presence of long-range interactions, interpreted as the lifting and extensions of stringlike arms, followed by further stretching until the elastic force of the cantilever equals the strength of the binding and the lever "jumps off". By contrast, using two control surfaces, without glyconectins, the adhesion took place directly at the surface and the shape of the curve indicates the presence of short-range forces. At the physiological Ca^{2+} concentration of 10 mM in seawater, multiple jump-offs were frequently observed, indicating polyvalent binding with an average adhesive force of 40 pN (Fig. 1).



F i g. 1. *A typical AFM approach-and-retract cycle for glyconectin 1-glyconectin 1 interaction.* The abscissa shows the vertical movement of the cantilever while the ordinate shows the bending of the cantilever and thus the force acting on it.



F i g. 2. *AFM measurements of glyconectin 1-glyconectin 1 binding strength.* Sequential measurements were carried out with the specified cation or monoclonal antibody (MAb). **a** - Ca^{2+} and Mg^{2+} dependence of the adhesive force in artificial seawater. **b** - Effect of antibodies on adhesive force. Fab fragments of MAb Block 2 or control MAb (both immunoglobulins G2b) were used at 20 μg/ml in artificial seawater containing 10 mM Ca^{2+}.

To characterise and verify that the measured forces originate from interaction between complementary glycans, beside the force necessary to separate the glycan functionalised sensor tip from the analogous glycan on the surface (final jump-offs) we have estimated also the percentage of interaction events under different ionic conditions. These two indicators of glycan activity were specifically dependent on the physiological Ca^{2+} concentration, essential for activation of glyconectin 1, accordingly to previous qualitative data [9, 15, 16, 29, 30]. At a Ca^{2+} concentration of 10 mM, the average force between glyconectins was 125 pN, ranging up to 400 pN, with a high probability of binding (60%). At a Ca^{2+} concentration of 2 mM, cell adhesion was sharply reduced and the force and probability were also decreased.

The interaction between glyconectins is Ca^{2+}-selective since 10 mM Mg^{2+} could not replace Ca^{2+} (Fig. 2a). Use of monoclonal antibody (MAb) Block 2 (Fab fragments), capable of blocking cell adhesion by recognising a carbohydrate epitope, provided accurate evidence that the AFM-measured interactions originate from glycan-glycan binding. This MAb reduced the interactive force at the level measured at 2 mM Ca^{2+}. Under similar conditions, a control MAb has no inhibitory activity on glyconectin-glyconectin interaction (Fig. 2b). Thus, during AFM measurements under all tested experimental conditions, glyconectin-glyconectin interactions resemble cell-cell adhesion events observed *in vivo*.

An AFM image of glyconectin 1 shows the rings with a diameter of 200 nm and about 20 irradiating arms, each 180 nm long [3]. The AFM observations are consistent with a model in which the glycan arms are responsible for glyconectin 1-glyconectin 1 cohesion. In the glyconectin 1 crosslinking to AFM surface only the protein part is involved and thus the glycan arms remained free to irradiate into the buffer. During each "approach and retract" cycle, multiple noncovalent bonds between facing glycan arms were formed and broken. Because the radius of curvature of an AFM tip is about 50 nm and the glyconectin backbone ring is approximately 200 nm in diameter, only a single glyconectin molecule could be attached. The multiple jump-off steps (Fig. 1) indicate that binding was polyvalent. Each step of 40 pN corresponds to the unbinding of a pair of glycan arms. The maximal measured adhesion force of 400 pN and the average force of 125 pN are thus interpreted as the binding between 10 and 3 pairs of glycan arms, respectively. These results indicate that the measured cohesive force between two individual glyconectin 1 molecules could theoretically hold the weight of 1600 cells in physiological solution. Thus the integrity of the multicellular sponge organism, with more than 1000 glyconectin molecules per cell, may be maintained by the multiplicity of glyconectin-glyconectin binding.

Electron microscopy, X-ray diffraction studies and biochemical analyses showed that, beside mucins, proteoglycans are the largest macromolecules extending above the cell surface many times higher than any other cell adhesion glycoprotein [5, 12, 32]. The glycans are sterically the most exposed and accessible molecules on the cell plasma membrane and in the extracellular matrix. This fact implies that

at least the initial cell-cell and cell-matrix contacts should take place through sugar-sugar interactions. Our initial investigations in marine invertebrates provided direct evidence that primordial proteoglycans can indeed mediate cellular interactions via a new alternative molecular mechanism of polyvalent carbohydrate-carbohydrate binding [15, 16]. The ability of this newly recognised molecular mechanism of cell recognition and adhesion is also supported by the following findings:

i) the oligomeric glycan structures are the biological molecules keeping the highest potential information, and

ii) the expression of specific glycan structures is timely and spatially regulated during both morphogenesis and renewal in adult organism.

These glyconectin arms are composed of glycans with a relative molecular weight of 200 000 D (g200) [16]. The functional assays provide direct evidence that homophilic carbohydrate-carbohydrate interactions of the g200 glycans mediate recognition and adhesion. The glass aminopropyl beads were coated with either glyconectin 1 or g200 glycan and their aggregation was monitored following addition of a physiological concentration of $CaCl_2$ (10 mM). Aggregation of coated glass beads occurred, as glyconectin 1 promoted cell or latex-amidine bead aggregation in the presence of 10 mM $CaCl_2$, but not with 2 mM $CaCl_2$ [16, 23].

Such calcium-dependent aggregation of g200 beads suggests that the g200 glycan is capable of mediating recognition and adhesion exclusively through homophilic sugar-sugar interactions. Also, the AFM experiments showed that stringlike structures, the g200 glycans, were responsible for polyvalent glyconectin-glyconectin interactions. This possibility is further supported by the fact that the length (180 nm) and the number (20 copies) of the g200 glycan per glyconectin molecule are similar to the length and number of glyconectin arms as measured by AFM and electron microscopy. At last, the inhibitory MAb Block 2 is directed against a self-association epitope located on the g200 glycan [3, 15]. Thus, highly polyvalent g200-g200 binding represents the basis for glyconectin 1-glyconectin 1 association, which by itself promotes cell recognition and adhesion.

Fab fragments of the Block 2 monoclonal antibody showed a concentration-dependent inhibition of glyconectin 1 and g200-coated glass bead agglutination. This antibody, recognising a sulphated carbohydrate epitope, appears to preclude cell adhesion through a direct inhibition of glyconectin 1 self-interaction, as shown previously [15].

Although the primary structure of the g200 glycan remains to be determined, our data indicate that this N-linked highly fucosylated and acidic polysaccharide, containing glucuronic acid, mannose, galactose, N-acetyl glucosamine, sulphate and pyruvate, belongs to a novel class of acidic glycans distinct from the classical glycosaminoglycans.

The cross-reactivity of the *Lytechinus pictus* polysaccharides with the Block 1 and Block 2 MAbs indicated similarity with the sponge glycans and thus could also be classified in the same group of large fucosylated acidic glycans [18, 19].

Immunofluorescence light microscopy of human colon carcinomas and healthy colon samples with Block 1 and Block 2 MAbs established that the carbohydrate structures resembling the invertebrate acidic glycan adhesion molecules are also expressed in humans. These results suggest that the acidic glycan adhesion molecules, originally found in sponges and sea urchin embryos, may represent a new class of carbohydrate carcino-embryonal antigens involved in cellular interactions associated with morphogenesis, metastasis, and maintenance and renewal of adult tissue [17].

Recently, two papers published in *Nature* remark the key role in signal transduction played by a cell surface heparan-sulphate-modified proteoglycan, named Dally, isolated from *D. melanogaster*. Dally, encoded by the *division abnormally delayed (dally)* gene, is a glycosyl-phophatidyl inositol-linked glypican and may act as a co-receptor for Wingless (Wg). Wg is a member of the Wnt family of growth factors, secreted proteins that control cell proliferation and differentiation during development [13, 31]. A few families of cell-cell signals dominate the decisions that cells make. Among these are members of the Wg signal-transduction pathway, inappropriate activation of which contributes to human cancers [20].

This sugar-sugar interaction is distinct from the higher affinity low valency protein-carbohydrate or protein-protein binding described for lectin-carbohydrate, integrin-extracellular matrix, immunoglobulin-immunoglobulin, and cadherin-cadherin adhesion molecules.

An open question concerning the role of carbohydrate-carbohydrate associations during cellular interactions is whether such an interaction provides the degree of specificity required for cell recognition. Our knowledge of noncovalent bonding suggests that many parameters determine selectivity in the binding of neighbouring carbohydrate structures [15, 24].

The absolute configuration of the majority of monosaccharide residues in a glycan chain is the $^4C_1$ D-configuration, except fucose, which exists in the $^1C_4$ L-configuration [8]. The fucose represents more than 60% of total carbohydrate content of g200 glycan. Because of its particular configuration, fucose could be also an important factor, which may determine the specificity and selectivity of glyconectin molecule interactions. At the same time, the presence of Ca^{2+} (at physiological concentration of 10 mM) is very important for this carbohydrate-carbohydrate interaction. The calcium ions are essential for cell recognition and adhesion in *M. prolifera* sponge. Recently, it has been demonstrated *in vitro* that calcium ions also mediate a heterophilic interaction between dextran sulphate and dimyristoyl-*sn*-glycero-3-phosphocholine via calcium bridges. Attractive forces between negatively charged polyelectrolytes and zwitterionic phospholipids arise from the assembly of Ca^{2+} bridges [10].

In this regard, the model for homophilic glyconectin-glyconectin interaction proposed by Simon [27] and Simon *et al.* [28] is to a great extent supported by our experimental data. Intercellular adhesion requires physiological Ca^{2+} concentration, and this suggests that pairs of saline bonds are formed between anionic groups

localised on opposite g200 glycan arms belonging to two different glyconectin molecules. Glycans should stem towards the exterior of each cell membrane and the model for the homophilic, autocomplementary interaction should explain the formation of large numbers of saline bonds in homophilic interaction, while only small numbers of such bonds should be possible in heterophilic interactions. The positioning and spacing between charged groups on such chains are essential. A maximal number of 20 contacts between the 20, relatively rigid, g200 glycan arms of the surface glyconectins of the superposed cells is possible only for identical positioning of these arms. This explains the homophilic character of glyconectin interactions on the surface of contacting cells. In terms of molecular symmetry concepts the g200 glycan arms have a C_2-autocomplementarity.

Conclusions and future prospects. 1. Cellular interactions are cardinal biological processes involved in the morphogenesis, tissue maintenance and renewal in multicellular organisms. In many pathological situations there is a strong relationship between distinctive modifications of surface carbohydrate structures and inappropriate functioning of cell adhesion and recognition. Identification, isolation and purification of specific functional glycans along with quantitative estimation of their adhesive/antiadhesive forces could improve our understanding of the cell-cell and cell-extracellular matrix interaction complexity.

2. Our results provide the first and essential evidence that a novel molecular mechanism of homophilic, species-specific, polyvalent, and Ca^{2+}-dependent glycan-glycan binding mediates cellular interactions in invertebrates. Such a carbohydrate-carbohydrate interaction can perform the cell recognition and adhesion functions that we have assigned to it. Future studies using a similar approach may verify whether carbohydrate-carbohydrate binding mediates cell recognition and adhesion during multistep processes of cell-cell or cell-extracellular matrix interactions in other Metazoans.

3. Further experiments and theoretical modelling are required to demonstrate the generality of our paradigm of glycan-glycan interactions involved in cell adhesion and recognition. At the conceptual and theoretical levels, it is fundamental to improve the current description of the molecular-scale properties of cell surfaces using as a reference glyconectin molecules. Theoretical approach of the surface interactions at short-range requires that the surfaces be treated, not just as hard or soft walls, but with the same molecular detail as are the intervening liquid molecules, including a correct balancing of the interplay between the long-range and short-range intermolecular forces [11].

4. It is evident that the spatial distribution of intermolecular forces controls macromolecular interactions. In this context, the AFM can be used to obtain essential data about charge density, adhesion, and stiffness of a determined biological surface.

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FUNCTIONAL AND ULTRASTRUCTURAL EFFECTS OF CLOFIBRIC ACID ADDITION TO ISOLATED MITOCHONDRIA AND PERFUSED LIVER OF RAT AND GUINEA PIG

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SUMMARY. – If concentrations of clofibric acid between 0.05 and 0.2 mM are added to isolated mitochondria or to the perfused liver of either rat or guinea pig, there are certain similar functional and ultrastructural effects in the two species, such as moderate uncoupling of oxidative phosphorylation, inhibition of both gluconeogenesis and ketogenesis, mitochondrial swelling, the presence of nuclei with an irregular outline and the disappearance of lipid droplets in the hepatocytes. However, the extents of the effects, at least in some cases, are different. While in isolated mitochondria the differences are generally small, they become more obvious in the perfused liver. For example, clofibric acid inhibits gluconeogenesis stronger in rat liver, while ketogenesis is inhibited stronger in guinea pig liver. Certain additional features can also be observed in the case of the perfused guinea pig liver, especially a beginning of cytoplasm vacuolisation, dilation of the perinuclear spaces and of the biliary canaliculi.

Clofibric acid belongs to a group of substances known as peroxisomal proliferators, so called because of their striking proliferative action on this organelle in certain mammals (see [6] for a general review). In man, however, clofibric acid and other fibrates are used for their hypolipidemic effect. In this capacity, fibrates have been studied extensively over the last 25 years, but, despite the multitude of results, their intimate mechanism of action has only recently begun to be understood [7,8]. The task was and remains very difficult due to the abundance of the effects produced by chronical treatment with fibrates. The most common pleiotropic responses induced by these drugs include hepatomegaly (produced through both hyperplasia and hypertrophy), polyploidy during the S phase of the cell cycle, peroxisome proliferation, inhibition or stimulation of several mitochondrial and cytosolic enzymes, stimulation of certain growth factors and oncogene activation [1, 2, 4, 11, 14-17].

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In an effort to assess the significance and the practical value of the proliferation effect, we have undertaken a comparative study of the structural-functional interrelations in the peroxisomal proliferation induced by fibrates in different organisms, including protists, plants and mammals. For the last 3 years we have been concerned with the effects of clofibric acid on rat and guinea pig hepatic metabolism and ultrastructure. Certain results of our studies have already been published [3, 5, 20]. The rat was chosen because of its responsiveness to fibrates in regard to peroxisome proliferation, whereas the guinea pig was used because of its alleged unresponsiveness, but also because it behaves metabolically closer to man than does the rat.

As a general strategy of our work, in order to discriminate between so many effects, we selected some simplified systems, *i.e*. isolated hepatic mitochondria and the perfused liver, adding first the drug (clofibric acid) directly to the suspending medium of the mitochondria or to the perfusing medium of the liver. In another series of experiments, the same relatively simple systems were used in conjunction with a subchronic treatment of animals with clofibric acid. The present article deals with the most important functional and ultrastructural effects observed after the direct addition of clofibric acid to isolated rat and guinea pig hepatic mitochondria and the corresponding perfused livers.

Material and methods. *Preparation of mitochondria*. Mitochondria were isolated from the livers of freshly decapitated male Wistar rats (200 g) and guinea pigs (250 g), essentially according to J o h n s o n and L a r d y [9]. The isolation medium consisted of 250 mM sucrose, 10 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA, while the washing and suspending medium lacked the chelating agent.

Measurement of respiration parameters. Respiration rates and oxidative phosphorylation were monitored polarographically, at 20 ^{O}C, in a 0.5-ml cell, with a Clark oxygen electrode (Yellow Springs, USA), in a phosphorylation medium usually consisting of 175 mM sucrose, 50 mM KCl, 10 mM phosphate, 10-20 mM Tris, buffered at pH 7.4, 0.5 mM EDTA and 2 mM $MgSO_4$. Either glutamate (10 mM) plus malate (5 mM) or succinate alone (10 mM) were used as respiratory substrates. Mitochondria (1 mg/ml with succinate and 2 mg/ml with glutamate+malate) were injected through the stopper capillary and the oxygraphic traces recorded in this way represented the basal respiration. After 1-2 min., 0.1-0.2 mM ADP was injected, which determined the transition to the so-called state 3 of respiration, characterised by a higher respiratory rate. When ADP was exhausted (*i.e.* completely phosphorylated to ATP), the oxygen consumption decreased, resulting in the so-called state 4 (similar to the basal state). The ratio between the respiration rates in state 3 and the basal state is known as the acceptor control ratio (ACR), while that between the state 3 and state 4 is called respiratory control ratio (RCR). Both parameters represent important indices of mitochondrial integrity and phosphorylation ability.

Clofibric acid was dissolved in a 1:1 mixture of absolute ethanol and water and added in the oxygraph cell from a stock suspension of 20 mM, so as to obtain the desired final concentration (0.05, 0.1 or 0.2 mM). The effect of ethanol was checked on parallel oxygraph traces and found to be insignificant.

Estimation of membrane potential. Membrane potential generated by succinate respiration and the kinetic behaviour of this potential following the addition of different concentrations of clofibric acid were monitored by the use of safranin or diS-C_2-(5) as potential probes, based on principles and details previously described [18, 19, 21], using a Jasco V-530 spectrophotometer or a Jasco FP-750 spectrofluorometer (2.5 μM diS-C2-(5); excitation at 636 nm and emission at 666 nm). Swelling was also monitored spectrophotometrically, at 540 nm.

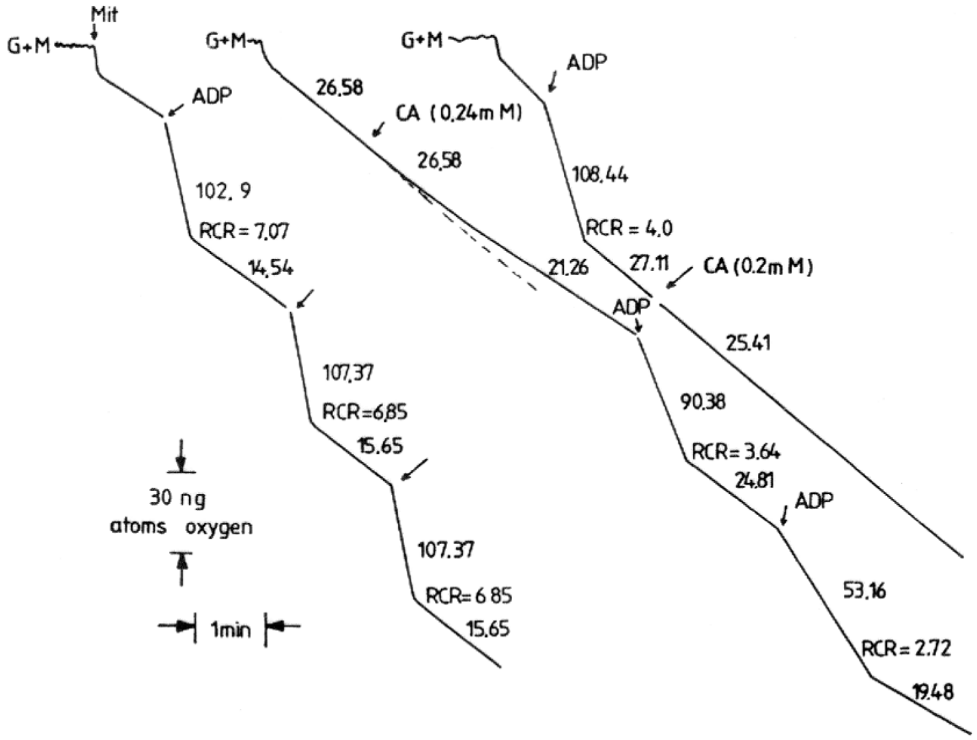
Liver perfusion: assays of glucose synthesis (gluconeogenesis) and ketone bodies (ketogenesis). The animals were anaesthetised by intraperitoneal injection of pentobarbital (50 mg/kg body weight) and after the removal of viscera the liver was perfused *in situ* with a Krebs-Henseleit-bicarbonate buffer (KHB), as previously described [10, 12, 13]. For the complete depletion of the glycogen reserve, the animals were starved for 48 hours and the glucose synthesis was initiated by introducing 4 mM lactate and 0.4 mM pyruvate into the perfusate, in the case of the rat, or half of these concentrations in the case of the guinea pig. Effluent samples were collected at 3-min. intervals and assayed spectrophotometrically for the presence of glucose, using the Biochemica Test-Combination kit (GOD-Perid). For the synthesis of ketone bodies, 2 mM octanoate was added (final concentration in the perfusate). Acetoacetate (AcAc) and β-hydroxybutyrate (β-OH-B) were determined from the effluent by an enzymatic method, using β-hydroxybutyrate dehydrogenase (Boehringer).

Electron microscopy. Mitochondrial and hepatic ultrastructure was studied with a TESLA-BS-500 electron microscope. For electron microscopic preparation of mitochondria, 0.5-ml samples were taken directly from the oxygraph or spectrophotometric cell at appropriate times, while for the hepatic tissue, small pieces of liver were cut and, in both cases, the material processed according to current techniques for electron microscopy or as described by us elsewhere [13, 19].

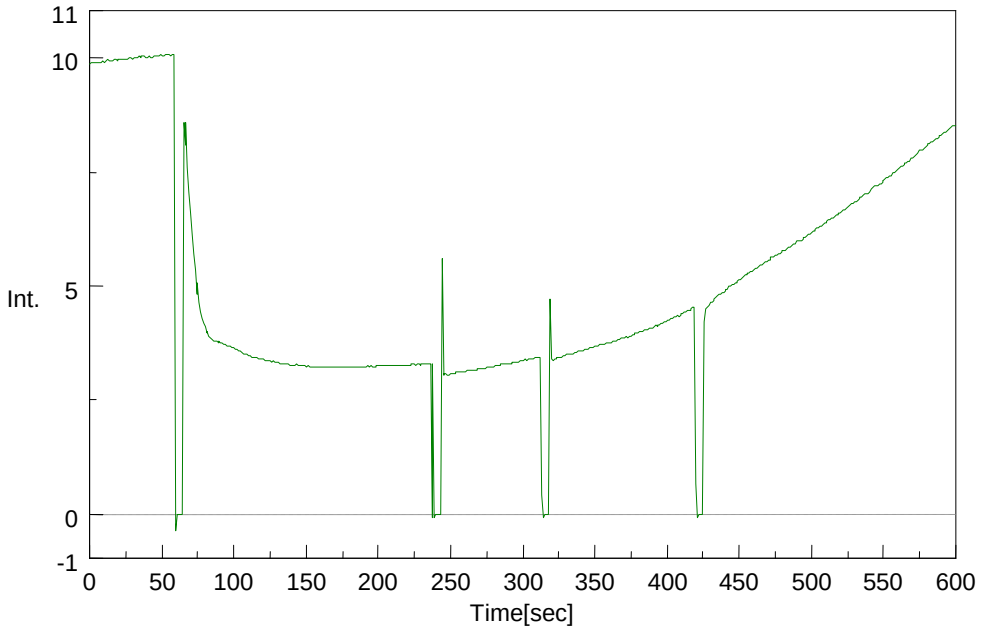
Results and discussion. *Effects on mitochondrial respiratory parameters and membrane potential*. Following the addition of clofibric acid to the suspending medium of the mitochondria, concentration-dependent effects can be observed. At 100 and 200 μM, the effects are significant for mitochondria of both animals. For example, in the case of guinea pig mitochondria, the addition of 200 μM clofibric acid in the oxygraph cell leads to a significant uncoupling of oxidative phosphorylation, RCR decreasing from an average of 4.5 with glutamate+malate to an average of 3.6, which

means a 20% decrease. A comparable phenomenon occurs for succinate-dependent respiration, the mean decrease being 21.6% (from 3.7 to 2.9). Similar results are obtained with rat mitochondria, especially in the case of succinate-dependent respiration. However, in the case of rat mitochondria respiring with glutamate+malate, the RCR decrease results more from a decrease of state-3 respiration rate than an increase of state 4 (see Fig. 1), suggesting an inhibitory effect on NADH dehydrogenase, the first respiratory complex of the inner mitochondrial membrane. Such a decrease of state 3 could not be clearly observed in the case of guinea pig mitochondria in the presence of glutamate+malate.

The membrane potential generated by succinate respiration is moderately affected by the addition of a total of 0.2 mM clofibric acid (CA) in the spectrofluorometer cell, regardless of the origin of mitochondria. Fig. 2 presents such a situation for guinea pig liver mitochondria. As can be seen, both the amplitude and the stability of membrane potential are influenced (decreased).



F i g. 1. *Effect of clofibric acid (CA) on rat liver mitochondria respiring with glutamate + malate* (see details in "Materials and methods").



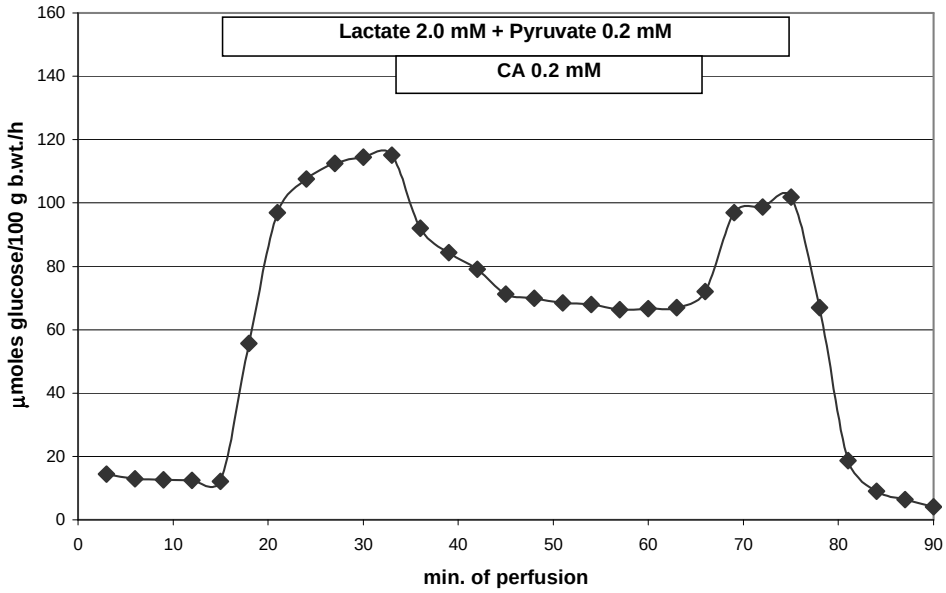
F i g. 2. *Effect of clofibric acid (CA) on the amplitude and stability of membrane potential.* Conditions are as described in text. Additions are as follows: 2.5 mM succinate (at 60 sec), 0.05 mM CA (at 240 sec), again 0.05 mM CA (at 320 sec) and 0.1 mM CA (at 425 sec).

Gluconeogenesis in the perfused liver. As can be seen from Figs. 3-4, glucose synthesis from lactate and pyruvate in the liver of the control rats covers an amplitude of about 100 μmoles/100 g body weight/h. If 0.2 mM clofibric acid (CA) is added (Fig. 3), there is a synthesis decrease of about 45 μmoles glucose/100 g body weight/h. However, about 1/3 of this effect is actually due to the solvent (ethanol) in which the drug is administered, as can be seen from Fig. 4.

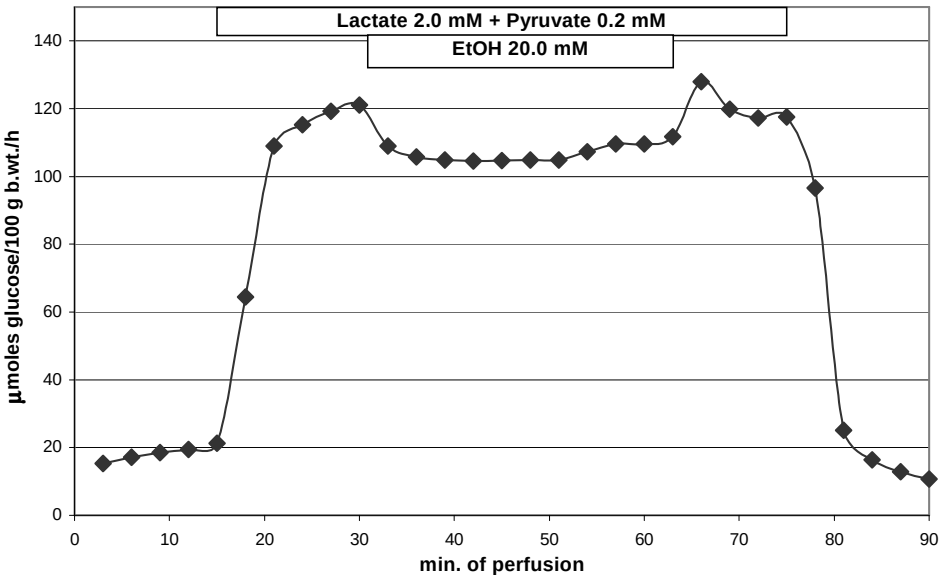
The gluconeogenic behaviour of guinea pig liver in the presence of clofibric acid is rather different. From an extent of about 75 μmoles glucose/100 g body weight/h, the apparent decrease due to clofibric acid is close to 30 μmoles glucose/100 g body weight/h (Fig. 5). However, most of this decrease is actually due to ethanol, as can be seen from Fig. 6.

Assuming a perfect additivity of the effects of the two drugs, only about 20-25% of the effect is due to clofibric acid. However, this may not be the case, since not all of the effects of the two compounds are synergistic, as we shall see from the ultrastructural studies.

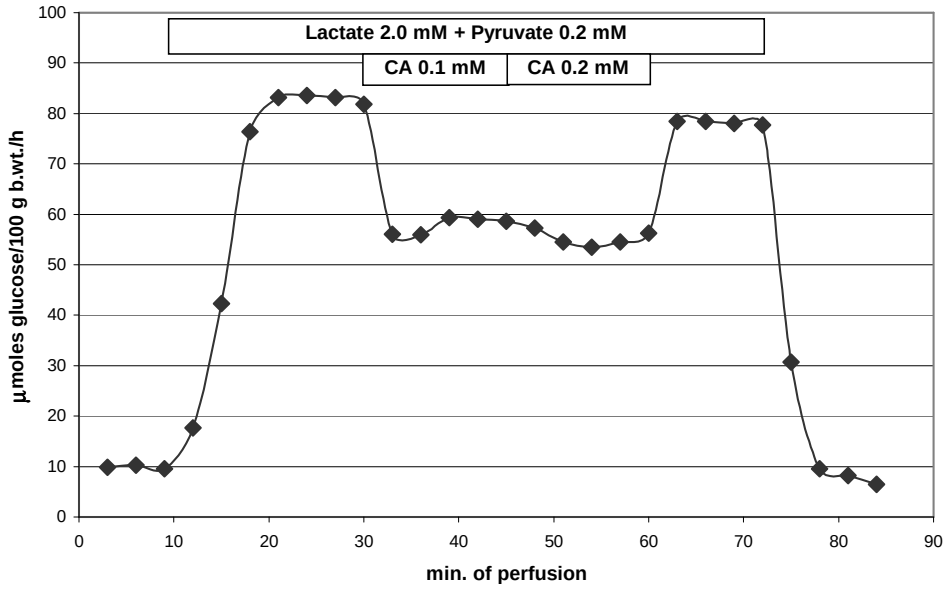
Judging by these results, it appears that rat liver is more reactive to the acute administration of CA than guinea pig liver. This difference may be due to species peculiarities of gluconeogenesis, in the first place to the different localisation of one of the key enzymes of this metabolic pathway: phosphoenolpyruvate carboxykinase. In both species, the enzyme has a double localisation, cytosolic and mitochondrial.



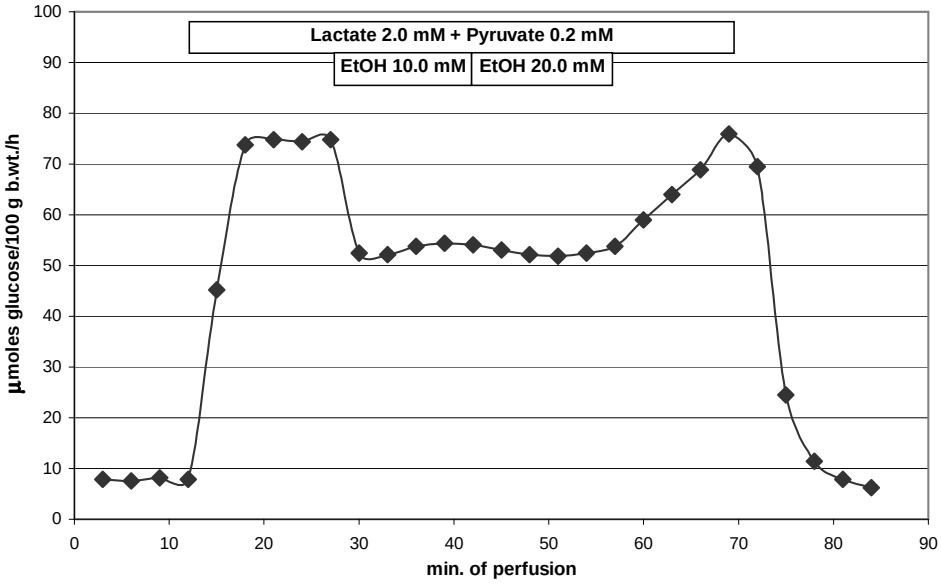
F i g. 3**.** *Gluconeogenesis in the rat liver perfused with clofibric acid (CA).*



F i g. 4. *Gluconeogenesis in the rat liver perfused with ethanol (EtOH).*



F i g. 5. *Gluconeogenesis in the guinea pig liver perfused with clofibric acid (CA).*



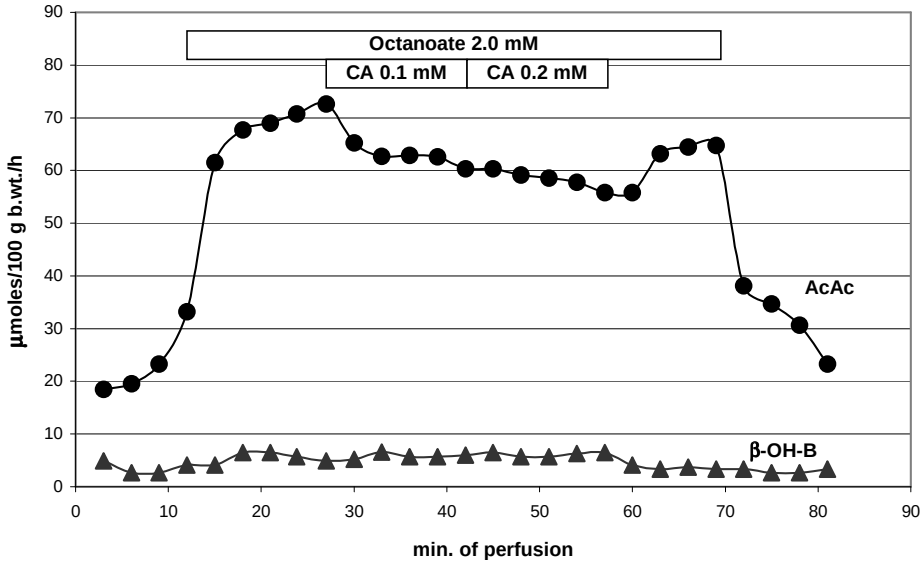
F i g. 6. *Gluconeogenesis in the guinea pig liver perfused with ethanol (EtOH).*

However, while in rat more than 90% of the enzyme is located in cytosol, in guinea pig 66% of the enzyme is found in the mitochondria. Taking into consideration that only the cytosolic enzyme participates directly in gluconeogenesis and is sensitive to the action of certain physiological (hormonal) or pharmacological regulators, it is reasonable to consider that, in this respect, the guinea pig liver has a lower sensitivity to CA than the rat liver.

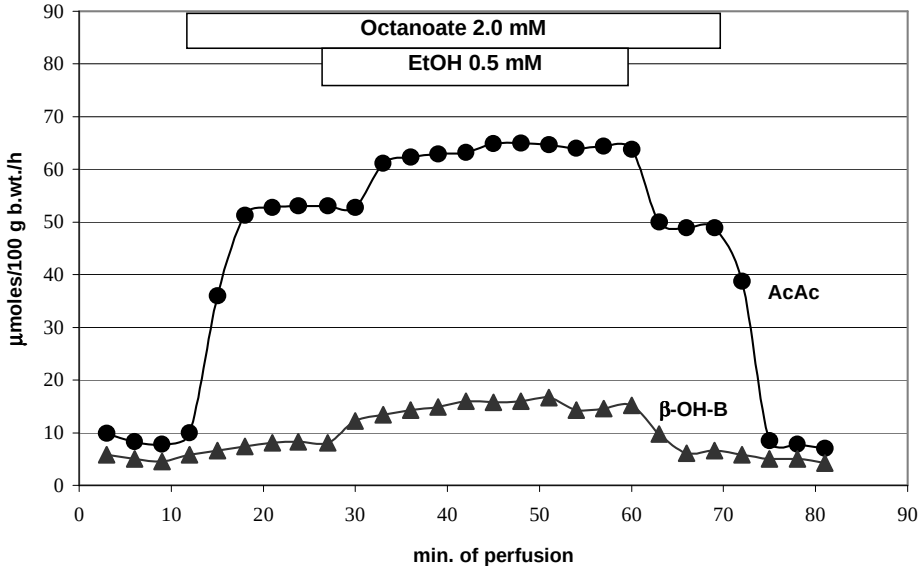
The synthesis of ketone bodies. Different aspects of ketogenesis in the perfused liver of guinea pig are presented in Figs. 7-8. The level of acetoacetate (AcAc) synthesised from octanoate varies between 50 and 55 μmoles/100 g body weight/h. In the presence of clofibric acid (Fig. 7), AcAc synthesis decreases apparently by 23% and 30%, for 0.1 and 0.2 mM CA, respectively.

However, the real decrease may be much larger, since the addition of equivalent concentrations of solvent (EtOH) as those used for CA infusion, as shown in Fig. 8, actually increases the synthesis of AcAc. The variation of β-OH-B, however, is much smaller and much less conclusive.

The stimulating effect of ethanol on AcAc synthesis by guinea pig liver is different from what occurs in the case of rat liver, where it has a marginal effect (not shown here). In order to explain this difference, one should remember that the generation of AcAc occurs from AcAc-CoA by two ways: direct deacylation by hydrolysis of AcAc-CoA and/or formation of β-hydroxy-β-methyl-glutaryl-CoA, which then yields AcAc and CoA.



F i g. 7. *Ketogenesis in the guinea pig liver perfused with clofibric acid (CA).*

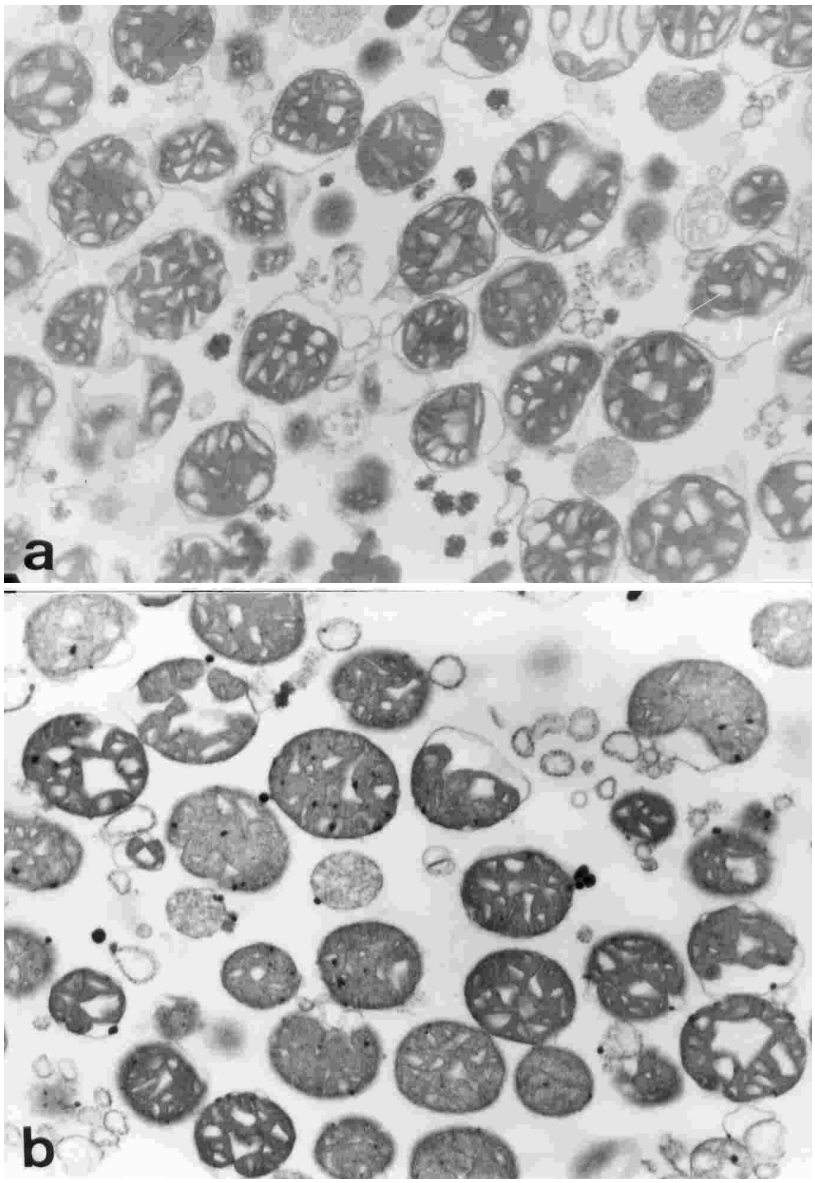


F i g. 8. *Ketogenesis in the guinea pig liver perfused with ethanol (EtOH).*

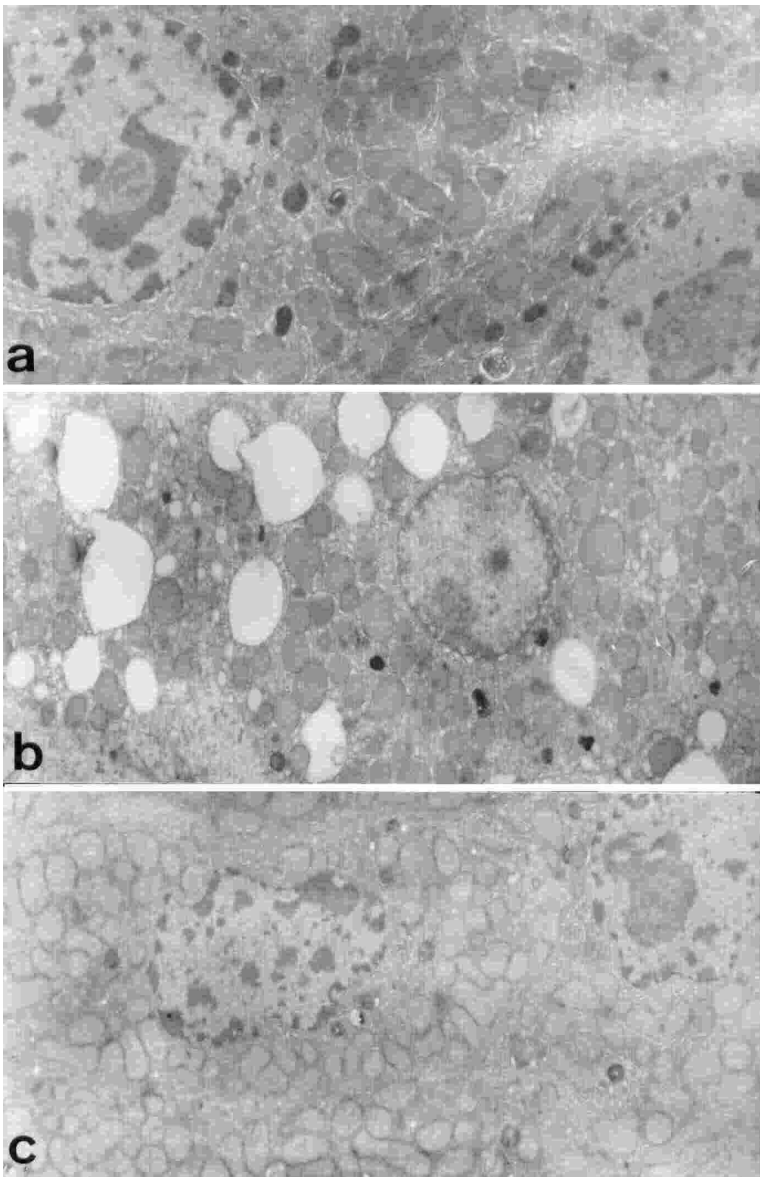
Therefore, the differences observed in the two species could be explained by different weights the two pathways may have in the synthesis of AcAc and a different impact of CA administration on the two synthesis pathways.

Ultrastructural results. Fig. 9a presents the ultrastructural aspect of control rat liver mitochondria under phosphorylating conditions (state 3) in the presence of glutamate+malate. Under these conditions, mitochondria display mainly a condensed (contracted) configuration, characteristic for coupled phosphorylating organelles, although a few ultracondensed mitochondria can also be seen, in agreement with a good but not excellent respiratory control ratio (RCR). Upon addition of 0.2 mM CA (Fig. 9b), the ultrastructural aspect changes, approaching an intermediate state between the condensed and orthodox configurations, with a slight tendency to swelling. This change is in perfect agreement with the over 20% decrease of RCR and the decrease of membrane potential described in the first section of our results. A somewhat similar situation can be observed in the case of isolated guinea pig liver mitochondria (not shown here).

The ultrastructural aspects of control rat liver are presented in Fig. 10a. The perfusion under gluconeogenic conditions with a medium containing 20 mM ethanol (Fig. 10b) has several ultrastructural effects, including a beginning of nuclear pycnosis and proliferation of smooth endoplasmic reticulum. The most important effect, however, is lipid accumulation in the cytoplasm (seen as white spots). The perfusion with a medium containing 0.2 mM clofibric acid in ethanol (Fig. 10c) does not allow lipid accumulation (antilipidemic effect of CA) but produces some effects of its own (nuclei with an irregular shape, mitochondrial matrix rarefaction etc.).



F i g. 9. *Ultrastructural aspects of rat liver mitochondria respiring with glutamate + malate, under phosphorylating conditions.* a - Control (X 32,000). b - In the presence of 0.2 mM CA (X 32,000).



F i g. 10. *Ultrastructural aspects of perfused rat liver*. a - Control (X 6,600). b - Perfused under gluconeogenic conditions in a KHB medium containing 20 mM ethanol (X 5,000). c - Perfused with 0.2 mM CA in 20 mM ethanol (X 5,100).

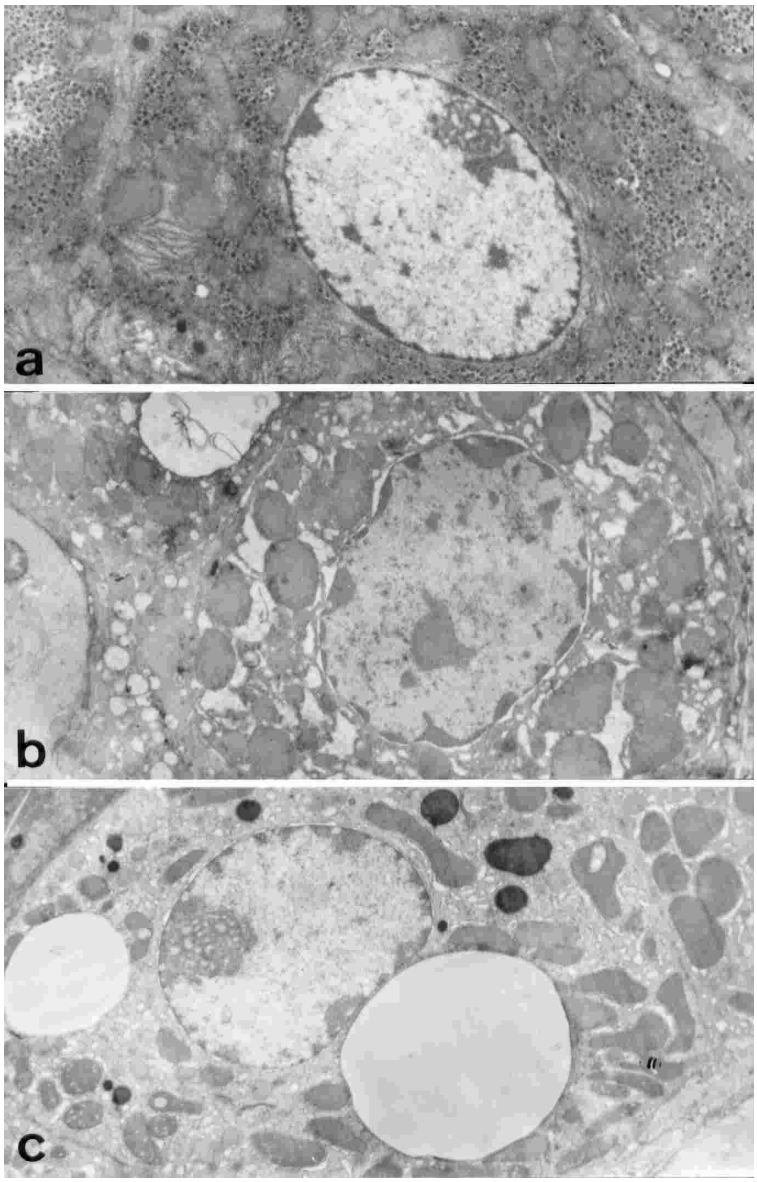
In the control guinea pig hepatic tissue (Fig. 11a), the hepatocyte has a nucleus (sometimes two) with an oval shape, containing one euchromatic nucleolus with a reticular structure. Slightly elongated mitochondria with evident cristae and of medium electron density are distributed throughout the cytoplasm. An abundance of glycogen particles, relatively uniformly distributed, can also be seen. Endoplasmic reticulum is present mostly as rough endoplasmic reticulum, especially in the perinuclear zones. Smooth endoplasmic reticulum and Golgi apparatus have a discrete presence. The lysosomes are present in small number, disposed around the biliary canaliculi. Lipid droplets are rare and peroxisomes can hardly be observed.

A 60-min. perfusion with gluconeogenic or ketogenic substrates (not shown here) leads to several changes which can be partly attributed to the perfusion flow. Thus, smaller or larger vacuolisations appear in the cytoplasm, while the sinusoidal and interhepatocyte spaces begin to dilate. The shape and electron density of mitochondria are less uniform. These and other small changes are more evident in the presence of the ketogenic substrates. As normally expected for a 48-hour pre-perfusion starvation, glycogen has disappeared.

If the perfusate also contains 0.2 mM CA, several other changes can be observed. Figs. 11b and 11c compare the effects produced by CA dissolved in ethanol (Fig. 11b) and ethanol itself (Fig. 11c), under gluconeogenic conditions. Negative effects can be observed even at the level of the nucleus, which has an irregular outline and rarefied chromatin, while the perinuclear space is dilated. Many smaller or larger vacuolisations are present in the cytoplasm, ER displays a series of small vacuoles, mitochondria are dilated and so are the biliary canaliculi. There is no glycogen, because of the 48-hour pre-perfusion starvation, and there is no clear presence of lipids (CA acts as a hypolipidemic drug). When only ethanol was used in the perfusate (Fig. 11c), lipids are present as large white drops, while the rest of the changes observed in Fig. 11b are either not present or less obvious.

Similar observations regarding different ultrastructural elements can be made under ketogenic conditions (not shown here), although due to the addition of the lipogenic substrate (octanoate) the existence of lipids can be observed even in the presence of CA. Of course, the quantity of lipids is much larger in the presence of ethanol alone and this is in perfect agreement with the stimulating effect of ethanol on ketogenesis observed by us (see Fig. 7).

In general, the ultrastructural modifications produced by CA infusion constitute the bases of the functional changes and confirm the results of the metabolic tests. Moreover, our results obtained following a subchronic administration of clofibric acid to rats and guinea pigs (to be presented in an accompanying paper) generally confirm and extend the present data.



F i g.11. *Ultrastructural aspects of guinea pig liver*. a - Unperfused control (X 6,200). b - Perfused under gluconeogenic conditions with 0.2 mM CA in 20 mM ethanol (X 6,000). c - Perfused with 20 mM ethanol (X 5,800).

Conclusions. 1. The present results demonstrate that the addition of clofibric acid (CA) to isolated mitochondria and especially to the perfused guinea pig liver has generally stronger effects than in rat liver.

2. There are certain similar characteristics in the two species: moderate uncoupling of oxidative phosphorylation, inhibition of both gluconeogenesis and ketogenesis as well as certain ultrastructural features, such as the presence of nuclei with an irregular outline, swollen mitochondria and disappearance of lipid droplets. However, the extents of the effects, at least in some cases, are different. For example, CA inhibits stronger the gluconeogenesis in the rat liver, while the ketogenesis is inhibited stronger in guinea pig liver.

3. Certain additional features can also be seen in the case of the guinea pig, especially a beginning of cytoplasm vacuolisation, dilation of the perinuclear space and of the biliary canaliculi, which may be partly due to an incompletely adapted perfusion flow.

4. Even though the results presented here are not sufficient for strong conclusions, we are now in the possession of the results obtained following a subchronic administration of clofibric acid to rats and guinea pigs and they confirm and extend the present results, *i.e.* the fact that there are important differences between the two species in regard to their reactivity to clofibric acid, although the hypolipidemic action is present in both cases.

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METABOLIC AND ULTRASTRUCTURAL EFFECTS RECORDED IN ISOLATED MITOCHONDRIA AND PERFUSED LIVER FOLLOWING THE SUBCHRONIC TREATMENT OF RATS AND GUINEA PIGS WITH CLOFIBRIC ACID

CORNELIU TARBA*, CORINA ROŞIORU*, CONSTANTIN CRĂCIUN*, CARMEN POPESCU* and VERONICA CRĂCIUN*

SUMMARY. − The results obtained in the present study demonstrate that clofibric acid (CA) has generally stronger effects on guinea pig liver metabolism and ultrastructure than on rat. Important differences can be seen following a subchronic treatment (7 days) of the animals with clofibric acid. Thus, mitochondria isolated from treated guinea pigs display a very low respiratory control ratio (1.2-1.5), while those isolated from treated rats seem to be very little affected. Ketogenesis and, to a lesser extent, gluconeogenesis in guinea pigs are more strongly affected (inhibited) by CA treatment. The differences are also confirmed by the ultrastructural results. In the case of guinea pig, isolated mitochondria are dominated by swollen or even disintegrated organelles, along with ultracondensed ones. In the hepatic tissue, one can observe polymorphous mitochondria with a rarefied matrix, dilated perinuclear spaces, enlarged lysosomes and an increased quantity of glycogen. These changes are much less visible in the case of the rat. What is striking for the rat liver after the CA treatment is the massive presence of peroxisomes (peroxisomal proliferation). Even though animal weight decreases in both species following the CA treatment, the mechanism by which it is achieved seems to be different. In the case of rat liver, the presence of peroxisomal metabolism, enhanced by the phenomenon of proliferation, is likely to represent a protective factor against CA effects, whereas the mitochondrial metabolism in guinea pig liver remains much more exposed to the action of CA.

It is generally known that the majority of xenobiotics have more than a single important effect, and this is especially true for fibrates, of which clofibric acid is a basic representative. Among the very many effects of fibrates [1, 2, 11, 12, 17, 18], peroxisomal proliferation in certain mammals is a striking phenomenon that occupies a special place [4, 12, 15-18]. However, its significance in relation to other effects has not been clearly established in all cases.

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Clofibric acid and related compounds have been used in man for their hypolipidemic effect (*i.e.* as fat lowering agents). This effect is a result of rather complex metabolic and structural changes, which are only now beginning to be understood [7, 8]. Even so, the interrelationship between peroxisome proliferation and the fat-lowering effect of fibrates is far from being clear.

As described in our previous paper [22], in order to assess the significance and the practical value of the proliferation phenomenon, we have undertaken a comparative study of the structural-functional interrelations in the peroxisomal proliferation induced by fibrates in different organisms [3, 5, 21].

We were actually able to show that such a phenomenon occurs even in certain plant cells [3], although our main target remained two laboratory mammals: rat and guinea pig. In both species, the hypolipidemic effect is present, whereas peroxisome proliferation is evident only in rat [5, 6, 21]. On the other hand, guinea pig is metabolically much closer to man than the rat and this makes it worthy of study.

Because of the pleiotropic responses to the drug, in order to discriminate between so many effects, we selected some simplified systems, *i.e.* the isolated hepatic mitochondria and the perfused liver. In a previous paper [22], we described the effects of the direct addition of clofibric acid to these systems, whereas the present article describes the results obtained following a subchronic treatment of animals with clofibric acid.

Material and methods. *Animal treatment and preparation of mitochondria*. Male Wistar rats and male guinea pigs of about 190 g each were treated with a daily dose of 20 mg clofibric acid/100 g body weight for 7 days. Clofibric acid (CA) was mixed with sunflower oil and administered in the morning, before the first feeding. The fine suspension of CA in oil was placed and absorbed onto a small piece of bread which was given individually to each animal. The piece of bread received by the animals in the control group contained only the proper amount of pure oil. Mitochondria were isolated from the livers of freshly decapitated animals, essentially according to J o h n s o n and L a r d y [9]. The isolation medium consisted of 250 mM sucrose, 10 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA, while the washing and suspending medium lacked the chelating agent.

Measurement of respiration parameters and membrane potential. Respiration rates and oxidative phosphorylation were monitored polarographically, at 20 ^{O}C, in a 0.5-ml cell, with a Clark oxygen electrode (Yellow Springs, USA), in a phosphorylation medium usually consisting of 175 mM sucrose, 50 mM KCl, 10 mM phosphate, 10-20 mM Tris, buffered at pH 7.4, 0.5 mM EDTA and 2 mM $MgSO_4$, as described in our previous paper [22]. Membrane potential generated by succinate respiration and the kinetic behaviour of this potential were monitored by the use of safranin or diS-C_2-(5) as potential probes, based on principles and details also described previously [19, 20, 23], using a Jasco V-530 spectrophotometer or a Jasco FP-750 spectrofluorometer.

Liver perfusion: assays of glucose synthesis (gluconeogenesis) and ketone bodies (ketogenesis). The animals were anaesthetised by intraperitoneal injection of pentobarbital (50 mg/kg body weight) and after the removal of viscera the liver was perfused *in situ* with Krebs-Henseleit-bicarbonate buffer (KHB), as previously described [10, 13, 14]. For the complete depletion of the glycogen reserve, the animals were starved for 48 hours and the glucose synthesis was initiated by introducing 4 mM lactate and 0.4 mM pyruvate into the perfusate, in the case of the rat, or half of these concentrations in the case of the guinea pig. Effluent samples were collected at 3-min. intervals and assayed spectrophotometrically for the presence of glucose as decribed [13, 14]. Ketogenesis was assayed by using the methods specified in [22].

Electron microscopy. Mitochondrial and hepatic ultrastructure was studied with a TESLA-BS-500 electron microscope. For electron microscopic preparation of mitochondria, 0.5-ml samples were taken directly from the oxygraph or spectrophotometric cell at appropriate times, while for the hepatic tissue, small pieces of liver were cut and, in both cases, the material processed according to current techniques for electron microscopy or as described by us elsewhere [14, 20].

Results. *Body weight evolution and relative liver weight.* Each animal was weighed at the beginning and the end of the treatment. The livers were also weighed at the time of the sacrifice or immediately after the perfusion. The results are presented in Table 1 as mean values of 3 individual measurements for each group of animals. In all cases, the differences are very significant ($p < 0.01$).

Table 1

Effect of a 7-day treatment with clofibric acid (CA) on the body and liver weight

Animal	Parameter	Mean body weight (b.w.)		Difference	Differential average	Mean relative liver
	Group	Initial (g)	Final (g)	(g)	change/animal (g)	weight (% of b.w.)
Rat	Control	188.7	201.0	+ 12.3	-	2.99
Rat	CA-treated	190.0	184.5	- 6.5	- 18.8	3.51
Guinea pig	Control	201.8	208.2	+ 6.4	-	2.65
Guinea pig	CA-treated	189.3	178.7	- 10.6	- 17.0	3.31

As can be seen from the table, following the CA treatment, there is a mean weight decrease of 6.5 g/rat and 10.6 g/guinea pig. If one considers the natural increase in the control groups, the differential average decrease is even larger: 18.8 g/rat and 17.0 g/guinea pig. In both species, a significant increase of the relative liver weight can also be observed. The percent increase is 16.7% for rat and 25% for guinea pig. These results suggest a hypertrophy of the liver, especially in the case of the guinea pig.

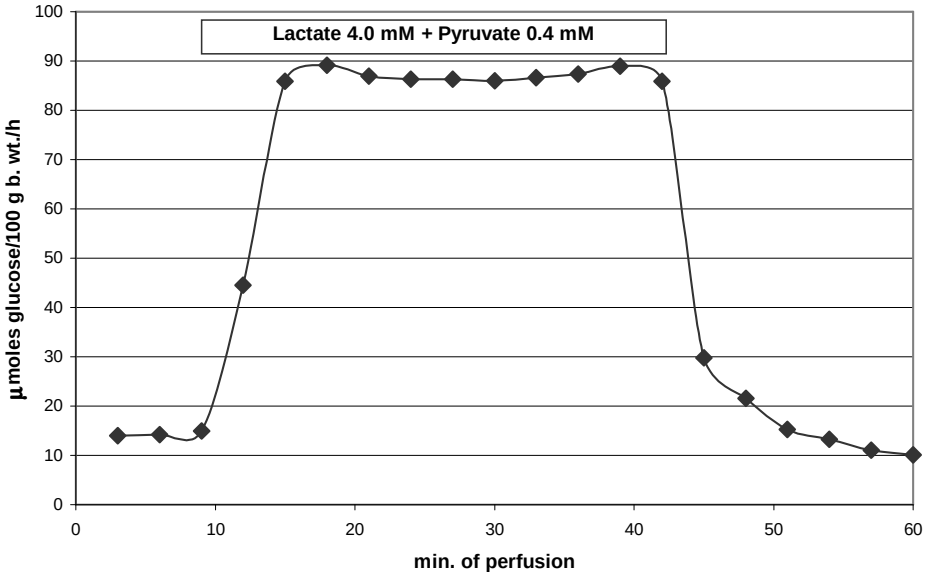
Effects on mitochondrial respiratory parameters and membrane potential. In our previous article [22], we reported slight differences between the behaviour of guinea pig and rat liver mitochondria as regards the effect of CA addition on the respiratory parameters. For mitochondria isolated from CA-treated animals, there is a much larger difference between the two species. While mitochondria from treated rats do not show significant differences from the control, guinea pig mitochondria are almost completely uncoupled (RCR = 1.2-1.5 with glutamate+malate). Likewise, in the case of guinea pig mitochondria, the membrane potential generated by succinate respiration has a small amplitude and collapses immediately after its formation, while for rat, there are no apparent differences in comparison with the control (not shown here).

Gluconeogenesis and the synthesis of ketone bodies in the perfused liver. As a general observation, we should mention that the aspects recorded in the control animals were not essentially different from what we presented in our previous paper [22] and, therefore, they will not be systematically shown here. Moreover, such a presentation is not always necessary, because the results reported here were obtained practically under the same conditions for both species. As can be seen from Fig. 1, glucose synthesis from lactate and pyruvate in the liver of CA-treated rats during perfusion reaches a steady state level close to 90 μmoles/100 g body weight/h, which is lower than in the control by about 30 μmoles/100 g body weight/h.

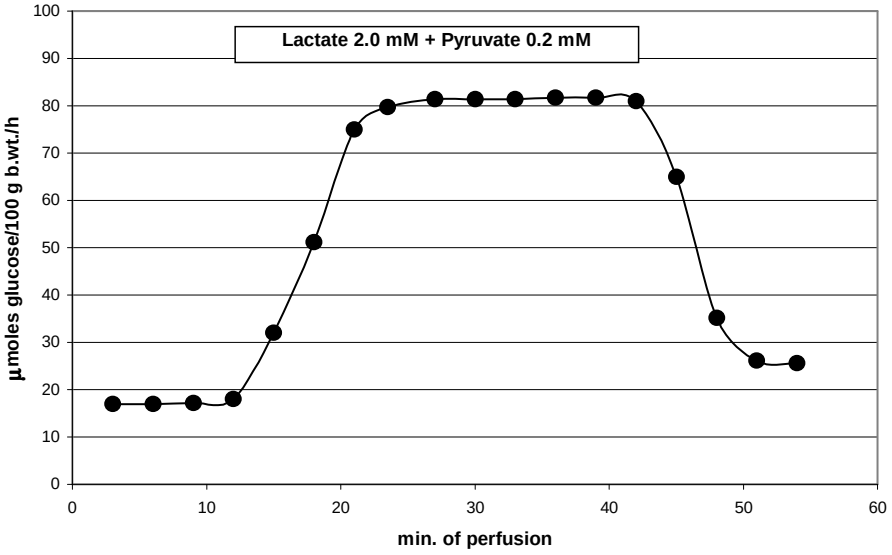
A slightly higher inhibitory effect can be detected in the case of gluconeogenesis occurring in the liver of CA-treated guinea pigs (Fig. 2). The steady state level of glucose here is close to 80 μmoles/100g body weight/h as compared to 115 in the liver of the control guinea pigs.

A much larger difference can be observed in the synthesis of ketone bodies during octanoate infusion in the livers of the two species, as illustrated in Figs. 3 and 4. While the maximum level of acetoacetate (AcAc) formation in the perfused liver of CA-treated rats reaches about 110 μmoles/100 g body weight/h (Fig. 3), the steady state level barely exceeds 45 μmoles/100 g body weight/h in the guinea pig perfused liver (Fig. 4), although in the corresponding control liver this level was close to 70 μmoles/100 g body weight/h.

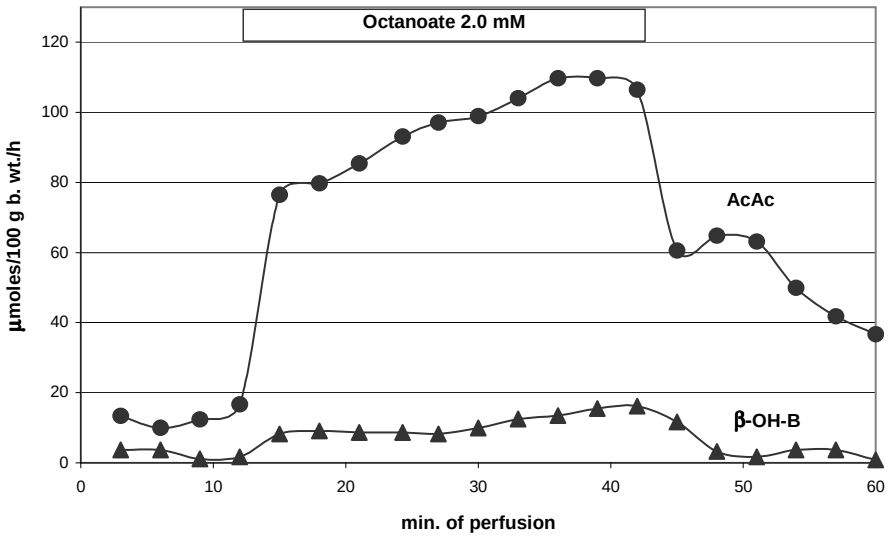
Electron microscopic results. The differences between the two species regarding the reactivity to clofibric acid are also confirmed by our electron microscopic results. Fig. 5a presents ultrastructural aspects of the control guinea pig liver mitochondria under phosphorylating conditions (state 3) in the presence of glutamate+ malate. Under these conditions, mitochondria display mainly a condensed (contracted) configuration, characteristic for coupled phosphorylating organelles, although a few ultracondensed mitochondria can also be seen, in agreement with a good but not excellent respiratory control ratio (RCR). Mitochondria isolated from the CA-treated guinea pigs have a totally different aspect. As can be seen from Fig. 5b, the electron micrograph is dominated by swollen or even disintegrated organelles. This is in contrast to the corresponding rat liver mitochondria which can hardly be differentiated from their control (not shown here).



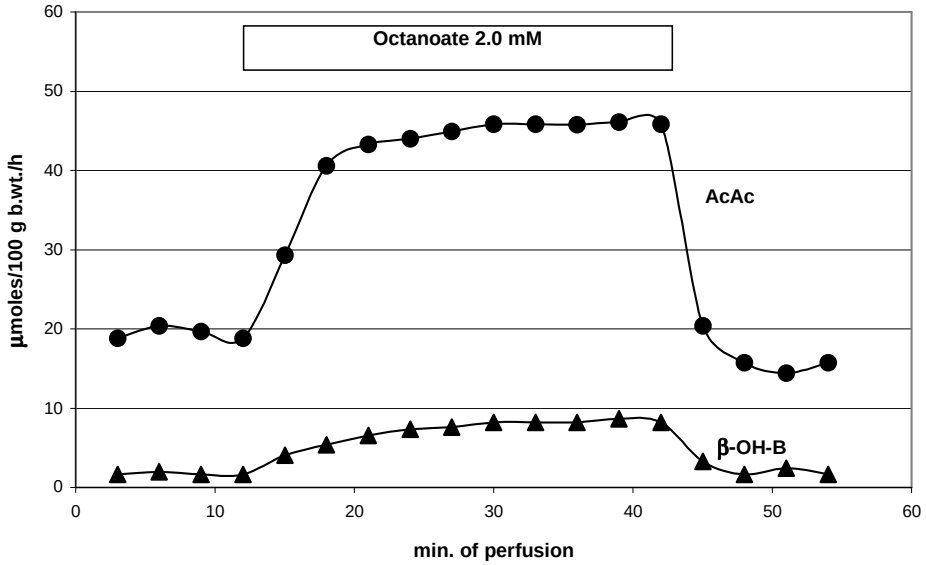
F i g. 1. *Gluconeogenesis in the perfused liver of CA-treated rats* (see details in text).



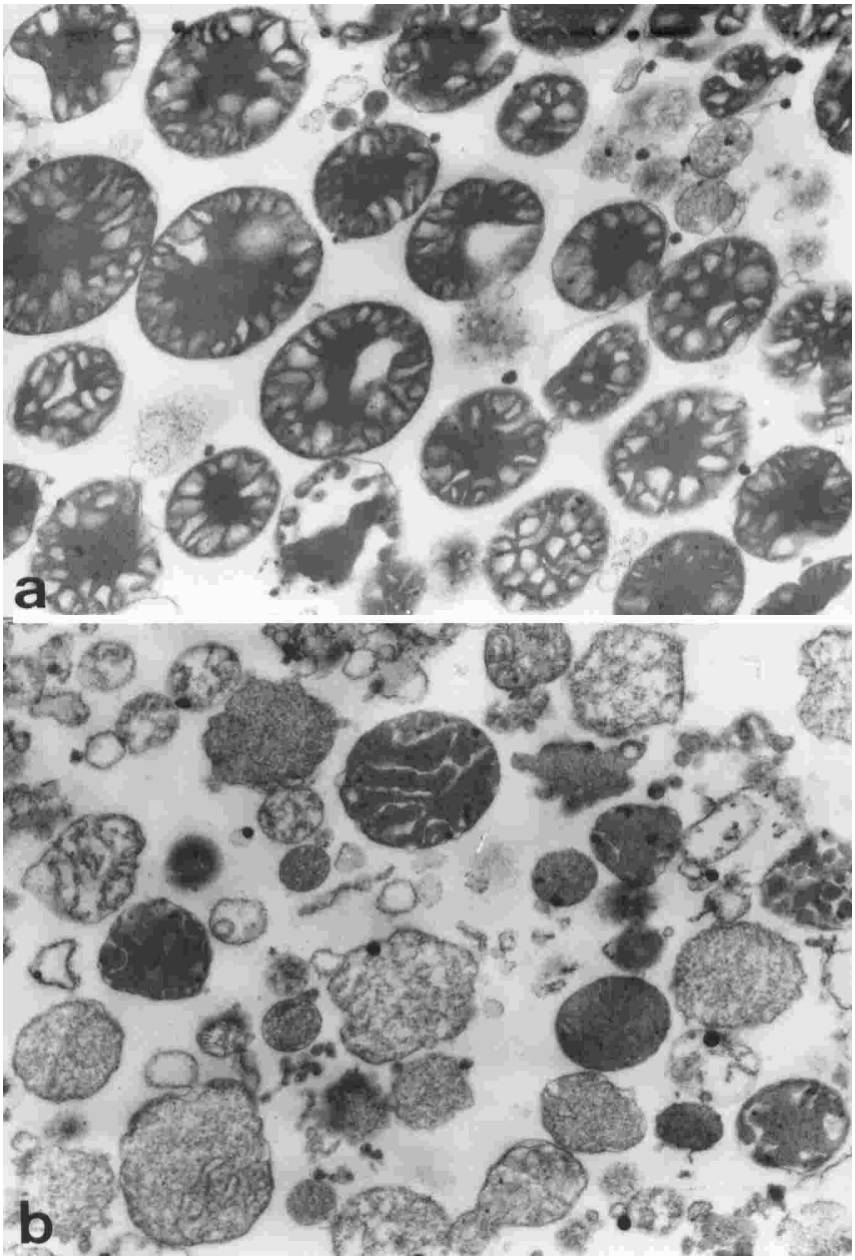
F i g. 2. *Gluconeogenesis in the perfused liver of CA-treated guinea pigs.*



F i g. 3. *Ketogenesis in the perfused liver of CA-treated rats.*



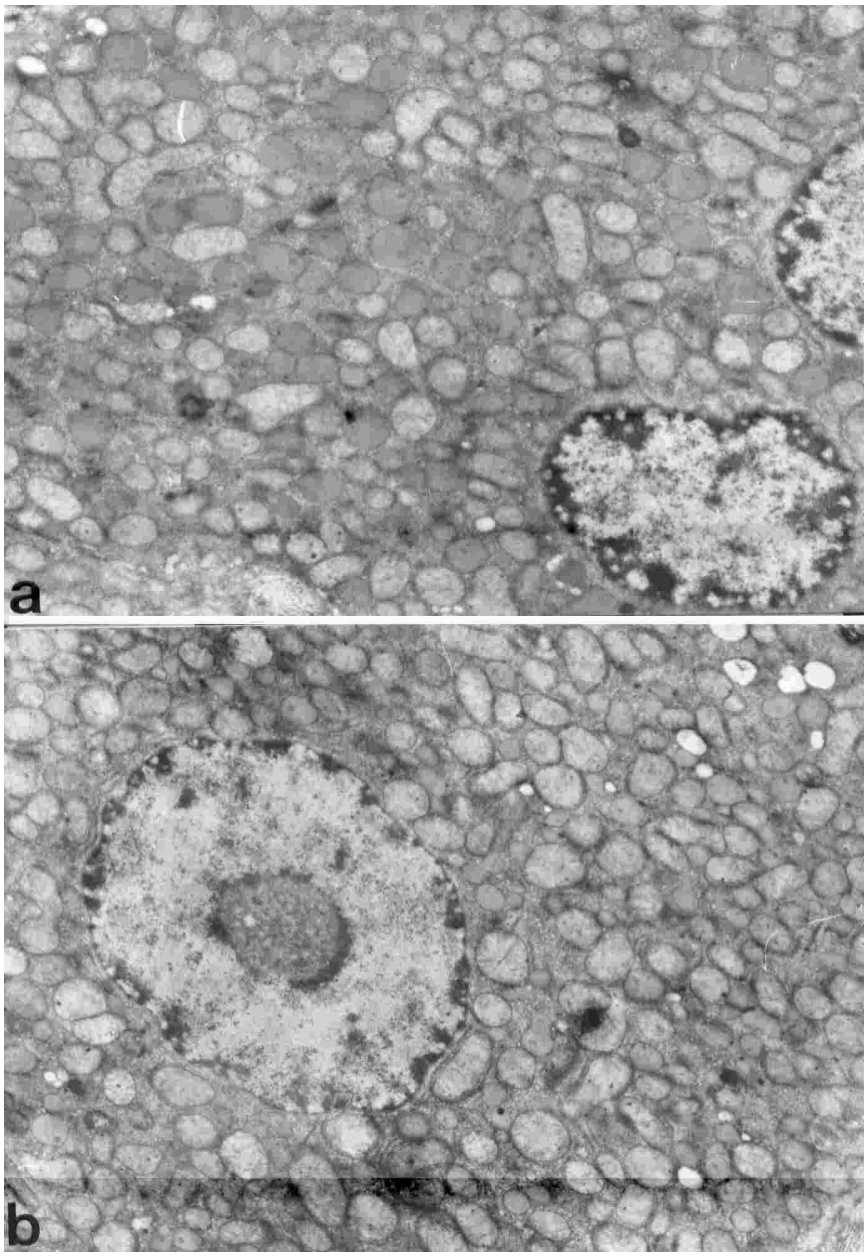
F i g. 4. *Ketogenesis in the perfused liver of CA-treated guinea pigs.*



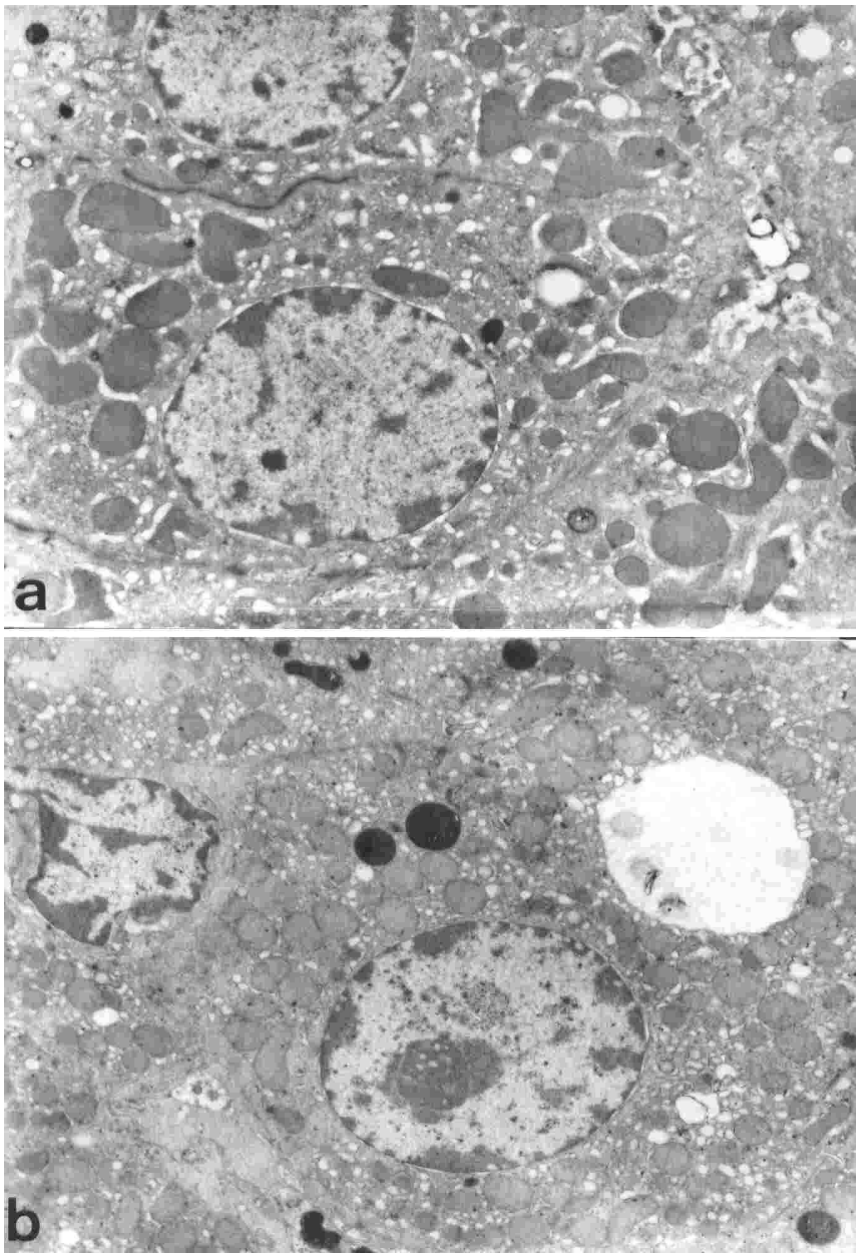
F i g. 5. *Ultrastructural aspects of guinea pig liver mitochondria under phosphorylating conditions.* a – Control (X 19,000). b - Obtained from CA-treated animals (X 19,000).

Differences between the two species can also be observed on liver sections. Fig. 6 compares the ultrastructural aspect of the hepatic tissue of the CA-treated rat (Fig. 6a) with that of the CA-treated guinea pig (Fig. 6b). The main difference between the two pictures is the massive presence of peroxisomes in the rat hepatocyte. This peroxisome proliferation is typical for rat, while, in the guinea pig hepatocyte, the presence of peroxisomes can hardly be identified. However, there are other more subtle changes present in the guinea pig hepatic tissue, which are much less obvious for the rat. Among such changes, we should mention: the diminution of the reticulate aspect of the nucleolus, the presence of polymorphous mitochondria with a rarefied matrix, of enlarged lysosomes and rarefied microvilli, as well as the occasional presence of dilated biliary canaliculi. Such changes become even more obvious under metabolic stress, when guinea pig liver is perfused under gluconeogenic (Fig. 7a) or ketogenic (Fig. 7b) conditions. Thus, the nuclei tend to become pycnotic, the perinuclear spaces and the endoplasmic reticulum are dilated, while the mitochondria have lost their cristae. The extension of these alterations may be influenced by the perfusion flow, but they are definitely more obvious than in the rat liver submitted to the same procedure (not presented).

Discussion. Despite the general hypolipidemic effect (which results in weight loss), observed following the subchronic treatment with clofibric acid of either rat or guinea pig, both the functional and the ultrastructural results seem to indicate a different type of reactivity of the two species towards this drug. It is interesting that in experiments where clofibric acid was added directly to the working systems (isolated mitochondria and perfused liver), the differences between the two species were less important (see [22]). Thus, in the simplest system used by us (isolated mitochondria), we could not detect significant functional or structural differences, while in the perfused liver, following a 30-60-min. infusion of clofibric acid, the differences started to appear, although to a lesser extent than in the subchronic treatment. The differences between the two types of methodological approach (direct addition of CA and subchronic treatment) were, however, largest in the simpler system, as demonstrated by comparing the results obtained in the present study to those reported in our previous study [22]. The functional incompetence associated with grave structural alterations observed in liver mitochondria isolated from CA-treated guinea pigs is in striking contrast with the functionally and structurally almost-perfect liver mitochondria obtained from CA-treated rats. All these observations have to be taken as a strong indication that CA effects are metabolically mediated in a different manner in the two species. The only structural feature observed by us which could have a positive biological significance in regard to this problem is peroxisome proliferation, which is significantly present only in the livers of CA-treated rats.



F i g. 6. *Ultrastructural aspects of the KHB-perfused liver in CA-treated animals.*
a - Rat liver (X 7,600). b - Guinea pig liver (X 7,600).



F i g. 7. *Ultrastructural aspects of livers obtained from CA-treated guinea pigs, perfused under gluconeogenic conditions (a)* (X 7,600) *or ketogenic conditions (b)* (X 7,600).

It is known that (in the eukaryotic cell) peroxisomes represent a secondary site of lipid oxidation. The peroxisomal β-oxidation of fatty acids differs from that of the mitochondrial β-oxidation in that it is not coupled to an electron transport chain and to ATP synthesis (see [24] for a review). Only about half of the energy liberated in this oxidation is finally conserved (into NADH) and this is one of the reasons why peroxisome proliferators can be used in diets for loosing weight. It has been demonstrated that the action of the peroxisome proliferators is actually mediated through several receptors known as PPAR (peroxisome proliferator-activated receptors) (see, for ex., [7, 25]), which are usually activated by their natural ligands (fatty acids) and capable of genetic induction of the necessary enzymatic systems.

Nevertheless, our results point to a hypolipidemic effect of clofibric acid not only in rat but also in guinea pig hepatocytes. From our functional and structural results, it appears that this is achieved mainly through a more-or-less direct action on mitochondria, which suffer a process of swelling and even disintegration, with the loss of respiratory control, collapse of the membrane potential and of the phosphorylation ability, leading to energy dissipation.

We showed in our previous article [22] that, by the direct addition of clofibric acid to isolated mitochondria, there was very little difference between the mitochondrial behaviour of the two species, a fact which raises the question regarding the mechanism by which the action of clofibric acid is alleviated in the case of rat liver mitochondria obtained from subchronically treated animals. A possible answer is that peroxisome proliferation in the rat hepatocytes, responsible for accomplishing the β-oxidation, is also responsible for the protective effect. One could speculate that clofibric acid and other fibrates induce not only peroxisome proliferation but also a set of peroxisomal enzymes capable of dealing with such drugs, fulfilling thus a protective effect.

Conclusions. 1. The results presented here, in corroboration with our previous results [22], indicate that the phenomenon of weight loosing determined by clofibric acid treatment has a different metabolic mechanism in rat and guinea pig.

2. Besides directing β-oxidation towards a less efficient utilisation, it seems that peroxisomal proliferation occurring in the rat liver may also constitute a protective phenomenon against the damaging effects that such a drug exerts on mitochondrial structure and metabolism in the guinea pig liver.

3. This observation may be very important in selecting the proper treatments for overweight persons, since humans (which also do not show an obvious peroxisomal proliferation) are generally known to behave metabolically closer to guinea pig than to rat.

R E F E R E N C E S

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI, BIOLOGIA, XLVI, 1, 2001

COMPARATIVE STUDIES OF THE ADRENAL CORTEX STRUCTURE AND ULTRASTRUCTURE IN MATURE RATS TREATED WITH TOPICAL DERMOCORTICOSTEROIDS

CONSTANTIN CRĂCIUN*, ERIKA KIS*, VICTORIA-DOINA SANDU*, CRISTINA PAŞCA*, CONSTANTIN PUICĂ, VERONICA CRĂCIUN* and IOSIF MADAR****

SUMMARY. - It is well established that synthetic topical corticosteroids, widely applied in human dermatology, exert, beside their excellent local action, adverse secondary systemic side effects, due to their epicutaneous absorption capacity. There are still very few correlative research works about the influence of glucocorticoids at systemic level, especially about those used in very common drugs, widely recommended to patients, such as Locoid, Dermovate and Fluocinolone-N, which were studied in our works. In order to obtain complete data about the action of these glucocorticoids upon some endocrine glands (thymus, adrenals, pituitary gland) especially of young animals, our research work was done primarily on prepubertal and pubertal rats. Our results indicate that Locoid has moderate potent actions, with reversible modifications, while Dermovate has potent effects and Fluocinolone-N superpotent effects, inducing the most severe modifications. Therefore, it is recommended that the topical use of this steroid class for long-term therapy be limited or to find possibilities for improving the benefit/risk ratio between their local and systemic adverse side actions.

In recent studies [3, 7] we have reported that the short-term epicutaneous application of some halogenated or unhalogenated topical glucocorticosteroids in young rats, exerts, depending on the age of individuals, steroid-diabetogen secondary side effects, manifested by hyperglycemia, hyperinsulinemia, hepatic glucose overproduction, elevated serum lipids and muscular resistance to insulin. All these endocrine-metabolic disorders were accompanied by pancreatic islet damage, thymolysis, intense lipid accumulation and several ultrastructural modifications.

Some recent experimental data suggest that the dermocorticosteroid action is facilitated by beta-adrenoreceptors lying in the keratinocytes of the stratum basal and in the Langerhans cells of epidermis [1, 11]. These receptors facilitate the epicutaneous absorption of glucocorticoids and their subsequent accumulation in the body, with negative side effects at systemic level.

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Starting from the above established data and from the important physiological roles of the adrenal glands in regulation of the metabolism, we investigated the adrenal reaction in mature rats, after a short-term epicutaneous treatment with the following dermocorticosteroids: Locoid, Dermovate and Fluocinolone-N.

Material and metods. The experiments were carried out on mature (60-day old) male Wistar rats. The animals were kept under standardised bioclimatic conditions and fed on a common rat chow, with water *ad libitum*. The animals were treated for 3 consecutive days, with Locoid, Dermovate and Fluocinolone-N.

Commercial formulations of 0.10% (w/w) hydrocortisone-17-butyrate containing Locoid cream ("Brocades Pharma" bv., Leidorp, Netherlands), of 0.05% (w/w) clobetasol-propionate containing Dermovate cream ("Glaxo Operations" UK Ltd., England), as well as of 0.025% (w/w) fluocinolone-acetonid-N containing ointment ("Antibiotics" S.A. Iaşi, Romania), were applied topically to the skin on 2 cm^2, for 3 consecutive days, by smearing 50 mg cream or ointment /100g b.w, on the inguinal region.

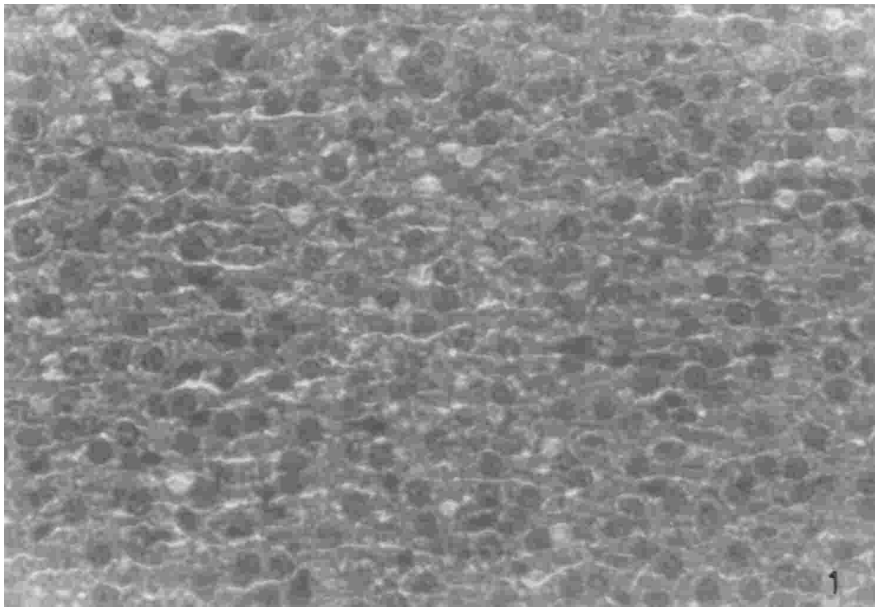
After 16 hours of fasting and 24 hours following the cessation of treatments, the animals (Locoid-treated, Dermovate-treated and Fluocinoline-treated groups) together with a control group, were sacrificed by exsanguination.

The adrenal fragments were quickly isolated and prepared for structural and ultrastructural examinations. For structural examinations, the adrenal was fixed in Bouin liquid and afterwards processed in view of being embedded in paraffin. The adrenal fragments were sectioned in the Reichert-Austria type microtome at a thickness of 7 μm, and the staining of the sections was made by means of hematoxylin-eosin method [9]. The histological preparations obtained were examined in the $IOTC_4$ light microscope.

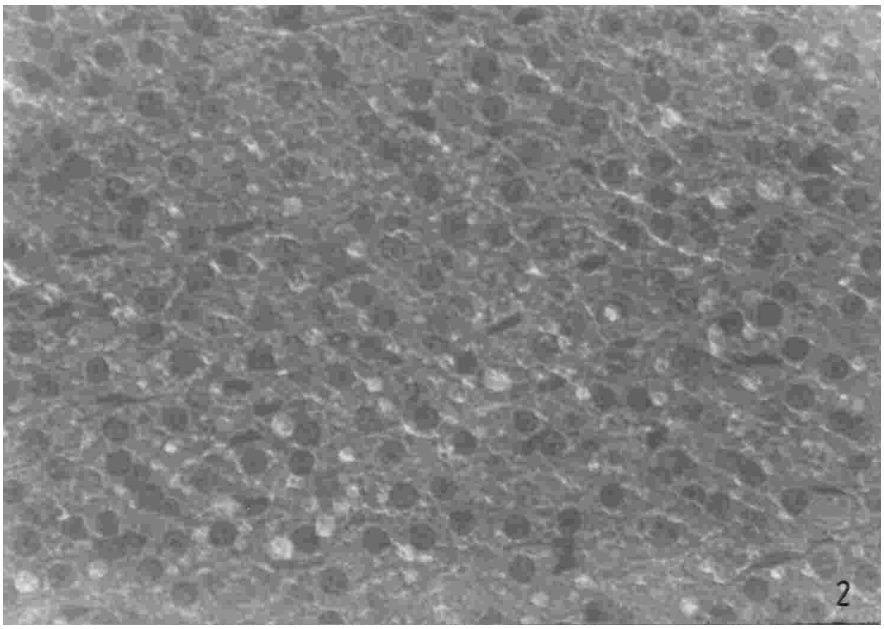
For ultrastructural examinations, the adrenocortical fragments were prefixed in a 2.7% glutaraldehyde solution and postfixed in a 2% osmic acid solution. The dehydration of samples was performed in acetone and then they were embedded in Vestopal W. The ultrathin sections were obtained using an LKB-III ultramicrotome and were contrasted with uranyl acetate and lead citrate. Examinations of sectiones were performed in a TESLA-BS-500 electron microscope.

Results and discussion. The histological examination evidenced significant morphological changes of the adrenal cortex in the Wistar rats treated with the three dermocorticosteroid formulations used. In the *Locoid-treated group* (L-group), the adrenal cortex presents an aspect which is close to that of the control group. However, a slight decrease can be seen in the zona fasciculata width, as compared to the medullar one (Fig. 1). Certain glandular cells of zona fasciculata have intensely vacuolated cytoplasm, showing a foamy aspect, due to the so-called spongiocytes which are present under normal conditions.

In the *Dermovate-treated group* (D-group), we observed more severe changes of the adrenal cortex (Fig. 2), compared to that treated with Locoid. The fasciculate zone is narrower but more compact, and the number of spongiocytes was more reduced than in the Locoid-treated group. The compact structure of the fasciculate zone suggests a moderate alteration of the secretory activity.

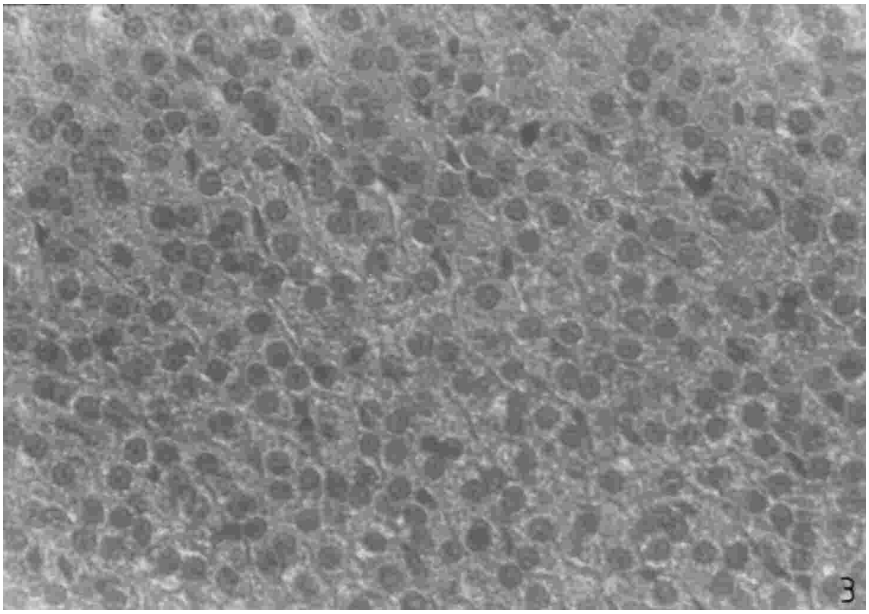


F i g. 1. *Adrenal cortex in L-group* (x 200).



F i g. 2. *Adrenal cortex in D-group* (x 200).

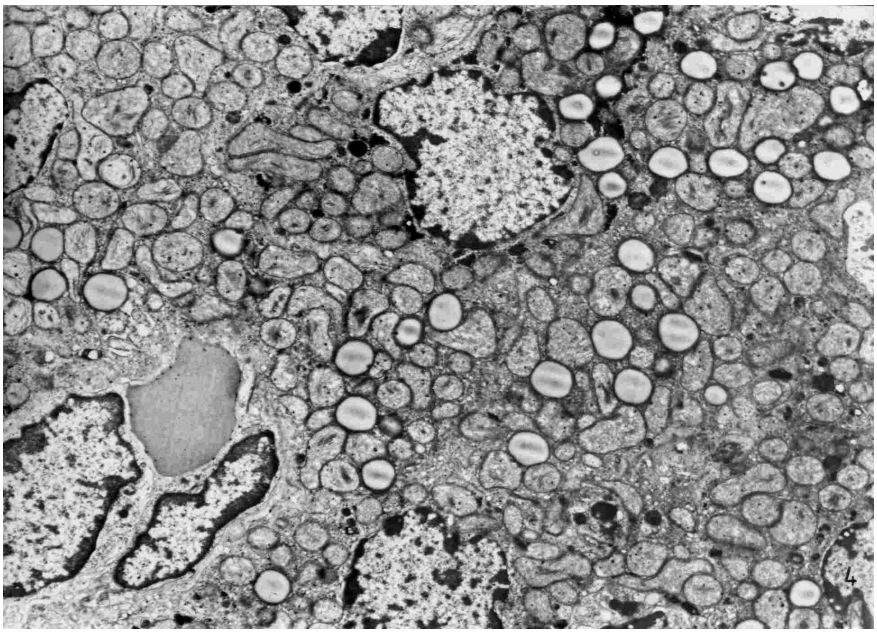
The examination of the histological aspect of the adrenal in *Fluocinolone-treated group* (F-group) allows us to notice the structural alterations induced by Fluocinolone-N ointment. Compared to the control group and the Dermovate- and Locoid-treated groups, a remarkable decrease in the width of adrenal cortex is noticed in F-group (Fig. 3), which seems to be due to an important loss of parenchymal cell number in the fasciculate zone and in the reticularis one. The fasciculate zone is more compact than in D-group and the spongiocytes are not present.



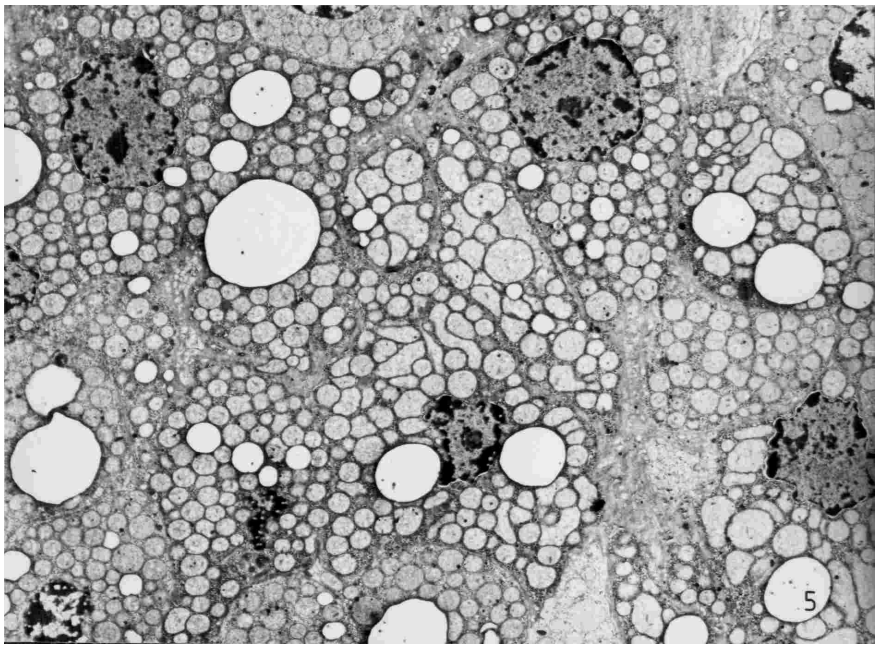
F i g. 3. *Adrenal cortex in F-group* (x 200).

The electron microscopic examination confirms the histological data mentioned above. Comparatively with the normal ultrastructural aspects observed in the adrenal cortex of the control group, the treated groups present different modifications. The ultrastructural analysis of the adrenal cortex of L-group showed the following changes: the presence of a cellular mosaic (Fig. 4), cells with an ultrastructure suggesting either a normal secretory activity or an alteration of this activity, a slight increase of the number of lysosomes and the presence of the myelinic figures. The cytoplasm contains a reduced number of free ribosomes and polysomes, relatively few vacuolated mitochondria with a rarefied matrix. The Golgi complex is less extensive, suggesting a moderate secretory activity.

In the D-group the number of secretory granules is more reduced (Fig. 5) than in the L-group. In contrast with the normal aspect of nucleus in controls, after epicutaneous treatment with Dermovate, unusually sizeable and irregularly shaped nucleus appeared. In some cells a slight nuclear chromatin condensation and an increase of heterochromatin quantity could be noticed. The heterochromatin is scattered through the entire nucleus or is packed in blocks near the nuclear membrane.

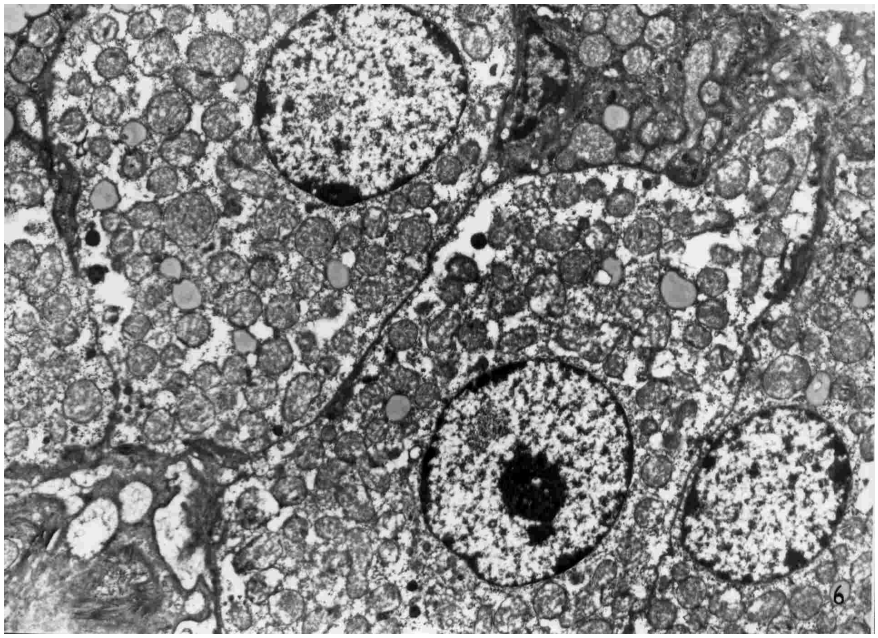


F i g. 4. *Zona fasciculata with moderate secretory activity in L-goup* (x 6,510).



F i g. 5. *Nuclear chromatic condensation, alterations of the mitochondria and of the smooth endoplasmic reticulum in D-group* (x 4,620).

Fluocinolone treatment of mature rats induces a severe secretory granule depletion in the entire zona fasciculata. The scarcity in the content of cytoplasmic organelles, mainly in the elements of smooth endoplasmic reticulum is also characteristic for the cells of this group. The mitochondrial matrix and cristae are more rarefied than in D-group (Fig. 6) and, in some cases, their membranes are completely destroyed.



F i g. 6. *Cellular vacuolisation in zona fasciculata in F-group* (x 6,510).

The hypothalamo-pituitary-adrenal axis and autonomic nervous system are major effector systems, that serve to maintain homeostasis during exposure to stressors [12]. The hypothalamus is generally believed to be the site of negative feed-back mechanism by which glucocorticoids counterregulate neuroendocrine responses to stressors. An excess of glucocorticoids inhibits the synthesis and release of corticotropin-releasing factor in hypothalamic paraventricular nucleus [2, 8, 10].

Literature data [5, 6] as well as our results have rendered evident the fact that glucocorticoid administration results in alteration of the hypothalamo-pituitary-adrenal axis activity. A considerable reduction in adrenal cortex width, accompanied by degranulation indicates a functional inhibition of the gland. Although the plasma corticosteroid concentration was not determined, the ultrastructural appearance of cortical cells also confirms that their activity was inhibited. This is consistent with the recent findings that dexamethasone (a syntetic glucocorticoid) administration inhibits the synthesis and release of cortisol [4,13].

Conclusions. 1. Epicutaneous administration of glucocorticoids to mature (60-day old) male rats over 3 days resulted in significant atrophic changes in adrenal cortex.

2. The structural and ultrastructural modifications of the adrenal induced by short-term epicutaneous treatments with Fluocinolone-N exert more severe actions than those observed in the case of treatments with Locoid or Dermovate.

3. The degree of adrenal atrophy and decrease of secretory activity are mainly dependent on the dose, composition and molecular structure of topical glucocorticoids present in these three dermocorticosteroids: Locoid exerts moderate potent actions, Dermovate has potent effects and Fluocinolone-N has superpotent effects, at the level of the adrenal cortex.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI, BIOLOGIA, XLVI, 1, 2001

COMPARATIVE STUDIES OF THE ULTRASTRUCTURE OF SOMATOTROPE, GONADOTROPE AND CORTICOTROPE CELLS IN MATURE RATS TREATED WITH TOPICAL DERMOCORTICOSTEROIDS

ERIKA KIS*, CONSTANTIN CRĂCIUN*, CRISTINA PAŞCA*, VICTORIA-DOINA SANDU*, VERONICA CRĂCIUN* and IOSIF MADAR**

SUMMARY. – Topical dermocorticosteroids have been used in treatment of skin diseases as well as in cosmetic products. Unfortunately, along with their undisputable efficacy, their use has been associated with unwanted secondary effects on some endocrine glands (thymus, adrenals, adenohypophysis). In this paper we present the ultrastructural modifications induced by topical glucocorticoid treatment in the somatotrope, gonadotrope and corticotrope cells.

Topical glucocorticoid therapy has been one of the most significant advances in dermatology. Glucocorticoids are potent antiinflammatory and immuno-suppressive agents widely used in the treatment of many skin diseases, but their mechanism of action, although known to be multifactorial, is not yet fully understood. Whereas some of the antiinflammatory effects of glucocorticoids have been attributed to the synthesis of lipocortins, the immunosuppressive effects are thought to be mediated through the inhibition of several immune functions, including chemotaxis, phagocytosis and cytotoxicity, by down-regulation of cytokine gene expression [9].

Despite their efficacy, the use of topical glucocorticosteroids is limited by the local and systemic side effects. Systemic absorption inevitably occurs to a variable degree depending on the pharmacokinetic properties of the drug, the area of skin on which it is applied.

In our experiments we intended to study the ultrastructural changes of the adenohypophysary cells in mature male rats, subjected to an acute epicutaneous treatment with the following dermocorticosteroids: Locoid, Dermovate and Fluocinolone-N.

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Materials and method. The experiments were carried out on mature (60-day old) male Wistar rats. The animals were kept under standardizd bioclimatic conditions and fed on a common rat chow, with water *ad libitum*. The animals were treated for 3 consecutive days, with Locoid, Dermovate and Fluocinolone-N.

Commercial formulations of 0.10% (w/w) hydrocortisone-17-butyrate containing Locoid cream ("Brocades Pharma" bv., Leidorp, Netherlands), of 0.05% (w/w) clobetasol-propionate containing Dermovate cream ("Glaxo Operations" UK Ltd., England), as well as of 0.025% (w/w) fluocinolone-acetonid containing ointment ("Antibiotics" S.A. Iaşi, Romania), were applied topically to the skin on 2 cm^2, for 3 consecutive days, by smearing 50 mg cream or ointment /100g b.w, on the inguinal region.

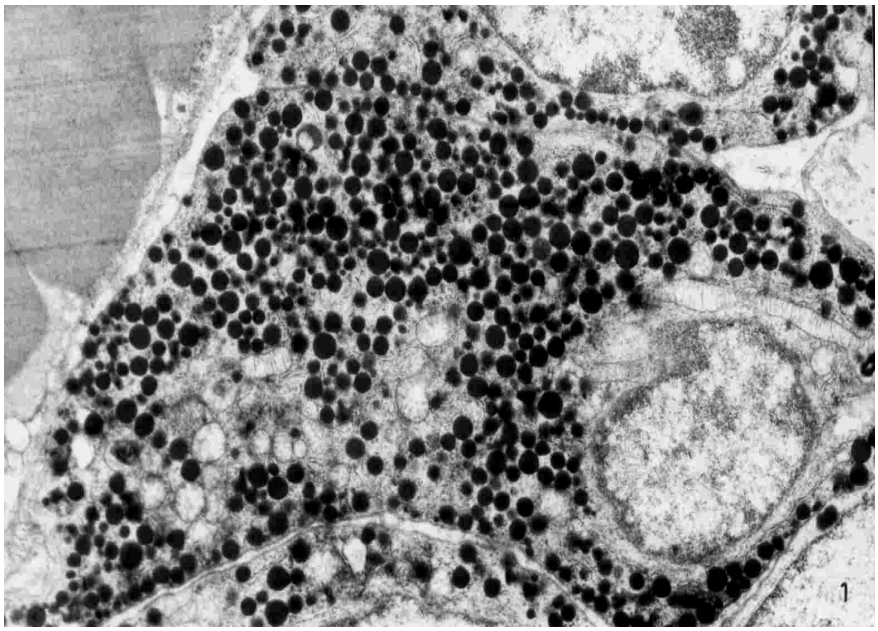
After 16 hours of fasting and 24 hours following the cessation of treatments, the animals (Locoid-treated, Dermovate-treated and Fluocinoline-treated groups), together with a control group, were sacrificed by exsanguination.

The anterior pituitary fragments were quickly isolated and prepared for ultrastructural examinations. The adrenohypophysary fragments were prefixed in a 2.7% glutaraldehyde solution and postfixed in a 2% osmic acid solution. The dehydration of samples was performed in acetone and then they were embedded in Vestopal W. The ultrathin sections were obtained using an LKB-III ultramicrotome and were contrasted with uranyl acetate and lead citrate. Examinations of sections were performed in a TESLA-BS-500 electron microscope.

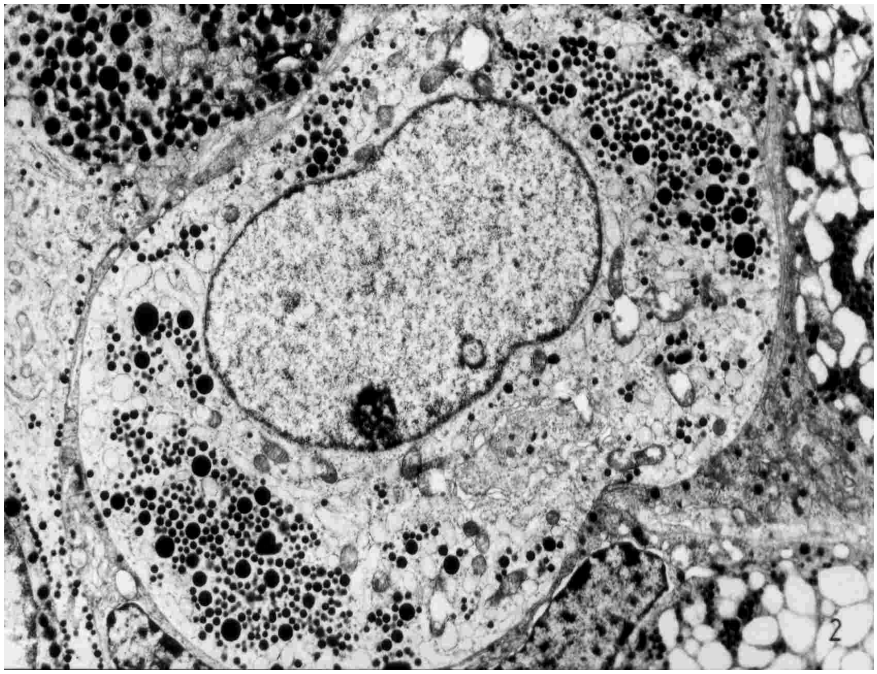
Results and discussion. *Control group* (C-group). The somatotropes (GH - growth hormone producing cells) are localised mostly in the lateral wings and, in contrast to all other pituitary cell types, are very stable in number, granule content and ultrastructure. Although GH is most important in the growth period, the structure of GH cells does not change from childhood to old age.

GH cells are acidophil, medium-sized or large, showing spherical shape and spherical nuclei. The ultrastructure of these cells demonstrated parallel areas of rough endoplasmic reticulum (RER), globular Golgi apparatus and many dense spherical granules with diameters mostly between 350 and 500 nm (Fig. 1).

The gonadotropes (GT) are scattered throughout the entire adenohypophysis, mostly in contact with capillaries and often adjacent to somatotropes and thyreotropes. They are medium-sized, oval or slightly irregular. The mostly oval nuclei are often excentrically located. By electron microscopy, the RER is well developed, with short, often slightly dilated profiles. The Golgi apparatus is prominent, with numerous sacculi and vesicles, and includes many immature secretory granules. The mature secretory granules vary considerably in size, structure and number. Two types of secretory granules exist: one type measures 150-250 nm, the other 350-600 nm (Fig. 2). Light bodies with spherical shape, granular content and dense core seem to be characteristic for gonadotrope cells.

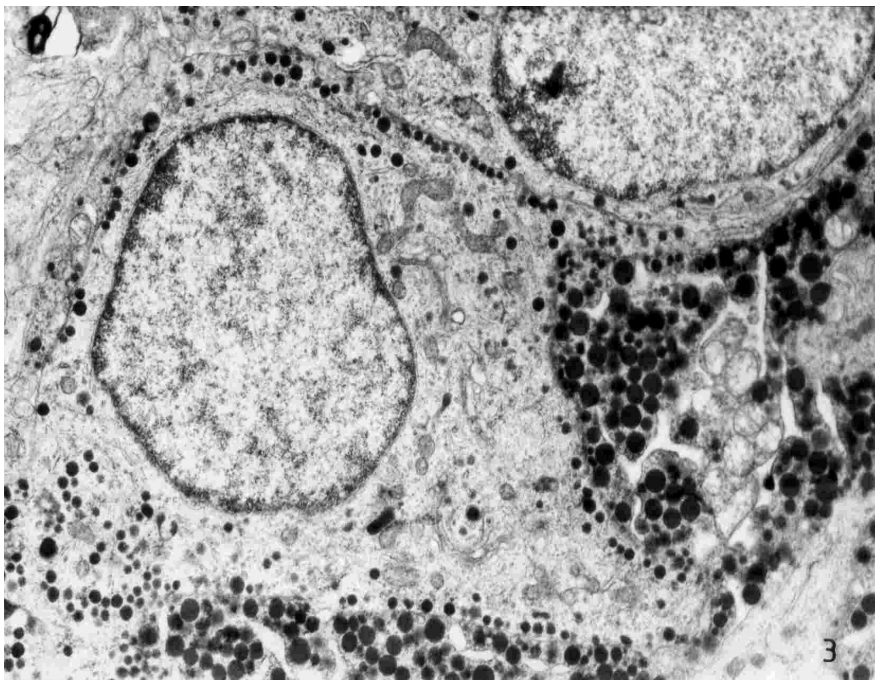


F i g. 1. *Somatotrope cell ultrastructure in C-group* (x 12,000).



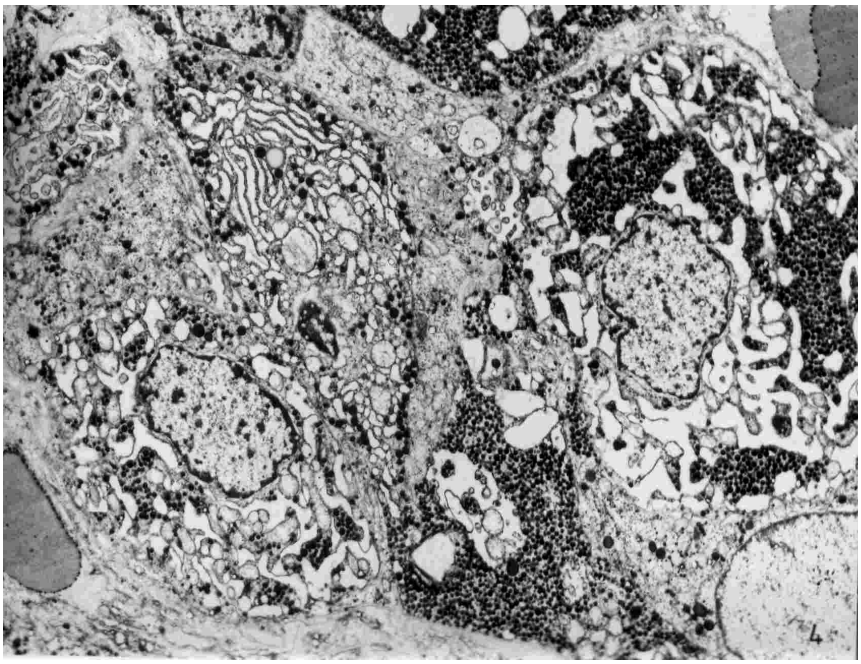
F i g. 2. *Gonadotrope cell ultrastructure in C-group* (x 8,400).

The main localisation of the corticotrope cells (ACTH) is the central mucoid wedge of the pituitary, where they comprise the majority of parenchymal cells. By ultrastructural examination, the ACTH cells have angular outlines, facing the capillaries. The nucleus lies excentrically and harbours a nucleolus in the vicinity of the nuclear membrane. In the relatively electron-opaque cytoplasm, a moderately developed and conspicuous Golgi complex with often dilated sacculi is found. The secretory granules are usually numerous, spherical, oval or slightly irregular and varying in electron density. They measure 200-400 nm (Fig. 3).



F i g. 3. *Corticotrope cell ultrastructure in C-group* (x 12,600).

In *Locoid-treated group*, the electron microscopic examination of the adenohypophysary fragments demonstrated that this glucocorticoid induces less severe ultrastructural changes than Dermovate or Fluocinolone, noticing, however, a moderate congestion of the blood vessels. The presence of vascular congestions explains the degenerative processes of some pituitary cells and the alteration of the cellular architecture. Examination of the sections showed that the most affected cells were the gonadotropes situated near the congested vessels. The cytoplasm contains dilatated RER and mitochondria (Fig. 4). These modifications suggest a moderate alteration of the secretory activity and a slight decrease of the secretory granule content of the gonadotrope cells.



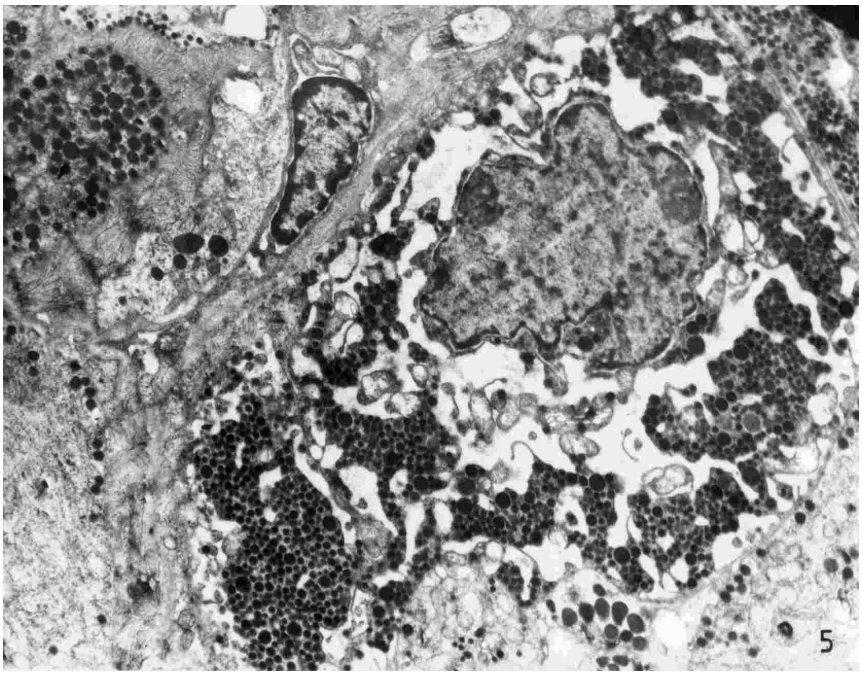
F i g. 4. *Gonadotrope, somatotrope and corticotrope cell ultrastructure in L-group* (x 7,600).

The ultrastructure of the GH cells is also affected. In some cells we could noticed a slight tendency of nuclear chromatin condensation, while other cells had extremely rarefied chromatin. Locoid treatment induced degranulation and a slight increase of the lysosome number. The perinuclear spaces and the RER present a moderate dilatation (Fig. 4).

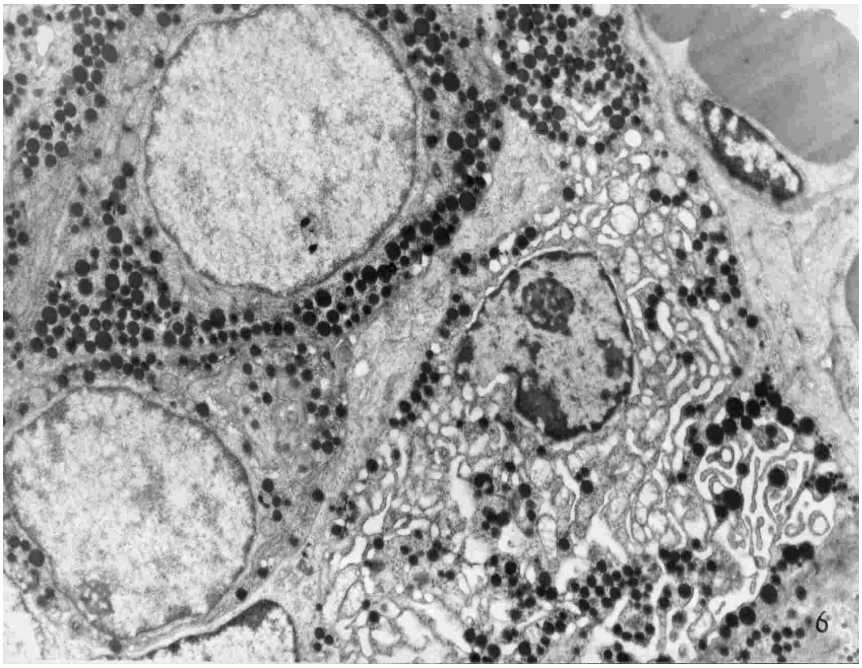
The corticotropes present an aspect which is close to that of the control group, a moderate alteration of the organelles being, nevertheless, observed (Fig. 4). The irregular shape of the nucleus and the slightly rarefied mitochondrial matrix suggest a moderate secretory activity of corticotropes.

In *Dermovate-treated group*, we observed severe ultrastructural modifications of the adenohypophysis compared to that treated with Locoid. A lot of gonadotropes suffered a process of nuclear pycnosis and even a gradual process of karyolysis. At the level of the cytoplasm, we could remark a perinuclear space and RER dilatation, more evident than in the L-group (Fig. 5). The mitochondria were completely vacuolised, thus suggesting a blockage of the hormonal biosynthesis.

In the GH cells the number of the secretory granules are more reduced than in the L-group. In contrast with the normal aspect of nucleus in controls, after epicutaneous treatment with Dermovate, unusually sizeable and irregularly shaped nuclei appeared (Fig. 6). Also, the characteristic disposition of the nuclear chromatin has been altered. In many somatotropes, we could see a severe vacuolisation of the cytoplasm, as well as rarefaction of its matrix due to the depletion of ribosomes, thus suggesting a decrease of the growth hormone synthesis.



F i g. 5. *Gonadotrope cell ultrastructure in D-group* (x 8,000).



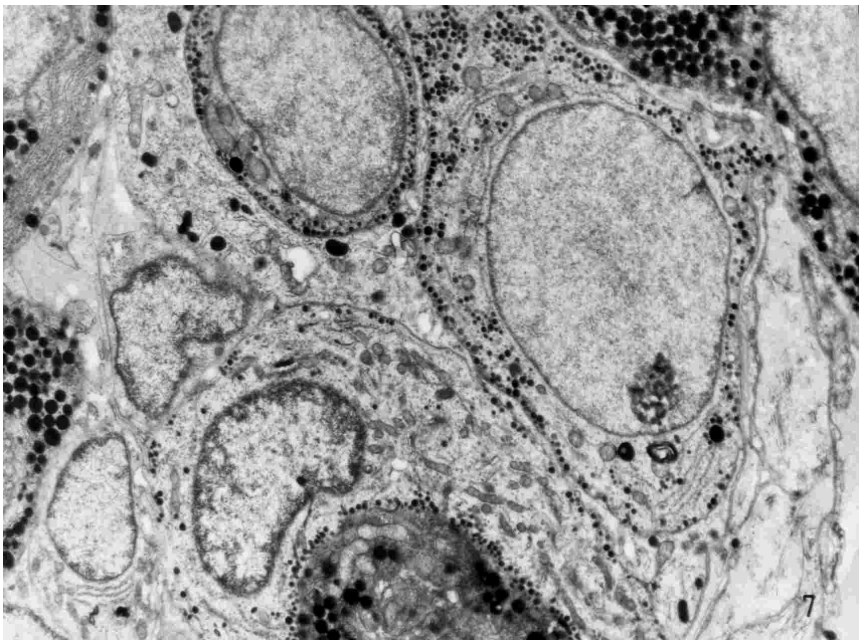
F i g. 6. *Somatotrope cell ultrastructure in D-group* (x 7,600).

The corticotrope ultrastructure was very close to that in the L-group (Fig. 7).

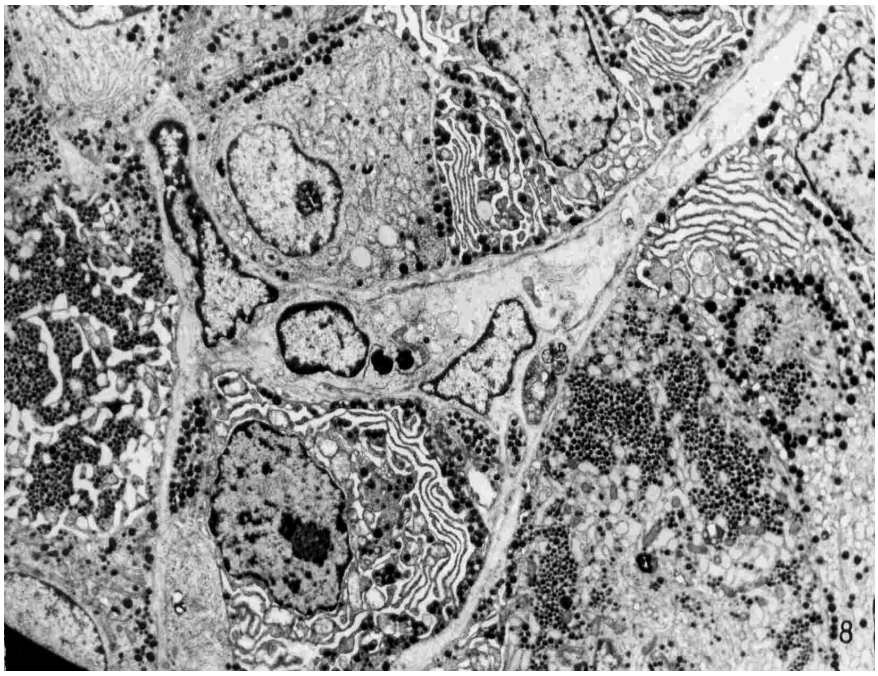
In *Fluocinolone-treated group*, the electron microscopic examination of the sections demonstrated that this glucocorticoid induces significant and more severe ultrastructural modifications, the gravity of the alterations depending on the age of the animals. The pituitary cell destructions were accompanied by a vascular response. The blood vessels were congested, with their lumen enlarged and loaded with erythrocytes. Congestion was always correlated with perivascular oedema and, after 3 days of treatment, with hemorrhages.The advanced disruption of the basal membrane facilitates the migration of erythrocytes between the adrenocorticotrope cells.

The vascular disturbances seem to have the most important role in the appearance and evolution of the ultrastructural modifications in the anterior pituitary gland.

The most affected cells were the somatotropes and the gonadotropes (Fig. 8). In the somatotropes, Fluocinolone induced changes in the shape of the nucleus and in the organelle content of the cytoplasm. In some cells, it could be noticed a moderate decrease of secretory granules and a slight alteration tendency of the nucleus. Other cells suffered a process of nuclear pycnosis. Also, the structure of the RER and mitochondria was altered. The swollen and elongated cisternae of RER are more evident around the nucleus, where they are placed parallel with the nuclear membrane, while the mitochondria are completely vacuolised. In some cells with pycnotic nuclei and vacuolised mitochondria, with rarefied matrix, we could see the appearance of intensive lysis of the cytoplasm and the degradation process of the secretory granules.



F i g. 7. *Corticotrope cell ultrastructure in D-group* (x 7,600).



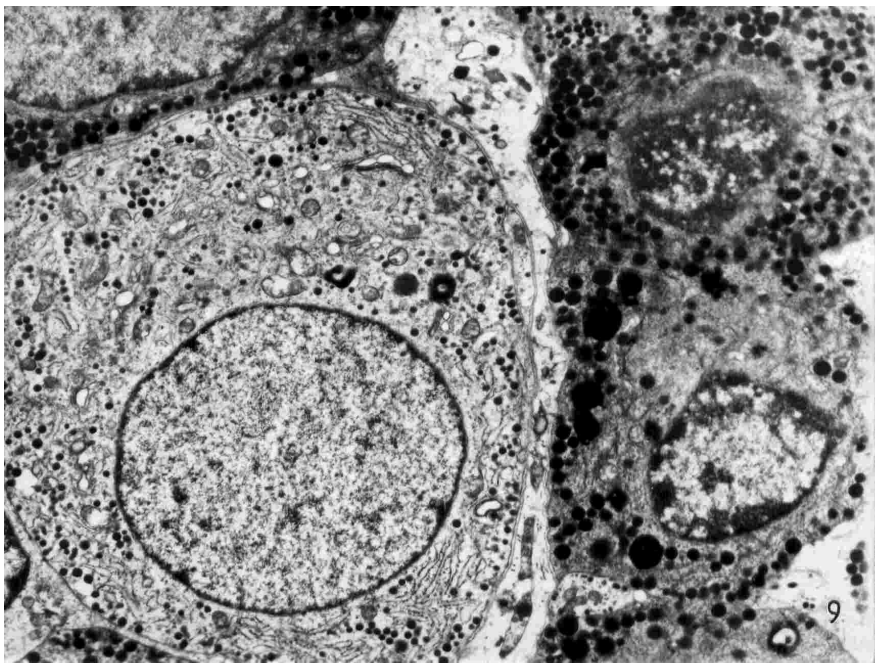
F i g. 8. *Gonadotrope and somatotrope cell ultrastructure in F-group* (x 4,560).

The Fluocinolone-acetonid N induced changes in the number of the gonadotrope cells. In some cells this glucocorticoid induced modifications in the shape and dimensions of the nucleus. Another ultrastructural characteristic of these cells is the presence of moderately vacuolised endoplasmic reticulum and mitochondria. In other cells, the nucleus suffered a process of pycnosis and even a gradual process of karyolysis. In these cells, the vacuolisation of the common organelles is more evident, giving a foamy aspect to the cytoplasm. In a lot of cells the number of secretory granules was more reduced than in controls, this fact suggesting a decrease of the glycoprotein synthesis.

The corticotrope cells present an aspect which is close to that of the control group; however, a slight decrease of the secretory granule content (Fig. 9) can be observed, which reflects a blockage of the hormonal release. Fluocinolone induced changes in the shape of the nucleus. Also, the characteristic disposition of the nuclear chromatin has been altered. In many cells, we could see a nuclear chromatin condensation and a moderate dilatation of the RER, these facts suggesting a reduced secretory activity.

Literature data [4, 6] as well as our results have revealed that an increase of plasma cortisol concentration, determined either by a treatment with glucocorticoid-based drugs or based on the action of different factors of stress, induces ultrastructural alterations of adenohypophysary cells in rats. A combined double immunohistochemical study revealed that the co-localisation of glucocorticoid receptor and anterior

pituitary hormones occurred in almost 99% of the growth hormone-producing cells and adrenocorticotropin hormone-producing cells [9]. Glucocorticoid receptor mRNA was abundant in the cytoplasm of anterior and intermediate pituitary cells but scattered sparsely in that of the posterior pituitary cells.



F i g. 9. *Corticotrope cell ultrastructure in F-group* (x 7,600).

Literature data [1, 3, 5, 6,12] as well as our results have rendered evident the fact that dermocorticoid administration results in alteration of the hypothalamic-pituitary-adrenal axis activity, in a decrease of corticotropin-releasing hormone (CRH) secretion from the hypothalamic paraventrivular neurons, which determined the blockage of the adrenocorticotropin hormone biosynthesis. It is well established that adrenal glucocorticoid hormones released in response to stress activation of the hypothalamic-pituitary-adrenal axis are powerful regulators of cellular function. In the anterior pituitary corticotrope cells, the *in vitro* glucocorticoid inhibition of ACTH secretion is best described as developing in two phases (early and late inhibition) that involve distinct genomic mechanism of action [11]. Analysis of early glucocorticoid inhibition of ACTH secretion from anterior pituitary corticotropes is providing insight into potentially genomic mechanisms by which glucocorticoids regulate cellular excitability. Early glucocorticoid inhibition is dependent upon activation of intracellular type II glucocorticoid receptors and induction of the synthesis of new proteins, including the calcium-binding protein calmodulin. Late inhibition of ACTH involves suppression of ACTH biosynthesis and down-regulation of CRH signalling pathways.

Excessive glucocorticoid concentrations *in vivo* inhibit somatic growth in both man and animals. Although this may be explained by the catabolic effects of glucocorticoids and a reduction in IGF I action, the role of GH remains unclear, since glucocorticoids can also affect GH secretion.

It is possible that the duration of glucocorticoid excess is important in the regulation of GH secretion in both pubertal and prepubertal rats. Administration of supraphysiological doses of dexamethasone (synthetic glucocorticoid) daily for a few days has been reported to inhibit GRH-induced (hypothalamic releasing factor for GH) GH secretion in rats [13], but the time-dependent effects of glucocorticoid on spontaneous GH secretion are unknown in rats. This observation aggrees with the clinical report showing that spontaneous and GRH-induced GH secretion is suppressed in patients with Cushing's disease [7]. F e r n a n d e z *et al.* [2] reported that corticosterone had a dual effect on hypothalamic GRH release. They have shown that a high concentration of corticosterone inhibited GRH release from the cultured fetal rat hypothalamic cells.

The precise mechanism which can account for the glucocorticoid-induced GRH inhibition is unknown. The presence of high density glucocorticoid receptors in GRH neurons [8] suggests that, at least partially, glucocorticoid can act directly on the hypothalamic neurons.

Our results have rendered evident the fact that synthetic glucocorticoid administration results in alteration of the hypothalamic-pituitary-gonadal axis activity, in a decrease of hypothalamic GRH (hypothalamic releasing factor) which determined the blockage of gonadotropin hormone biosynthesis, a fact illustrated by the alteration of the structure and ultrastructure of the gonadotrope cells. The mechanism of glucocorticoid action upon the spontaneous and GRH-induced gonadotropin secretion is yet unknown in rats.

Conclusions. 1. Exposure of mature Wistar rats to the action of dermocorticosteroids determined modification in the ultrastructure of some adenohypophysary cells, manifested by a decrease of secretory activity of somatotrope, gonadotrope and corticotrope cells.

2. The degree of pituitary structure and ultrastructure modification and decrease of secretory activity are mainly dependent on the dose, composition and molecular structure of the topical glucocorticoids present in these three dermocorticosteroids: Fluocinolone-acetonid N has superpotent effects, Dermovate has potent effects, while Locoid exerts moderate potent actions.

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HISTOLOGICAL AND ULTRASTRUCTURAL ASPECTS OF THE MYOCARDIUM OF RATS TREATED WITH AN ANTHRACYCLINE ANTIBIOTIC - EPIRUBICIN

CRISTINA PAŞCA*, VIOREL MICLĂUŞ, CONSTANTIN CRĂCIUN*, ERIKA KIS*, VICTORIA-DOINA SANDU*, IONEL PAPUC***and VERONICA CRĂCIUN***

SUMMARY. - The light microscopic characteristics and ultrastructure of the muscle cells of the left ventricular wall of the rat heart have been studied during certain pathologic processes induced by hypoxia or some toxic drugs. Epirubicin is an anthracycline antibiotic, a cytostatic drug widely used in the chemotherapy of many types of cancer in humans, which is able to determine significant toxic injury at the level of myocardium. Cardiotoxicity is one of its side effects, which, according to previous studies, is a significant feature. Our studies tried to evaluate the histological and ultrastructural modifications induced by a single dose of 89 mg Epirubicin/m^2 body surface on the rat myocardium. By light microscopy, it could be seen that this cytostatic caused circulatory disturbances consisting of congestion, stasis, changes of the vascular permeability correlated with the appearance of a significant perivascular and interfascicular oedema. Epirubicin affected both the vessels and the myocytes, inducing a granular degeneration and myolysis. In addition, this drug caused many severe interfascicular haemorrhages.The electron microscopy showed that the lesions are determined by the alterations of the vascular permeability. The oedema progressed between the myocytes, broke the intercellular junctions and affected the cell membrane, inducing swelling and, finally, its breaking. Then, the oedema progressed between the myofibrils and determined the myocyte disorganisation. In the areas with an advanced lysis, a collagenous proliferation could be seen.

It is known that the administration of some anthracycline antibiotics may induce significant toxic myocardial injury. According to previous studies, cardiotoxicity of the anthracycline antibiotics is a significant feature consisting of the appearance of some ultrastructural myocardial cell alterations such as dilatation of the sarcoplasmic reticulum and of the T-tubules, lysis of myofibrils

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and degeneration of mitochondria [3, 21]. Epirubicin (4'-epidoxorubicin) belongs to this drug family, its toxicity being lower than that of the other cytostatics included in this anticancer drug group, such as Doxorubicin [1]. The dose-limiting effect of Epirubicin is mainly myelotoxicity, particularly leukopenia [8]. Besides, it has a cardiotoxic effect but this is lower as compared to that of Doxorubicin, the analogue of Epirubicin [3, 21]. The previous morphological studies of the myocardium showed that this drug induces a severe cardiomyopathy manifested by myofibrillar loss, vacuolisation and swelling of myocardial cells and dilatation of the sarcoplasmic reticulum. The loss of the myofilaments is correlated with the appearance of some contractile alterations [4, 10, 16, 20]. Therefore, our studies tried to evaluate the histological and ultrastructural modifications induced by a single dose of Epirubicin (89 mg/m^2 body surface) administrated i.v. on the rat myocardium in concordance with the moment of sacrification.

Material and methods. Our experiments were carried out with the following four groups of healthy adult male Wistar rats, weighing 190 $\pm$ 10 g, and maintained under bioclimatic laboratory conditions, with no food for 18 hours before the treatment, but having water *ad libitum*:

-group U – untreated (control) group;

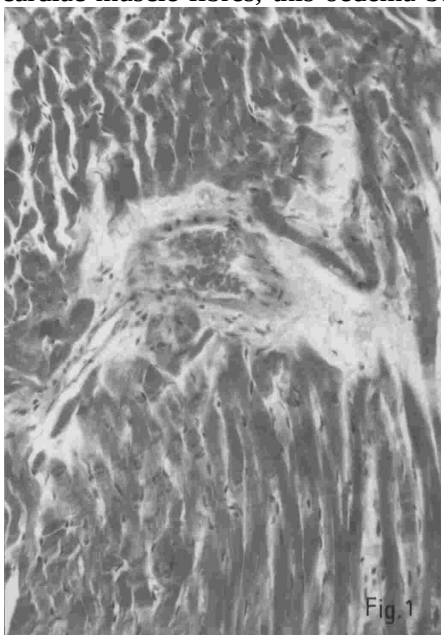
-group E_1, E_2 and E_3 - treated i.v. with 89 mg Epirubicin/m^2 body surface and sacrificed 24 hours, 4 and 6 days after the treatment.

Initially, our intention was to have four treated groups to be sacrificed after 24 hours, 4, 11 and 18 days, but after 5 days following the treatment the rats suddenly became sleepy and listless and, in the 6th day, 30% of them died, so we had to sacrifice the rest of them. The animals were not fed for 18 hours before the sacrification. Having sacrificed the animals, we took fragments from the myocardium. For microscopic examination, the fragments were fixed in 10% neutral formol, processed by the paraffin technique and the sections of 6 μm were stained by the hematoxylin-eosin and Masson-Goldner trichrome [15]. For ultrastructural investigations, fragments of kidney were prefixed in 2.7% glutaraldehyde solution and postfixed in 2% osmic acid solution. The fragments were dehydrated in acetone and then embedded in Vestopal W. The ultrathin sections were obtained using an LKB III ultramicrotome and were contrasted with uranyl acetate. Examination of the sections was performed in a TESLA-BS-500 electron microscope [2, 12, 17].

On the stained and contrasted sections we studied, by light and electron microscopic examinations, the histological and ultrastructural modifications induced by Epirubicin on the myocardium and its structural components in concordance with the moment of sacrification and compared to the untreated group.

Results and discussion. The light and electron microscopic examinations of the sections obtained from the treated rats demonstrated the existence of some obvious histological and ultrastructural alterations, the intensity, gravity and extension of which were different, depending on the moment of sacrification.

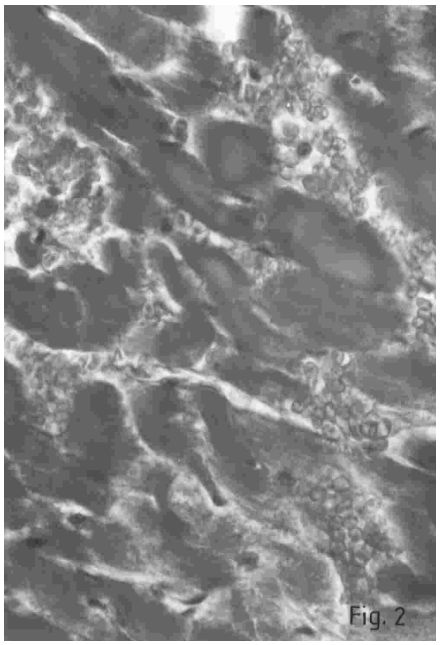
The first histological modifications appeared 24 hours after the treatment (group E_1). They were obvious enough and consisted of the appearance of an extensive congestion and interfascicular microhaemorrhages which had a zonal character. Besides, a moderate oedema could be noticed among the fasciculi of cardiac muscle fibres, this oedema being more increased in the perivascular zone. The oedema was not correlated with the presence of a cellular infiltration (Fig. 1). At the level of many cardiac muscle fibres some nuclear modifications appeared consisting of hypertrophy, the presence of an increased number of nucleoli, a peculiar arrangement of chromatin in groups. A few nuclei were round shaped and very intensely stained. Such phenomena of anisocaryocytosis and anisochromy were not noticed with the untreated group. All these histological modifications were very well pointed out on the sections stained with hematoxylin-eosin. The interfascicular oedema was more obvious on the sections stained with Masson-Goldner, the oedema having a serous character. In addition, discrete processes of myolysis already occurred, but they affected small areas.



F i g. 1. *Congestion and perivascular and interfascicular oedema in the myocardium* (x 512).

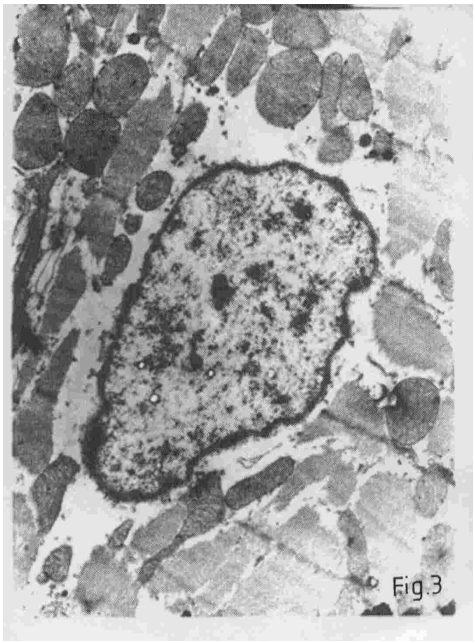
All these modifications were still present after 4 days (group E_2), their intensity being significantly increased, especially by congestion and haemorrhages (Fig. 2). Besides, discrete processes of myolysis already occurred (they affected small areas) and a collagenous proliferation also took place.

The toxic myocardial injury could be remarked in the electron microscopic examination, too, which showed that the ultrastructural modifications were determined by the alterations of the vascular permeability and by the hypoxic effect induced by Epirubicin.



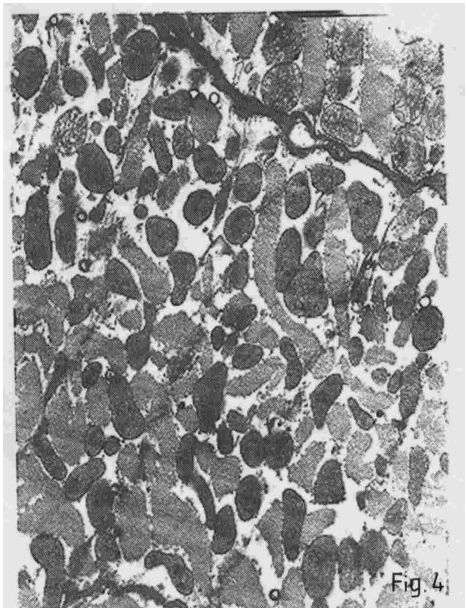
F i g. 2. *Massive interfascicular haemorrhages and wide areas of myolysis in the myocardium* (x 1,380).

This drug induced severe myocardial modifications which affected both the myocytes and the vessels in the myocardium. They consisted of the appearance of a cell swelling, the sarcolemma being lifted off the cell s involved, which exhibit large empty bleb-like spaces and small defects in the plasma membrane. The glycocalyx appeared to be separated from the surface bilayer membrane. A massive myofibrillar lysis appeared, which was more pronounced in the perivascular zone accompanied by a coarse aggregation of nuclear chromatin. The mitochondria were swollen and showed destroyed cristae and intramitochondrial amorphous inclusion bodies, some mitochondria being degenerated. Besides, the dilatation of the sarcoplasmic reticulum and of the T-tubules could be noticed (Figs. 3-6).



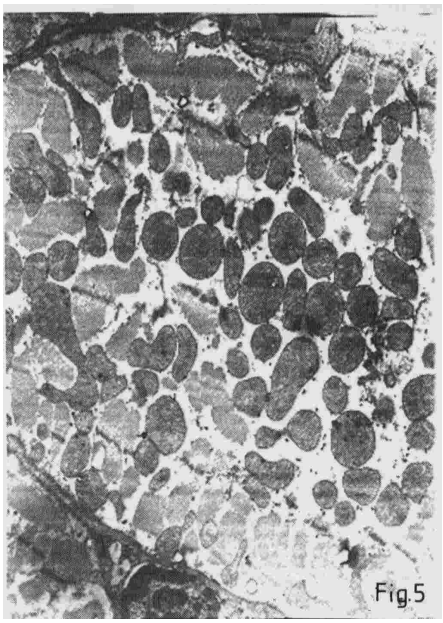
These histological aspects previously presented persisted in group E_3 sacrificed 6 days after the treatment, they being a little more obvious, affecting wide areas (both isolated and grouped myocytes).

F i g. 3. *Thickened nuclear membrane, significant nuclear swelling and margination and disorganisation of the nuclear chromatin in the myocytes* (x 16,800).



All the modifications previously presented confirm the cardiotoxicity of this anticancer drug. The intensity, gravity and spreading of the lesions were graver and graver during the 6 days of the experiment. Our histological and ultrastructural studies of the myocardium sections demonstrated that Epirubicin caused circulatory disturbances consisting of the appearance of congestion, stasis and modification of the vascular permeability, which induced a significant perivascular and interfascicular oedema.

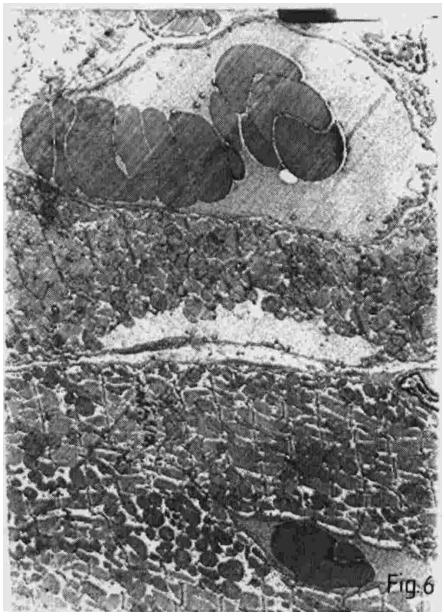
F i g. 4. *Massive lysis of the myofibrils which have a pulverised aspect among the mitochondria at the level of the myocardial cells* (x 12,600).



Our results, which are in agreement with previous studies, demonstrate that Epirubicin induces primary damage to the cell membranes, including the sarcolemma, which leads to cell death. The functional consequences of the altered sarcolemmal permeability involve a modified flux of electrolytes and water, leading to cell swelling and increased transport of Ca^{2+} from the extracellular space to the cardiac muscle cell, which produces a devastating effect on cardiac cell structure and function [7].

Vesiculation and disruption of the sarcoplasmic reticulum and transverse tubules have been documented to be associated with a progressive disturbance of the events involved in excitation-contraction coupling [14].

F i g. 5. *Thickened Eberth jonction, vacuolisation and swelling of the myocardial cells* (x 12,600).



The moderate hypoxic effect of this anticancer drug could be noticed on the myocardium sections, where significant nuclear swelling, margination of the nuclear chromatin and disorganisation and rupture of inner mitochondrial membranes appeared. Besides, the relaxation of the myofibrils may be caused by increased connective tissue content that may restrict fibre shortening, since hypoxic conditions are known to stimulate collagen synthesis by fibroblasts [11].

F i g. 6. *Congestion and an obvious oedema which determines the detachment of the myocardial sarcolemma* (x 5,250).

According to previous studies, the increased condensation and reorganisation of gap junction particles during hypoxia induced by Epirubicin provide evidence for an enhanced electrical resistance between cardiocytes [5, 18].

Although, we could not notice any macrophage activity at the level of the myocardium, H i b b s *et al.* [9] and S c h m a l b r u c h and D u m e [19] demonstrated that destroyed myofibrils are finally phagocyted by the macrophages.

Concerning the dynamics of the circulatory disturbances (congestion, stasis, haemorrhages and oedema) and the myocyte modifications (nuclear, mitochondrial and membranar modifications, anisocaryocytosis, anisochromy, myolysis etc.) it must be emphasised that they already appeared 24 hours after the treatment and got worse significantly during the 6 days of the experiment, no recovery tendency being remarked. All these aspects are in concordance with the results of previous studies, according to which Epirubicin induces severe histological myocardial modifications which affect both the myocytes and vessels in the myocardium [4, 6, 10, 13, 16, 20].

Conclusions. 1. Epirubicin induces primary damage to the cell membranes, including the sarcolemma, which leads to cell death.

2. Epirubicin causes grave circulatory disturbances consisting of congestion, stasis, haemorrhages, modification of the vascular permeability correlated with the appearance of a significant perivascular and interfascicular oedema.

3. Epirubicin disturbs both the vessels of the myocardium and myocytes, inducing a granular degeneration and myolysis, phenomena which affected wide areas and had an irreversible character.

4. Ultrastructurally, Epirubicin determines cell swelling, structural alterations of the sarcolemma, massive myofibrillar lysis which was more pronounced in the perivascular zone, a coarse aggregation of nuclear chromatin, the swelling and even degeneration of the mitochondria and the dilatation of the sarcoplasmic reticulum and of the T-tubules.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI, BIOLOGIA, XLVI, 1, 2001

DOES A SINGLE THERAPEUTIC DOSE OF CYCLOPHOSPHAMIDE INDUCE THE APOPTOSIS OF THE LYMPHOCYTES ?

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SUMMARY. - Among alkylating agents, Cyclophosphamide is a chemotherapeutic drug widely used in the treatment of many malignant and autoimmune diseases. Apoptosis is recognised as being the main type of cellular death observed during the evolution, which allows the exact regulation of the cell number. Generally, apoptosis is welcome for the organism, but its inadequate activation leads to different pathological states. Our histological investigations intended to emphasise the apoptotic effect of a single therapeutic dose of Cyclophosphamide on the lymphocytes in the spleen during 21 days after the chemotherapy. Our results demonstrate that, at the cellular level, Cyclophosphamide may selectively affect the mature lymphocytes and their precursor cells. Its apoptotic effect could be noticed even after 24 hours since the treatment. This effect had a zonal character, some cellular clones being more quickly affected than others. The apoptotic processes occurred in a different way in the two components of the spleen, the white pulp being earlier affected. Although this kind of process had a different intensity during the 21 days of the experiment, it persisted all this period of time. Histologically, the apoptotic effect consisted of the appearance of nuclear condensation, morphological changes of the cells, nucleolar distorsions, nuclear and cell fragmentation.

Nowadays, several types of cellular death are known: necrosis, oncosis, apoptosis, autophagic death and death by histologic staining. Apoptosis represents a form of death often qualified as active death or programmed cell death [8, 16]. Unlike necrosis, which is an accidental death, apoptosis needs the active participation of the cell, involving a succession of cellular events which are the object of a fine genetic regulation. Apoptosis is involved in normal development of the organism, assuring the elimination of the excess cells during organogenesis, metamorphosis or involution and replacement of cells in the adult organism. It is

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also observed in tissues treated with relatively low doses of noxious agents including cytostatics. At the tissular level, this process consists of the appearance of some changes: specialised surface structures are lost, membrane surface becomes smooth and the cells become separated from their neighbours, followed by the reduction of the cell volume, while the cytoplasm retracts, plasma membrane loses its normal contour, organelles aggregate, although the integrity of both plasma and organelle membranes is preserved [4, 6].

Material and methods. Our experiments were carried out with the following six groups of healthy adult male Wistar rats, weighing 190 $\pm$ 10 g and maintained under bioclimatic laboratory conditions, with no food for 18 hours before the treatment, but having water *ad libitum*:

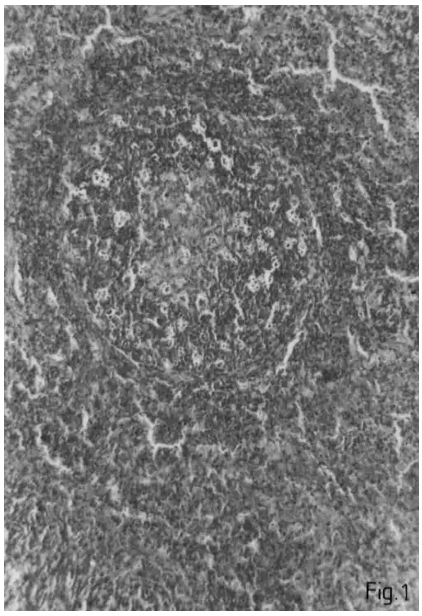
-group U – untreated (control) group;

-groups T_1, T_2, T_3 and T_4 treated i.v. with 40 mg Cyclophosphamide / kg body weight and sacrificed 24 hours, 4, 11, 18 and 21 days after the treatment.

The animals were not fed for 18 hours before the sacrification. Having sacrificed the animals, we took fragments from the spleen. For microscopic examination the fragments were fixed in 10 % neutral formol, processed by the paraffin technique and the sections of 6 μm were stained by the hematoxylin-eosin and Masson-Goldner trichrome [13].

On the stained sections, we studied, by microscopic examination, the histological modifications induced by Cyclophosphamide on the lymphocyte populations in the spleen in correlation with the moment of sacrification.

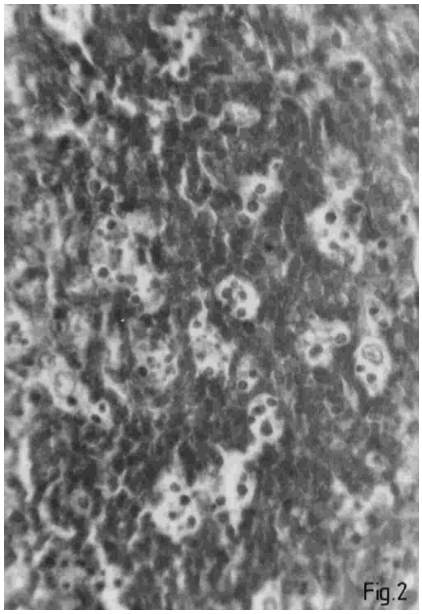
Results and discussion. The histological examination evidenced significant morphological changes even after 24 hours since the treatment. They consisted of a serious decrease in the dimensions of the splenic nodules in the white pulp. Most of the nodules still had a germinal centre, where the lymphocyte density was decreased as compared to the control group, and some cells presented pycnotic, heterochromatic and even fragmented nuclei. All around, on the spleen surface, a zonal lysis process of the lymphocytes could be noticed. These zones were exclusively made up of cells which were in different stages of lysis or even of cell fragments. The density of the lysis zones was higher at the level of the marginal zone and at the periphery of the germinal centres. The dimension of the lysis areas was different, some of them including 1-2 degenerated cells and others including tens of such cells (Fig. 1). Not all cells in such a lysis zone were in the same stage of degeneration or alteration. Thus, in some cells it could be noticed a slight pycnosis tendency and nuclear chromatin condensation, whereas other cells had extremely condensated nuclei. Few nuclei presented an obvious tendency of chromatin fragmentation, while other nuclei were already completely fragmentated. Such fragments, having different dimensions, were found in a larger or smaller amount in most of the lysis zones. Here and there, inside these zones, it could be noticed many macrophages full of cellular remains. Outside the lysis areas, most of the lymphocytes had a normal aspect. There was no transitional area between the two zones.



The lysis processes of the lymphocytes were noticed in the red pulp, too, but usually they affected only isolated cells or small cellular groups made up of 2-4 cells.

After 4 days from the treatment, the splenic nodules were much smaller than in the group sacrificed after 24 hours, and they had a very different aspect. Thus, they did not present a germinal centre anymore, the lysis zones were rare, present here and there, and they had smaller dimensions than in group T_1. The splenic nodules also presented a significant decreased cellular density as compared to the untreated group. Besides, in the structure of these nodules we could notice a lot of macrophages, which were

F i g. 1. *Splenic nodule having a "starry sky" aspect as a consequence of the presence of many areas full of apoptotic bodies* (x 360).

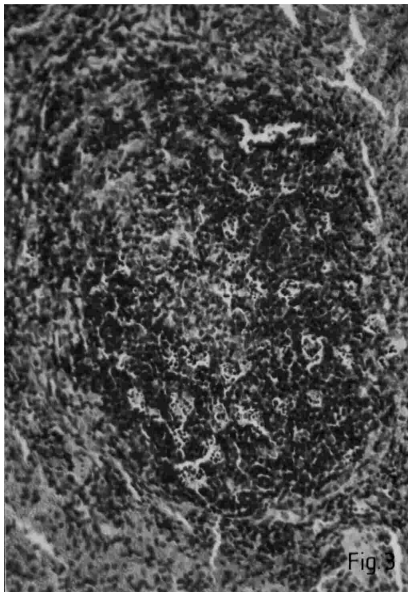


much more numerous than in the control group. The cytoplasm of many macrophages seemed to be full of a granular material. The number of the cells which presented pycnotic nuclei was not predominant at the level of the splenic nodules (Fig. 2). But, in the red pulp, there was a large number of such cells which were spread, isolated or in groups, all around on the section surface, without a zonal character.

After 11 days, the splenic nodules had smaller dimensions and their aspect was comparable with that in the group sacrificed 4 days after the chemotherapy. However, in the red pulp, the number of the cells with a degenerate or altered aspect was larger than after 4 days (Figs. 3, 4).

F i g. 2. *Many macrophages and apoptotic bodies at the level of the lysis areas in the splenic nodule* (x 1,320).

After 18 days, the splenic nodules were larger than after 11 days, and just a few small zones of lysis could be noticed in their structure. The number of the cells with euchromatic nuclei was certainly higher than in the previous group, this aspect demonstrating the decreasing of the cytotoxic effect of this cytostatic. In the red pulp, there were many lymphocytes with euchromatic nuclei, too, although the number of

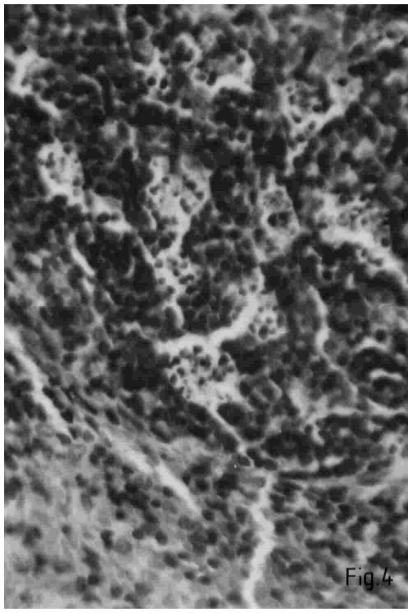


the cells with pycnotic and hyperchromatic nuclei was still significant. The increasing of the dimensions of the splenic nodules and the number of lymphocytes with euchromatic nuclei suggest the starting of a recovery process at the level of the spleen, and the presence of some lysis zones and of many cells with pycnotic nuclei demonstrates that the toxic action of Cyclophosphamide decreased, but did not completely stop.

This recovery process increased, being more obvious after 21 days from the treatment, when the dimension of the splenic nodules was comparable with that in the control group. In addition, a few of them presented a certain recovery tendency of their germinal centres.

F i g. 3. *Numerous areas full of apoptotic bodies in the outer cortex of the splenic nodule* (x 512).

Most of the nodules had a homogeneous structure and contained cells with euchromatic nuclei. But, small lysis areas could be still noticed, while in the red pulp there were still many cells with pycnotic nuclei, although their number was significantly decreased as compared to the group sacrificed after 18 days.



Cyclophosphamide, a cytostatic drug, an alkylating agent belonging to the family of nitrogen mustards, is commonly used to treat many types of cancer and autoimmune diseases in humans. At the molecular level, its cytotoxicity results from DNA double strand cross-links and, at higher concentrations, from DNA strand breaks [3, 11, 14, 15]. At the cellular level, Cyclophosphamide may selectively affect mature lymphocytes with a relative sparing of the respective precursor cells [13].

F i g. 4. *Wide lysis areas including many apoptotic bodies and some macrophages in the white pulp of the spleen* (x 1,320).

All these effects induce the appearance of the leukopenia, which, according to previous studies, is the limiting dose factor in the Cyclophosphamide chemotherapy, the most affected cells being T and B lymphocytes and monocytes

[1, 2, 4, 5, 7, 9, 10, 12, 16]. Besides, it is known that in the white pulp of the spleen, the T and B lymphocytes are generally segregated at two different sites. The T lymphocytes populate the periarterial sheaths, whereas the B lymphocytes are concentrated in the marginal zones and in the nodules.

All these aspects are in concordance with our histological results, according to which Cyclophosphamide strongly affects the lymphocyte populations in the spleen, both in the white and red pulp. The cytotoxic effect of Cyclophosphamide has a particular evolution. The lysis process of the lymphocytes started earlier in the white pulp than in the red one, where the phenomenon started later. The lymphocytes in the spleen nodules were not all affected with the same promptitude, some of them being more sensitive than others. This explains why after 24 hours since the treatment, in the same splenic nodule, some lymphocytes had a normal aspect, while others were in different degenerative or alterative stages. Besides, it could be noticed that these processes affected compact lymphocyte groups which occupy well delineated zones in the nodule.

Although we expected that the number of the affected cells be larger in the median zone of the germinal centre, where normally the division rate is more increased, we could notice that the number of lysis areas was much increased at the periphery of the germinal centres. All these cytological aspects determined us to think that Cyclophosphamide could affect the cell populations not only through the blocking of the cellular mitosis. We are tempted to consider that this cytostatic is able to induce the apoptosis of the lymphocytes. Unfortunately, our histological investigations did not allow us to establish the ways by which different lymphocyte types or cellular clones are affected.

Curiously, the lysis affected wider or smaller cell groups in which all the cells were affected in different ways. These results suggest a higher sensitivity of some lymphocyte clones as compared to others.

Between the 4th and 11th days since the chemotherapy, the destructive processes had an obvious intensifying tendency, and after 18 days it could be noticed that the lysis processes disappeared progressively, although they still persisted even after 21 days since the treatment.

Conclusions. 1. The particular and complex aspects of the spleen in the rats sacrificed 24 hours after the treatment determined us to consider that this cytostatic stops the mitosis and also starts the lymphocyte apoptosis.

2. The apoptotic effect seems to start earlier in some cellular groups and later in others.

3. The lysis process and apoptosis occurred in a different way in the two components of the spleen, more exactly, they appeared earlier in the white pulp.

4. The cells affected by Cyclophosphamide were in different stages of apoptosis: nuclear condensation, morphological changes of the cells, nucleolar distortions, nuclear fragmentation and cellular fragmentation.

5. Although the intensity of the destructive processes was different during the experiment, these processes persisted even after 21 days from the chemotherapy.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI, BIOLOGIA, XLVI, 1, 2001

INFLUENȚA UNOR METALE GRELE ASUPRA METABOLISMULUI CELULAR AL DROJDIILOR

LETIȚIA OPREAN*

SUMMARY. - Influence of Some Heavy Metals on the Metabolism of Yeast Cells. The paper presents the results of a comparative research on the influence of some heavy metals (Cd, Pb, Cu, Mn and Zn) on the cell metabolism of the beer yeast *Saccharomyces carlsbergensis.* During the alcoholic fermentation we have examined the dynamics of the wort fermentation due to the yeast, in the presence of these metals, measuring the amount of CO_2 produced in 24 hours and the number of the living yeast cells in the wort, using standard methods.

The results have shown that the heavy metals have a differentiated toxic effect on the yeast cell metabolism. The toxic effect can be correlated both to the specific action of each metal and to the metal amount in the fermentation medium. It can be determined a certain order of the toxicity degree of the studied metals on the fermentation dynamics of the wort and on the number of the living yeast cells in wort. Thus, the decreasing order of the toxicity degree of the studied metals is: Cd>Pb>Cu>Mn>Zn. The beer yeast *Saccharomyces carlsbergensis* can be used as a bioindicator for the heavy metals existing in food**.**

Poluarea atmosferei, apei, solului şi a produselor alimentare cu o gamă tot mai largă de substanțe chimice reprezintă un fenomen în progresivă amplificare la scară mondială, ca rezultat al dezvoltării industriale, măririi traficului rutier, chimizării agriculturii etc.

O sursă importantă de poluanți este industria de prelucrare a minereurilor din care rezultă zgură şi pulberi cu un conținut ridicat în metale grele: Pb, Zn, Cu, Co, Cd, Mn, Sn etc.

Produsele alimentare de origine vegetală se contaminează cu metale grele din sol şi atmosferă. Animalele hrănite cu furaje contaminate cu metale grele acumulează aceste metale în carne, dar mai ales în organe.

În termeni comuni, în categoria metalelor grele sunt cuprinse metalele cele mai toxice care sunt responsabile de anumite tulburări, intoxicații şi uneori de accidente mortale. Toxicitatea metalelor grele este rezultatul legării lor de sistemele enzimatice importante din celula animală sau de anumite componente ale membranelor celulare [1, 3, 4].

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Specialistul în industria alimentară este interesat să beneficieze de metode eficiente şi rapide de depistare a prezenţei substanţelor poluante din produsele alimentare. O asemenea metodă trebuie să aibă mai mult un aspect cantitativ, pornind de la ideea că în primul rând interesează dacă produsul respectiv este nociv pentru organismul uman şi apoi interesează natura agentului toxic [7].

Pornind de la ideea că substanţele toxice acţionează la nivelul celulei prin declanşarea unui efect deprimant general asupra metabolismului celular, indiferent dacă este vorba de organisme monocelulare sau pluricelulare, am studiat posibilitatea folosirii drojdiei de bere *Saccharomyces carlsbergensis* ca bioindicator simplu şi eficient al prezenţei substanţelor poluante în produsele alimentare.

În lucrarea de faţă descriem cercetări comparative privind influenţa unor metale grele (Cd^{2+}, Pb^{2+}, Cu^{2+}, Mn^{2+} şi Zn^{2+}) asupra metabolismului celular al drojdiei de bere *Saccharomyces carlsbergensis*, în mustul de bere în fermentaţie.

Materiale şi metode. Pentru cultivarea drojdiei de bere *Saccharomyces carlsbergensis* a servit, drept mediu nutritiv, mustul de bere industrial preparat din malţ, sterilizat în prealabil, nehameiat şi cu un extract real de 13%. Mustul de bere a fost distribuit în cantităţi de 150 ml în baloane cu fund plat de 250 ml, sterilizate şi închise cu ventile de fermentaţie cu acid sulfuric concentrat.

În experiment s-au utilizat următoarele metale grele sub formă de săruri, în cantităţi diferite, similare celor întâlnite adeseori în produsele alimentare:

- Cd^{2+} sub formă de $CdCl_2$ (în doze de 0,005, 0,01 şi 0,1 mg/l);
- Pb^{2+} sub formă de $Pb(NO_3)_2$ (în doze de 0,01, 0,05 şi 1 mg/l);
- Cu^{2+} sub formă de $CuS0_4 \cdot 5\ H_2O$ (în doze de 0,5, 5 şi 10 mg/l);
- Mn^{2+} sub formă de $MnS0_4$ (în doze de 0,5, 5 şi 10 mg/l);
- Zn^{2+} sub formă de $ZnCl_2$ (în doze de 0,5, 5 şi 10 mg/l).

Determinările s-au efectuat în serii paralele de probe şi în prezenţa unei probe martor, în care adaosul de metal sub formă de sare a fost exclus.

Pentru inocularea mustului de bere, din cultura stoc s-a preparat cultură tot pe mustul de bere sterilizat. Fermentaţia mustului de bere a fost condusă la temperatura camerei (20^0C), timp de 9 zile (192 ore).

În cursul fermentaţiei mustului de bere în prezenţa metalelor grele, am urmărit dinamica degajării CO_2 de către drojdie şi dinamica numărului celulelor vii de drojdie.

În acest scop, am determinat zilnic cu metodele de analiză curente, în conformitate cu STAS-ul în vigoare [5], masa de CO_2 degajată în 24 ore şi exprimată în % masice (CO_2/100 g) şi dinamica numărului de celule vii de drojdie prin numărarea cu camera Thoma, după colorare cu soluţie de albastru de metilen [2, 6].

Rezultate. Rezultatele obținute în studierea dinamicii fermentației mustului de bere de către drojdia de bere *Saccharomyces carlsbergensis* în prezența sărurilor metalelor grele testate sunt trecute în Tabelul 1.

Tabel 1

Dinamica fermentației mustului de bere de către drojdia de bere *Saccharomyces carlsbergensis*, în prezența unor metale grele

Metale grele	Doze (mg/l)	Masa CO_2 degajată în 24 ore (% masice)								CO_2 total degajat	Scădere % față de martor
		Durata fermentației (ore)									
		24	48	72	96	120	144	168	192		
Cd^{2+}	0,005	0,26	0,83	0,90	1,00	1,02	1,02	1,02	1,02	1,02	68,12
	0,01	0,14	0,35	0,40	0,45	0,50	0,50	0,50	0,50	0,50	84,37
	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	96,88
Pb^{2+}	0.01	0,25	0,90	1,00	1,10	1,20	1,20	1,20	1,20	1,20	62,50
	0,05	0,20	0,55	0,66	0,66	0,66	0,66	0.66	0,66	0,66	79,37
	1,00	0,10	0,20	0,20	0,20	0,20	0,20	0,20	0,20	0,20	93,75
Cu^{2+}	0,50	0,40	0,91	1,25	1,30	1,35	1,45	1,45	1,45	1,45	54,69
	5,00	0,30	0,30	0,75	1,10	1,15	1,25	1,25	1,25	1,25	60,94
	10,00	0,15	0,15	0,25	0,25	0,25	0,25	0,25	0,25	0,25	92,19
Mn^{2+}	0,50	0,45	1,40	1,80	1,90	2,20	2,20	2,20	2,20	2,20	31,25
	5,00	0,30	1,25	1,60	1,70	1,75	1,75	1,75	1,75	1,75	43,75
	10,00	0,20	0,65	1,00	1,05	1,10	1,10	1,10	1,10	1,10	65,27
Zn^{2+}	0,50	0,50	1,60	2,25	2,50	2,60	2,60	2,60	2,60	2,60	16,90
	5,00	0,40	1,45	1,95	2,05	2,10	2,10	2,10	2,10	2,10	34,38
	10,00	0,35	0,80	1,43	1,55	1,60	1,60	1,60	1,60	1,60	43,80
Martor (fără metal)	—	0,65	1,70	2,85	3,10	3,15	3,20	3,20	3,20	3,20	

Sărurile metalelor grele testate - după cum se poate constata din Tabelul 1 – au prezentat un efect toxic asupra dinamicii fermentației mustului de bere de către drojdie. Acest efect poate fi corelat atât cu acțiunea specifică a fiecărui metal, cât şi cu cantitatea de sare a metalului prezentă în mediul testat. Ca metale intens toxice se remarcă în ordine descrescătoare Cd^{2+}, Pb^{2+} şi Cu^{2+}.

Cd^{2+}, sub formă de $CdCl_2$, la doza de 0,005 mg/l, produce o scădere a masei de CO_2 degajată de drojdie, valoarea obținută fiind cu 68,12% mai mică decât cea a probei martor. Acelaşi metal, la doza de 0,01 mg/l, produce o scădere şi mai mare a masei de CO_2 degajată, valoarea obținută fiind cu 84,37% mai mică decât cea a probei martor. La doza de 0,1 mg/l, Cd^{2+} produce o inhibare a fermentării mustului de bere de către drojdie, masa de CO_2 degajată fiind aproape nulă (valoarea obținută fiind cu 96,88% mai mică decât cea a probei martor).

Toxicitate mare prezintă şi Pb^{2+}, care sub formă de $Pb(NO_3)_2$, la doze de 0,01 şi 0,05 mg/l, produce scăderi mari ale masei de CO_2 degajată de drojdie, valorile obținute fiind cu 62,5% şi respectiv 79,37% mai mici decât cea a probei martor. La doza de 1 mg/l, Pb^{2+} produce o inhibare a fermentării mustului de bere de către drojdie, masa de CO_2 degajată fiind aproape nulă (valoarea obținută fiind cu 93,75% mai mică decât cea a probei martor).

De asemenea, Cu^{2+}, sub formă de $CuSO_4 \cdot 5\ H_2O$, la doze de 0,5 şi 5 mg/l, prezintă acțiune toxică mare, valorile masei de CO_2 degajată de drojdie fiind cu 54,69% şi respectiv 60,94% mai mici decât cea a probei martor. La doza de 10 mg/l, Cu^{2+} produce o inhibare a fermentării mustului de bere de către drojdie, masa de CO_2 degajată fiind aproape nulă (valoarea obținută fiind cu 92,19% mai mică decât cea a probei martor).

Comparativ cu aceste metale, Mn^{2+} şi Zn^{2+} prezintă o toxicitate mai redusă asupra fermentării mustului de bere de către drojdie. Mn^{2+}, sub formă de $MnSO_4$, la doze de 0,5, 5 şi 10 mg/l, produce o scădere a masei de CO_2 degajată de drojdie, valorile obținute fiind cu 31,25, 43,75 şi 65,27% mai mici decât cea a probei martor. De asemenea, Zn^{2+}, sub formă de $ZnCl_2$, la doze de 0,5, 5 şi 10 mg/l, produce o scădere a masei de CO_2 degajată de drojdie, valorile obținute fiind cu 16,9, 34,38 şi 43,8% mai mici decât cea a probei martor.

Rezultatele obținute în studierea dinamicii numărului celulelor vii de drojdie în mustul de bere în fermentație în prezența sărurilor metalelor grele testate sunt trecute în Tabelul 2.

Numărul inițial al celulelor de drojdie este de $1{,}6 \times 10^6$ celule/ml must de bere.

Tabel 2

Variația numărului celulelor vii de drojdie în mustul de bere în prezența unor metale grele

Metale	Doze (mg/l)	Număr celule vii de drojdie $\times 10^6$/ml must								Scădere % față de martor
		Durata fermentației (ore)								
		24	48	72	96	120	144	168	192	
Cd^{2+}	0,005	1,80	30,40	56,20	15,30	7,25	3,80	1,80	0,70	72,00
	0,10	1,65	1,75	1,10	0,20	0,10	0,10	0,10	0,10	96,00
Pb^{2+}	0,01	1,95	21,35	58,30	11,70	7,20	3,35	1,24	0,75	70,00
	1,00	1,70	1,80	1,25	1,15	0,15	0,15	0,15	0,15	94,00
Cu^{2+}	0,50	1,90	23,85	60,35	12,00	7,90	3,40	1,10	0,80	68,00
	10,00	1,75	2,10	1,50	0,40	0,20	0,20	0,20	0,20	92,00
Mn^{2+}	0,50	3,40	80,20	100,4	35,36	15,30	7,25	2,00	1,80	28,00
	10,00	2,50	55,80	80,50	20,40	10,60	5,10	1,60	1,20	52,00
Zn^{2+}	0,50	4,50	100,5	132,1	50,10	19,45	11,10	2,25	2,20	12,00
	10,00	2,80	71,00	100,6	30,65	15,20	8,25	1,90	1,60	36,00
Martor (fără metal)	-	6,35	148,2	170,2	86, 70	31,75	15,60	3,80	2,50	

Se poate vedea din acest tabel că în condițiile fermentării mustului de bere în absența metalelor grele (în proba martor), drojdia de bere *Saccharomyces carlsbergensis* se înmulțește activ, valoarea maximă a numărului celulelor vii de drojdie fiind obținută în a treia zi de fermentație ($170,20 \times 10^6$ celule/ml must).

Prezența metalelor grele în mustul de bere are o acțiune toxică asupra dinamicii multiplicării şi viabilității celulelor de drojdie. În toate probele cu metale grele testate, multiplicarea celulelor de drojdie este mult mai lentă. Se constată că acțiunea de frânare a procesului de multiplicare al celulelor de drojdie în mustul de bere în fermentație este mai intensă în probele cu Cd^{2+}, Pb^{2+} şi Cu^{2+}.

În prezența acestor metale toxice, numărul celulelor vii de drojdie în a treia zi de fermentație este evident inferior celui obținut în proba martor. Toxicitate mare prezintă Cd^{2+}, care sub formă de $CdCl_2$, la doze de 0,005 şi 0,1 mg/l, produce o scădere a numărului de celule vii de drojdie, valorile obținute fiind cu 66,99% şi respectiv 99,36% mai mici decât cea a probei martor. De asemenea, Pb^{2+}, sub formă de $Pb(NO_3)_2$, la doze de 0,01 şi 1 mg/l, produce o scădere a numărului de celule vii de drojdie, valorile obținute fiind cu 65,75% şi respectiv 99,27% mai mici decât cea a probei martor. Cu^{2+}, sub formă de $CuSO_4 \cdot 5H_2O$, la doze de 0,5 şi 10 mg/l, produce o scădere a numărului de celule vii de drojdie, valorile obținute fiind cu 64,55% şi respectiv 99,22% mai mici decât cea a probei martor. Se constată că sărurile de Cd^{2+}, Pb^{2+} şi Cu^{2+}, în doze maxime, produc o inhibare a multiplicării celulelor de drojdie, numărul de celule vii de drojdie fiind aproape nul.

Comparativ cu aceste metale, Mn^{2+} şi Zn^{2+} prezintă o toxicitate mai redusă asupra capacității de multiplicare a celulelor de drojdie în mustul de bere în fermentație. Astfel, Mn^{2+}, sub formă de $MnSO_4$, la doze de 0,5 şi 10 mg/l, în a treia zi de fermentație, produce o scădere a numărului de celule vii de drojdie, valorile obținute fiind cu 52,7% şi respectiv 30,42% mai mici decât cea a probei martor. Zn^{2+}, sub formă de $ZnCl_2$, în doze de 0,5 şi 10 mg/l, în a treia zi de fermentație, produce o scădere a numărului de celule vii de drojdie, valorile obținute fiind cu 22,4% şi respectiv 50% mai mici decât cea a probei martor.

La sfârşitul fermentației mustului de bere – după cum se poate constata din Tabelul 2 – metalele prezintă un efect toxic diferențiat asupra dinamicii numărului celulelor vii de drojdie. Astfel, în ordinea descrescătoare a gradului de toxicitate, metalele se pot aranja în felul următor: $Cd^{2+} > Pb^{2+} > Cu^{2+} > Mn^{2+} > Zn^2$.

Cd^{2+} sub formă de $CdCl_2$, chiar la doza de 0,005 mg/l produce o scădere a numărului de celule vii de drojdie în mustul de bere fermentat, valoarea obținută fiind cu 72% mai mică decât cea a probei martor, iar la doza de 0,1 mg/l produce o inhibare a multiplicării celulelor de drojdie, numărul celulelor vii de drojdie fiind aproape nul (valoarea obținută fiind cu 96% mai mică decât cea a probei martor).

Toxicitate mare prezintă şi Pb^{2+}, care sub formă de $Pb(NO_3)_2$, la doza de 0,01 mg/l, produce o scădere a numărului de celule vii de drojdie, valoarea obținută fiind cu 70% mai mică decât cea a probei martor. La doza de 1 mg/l, Pb^{2+} produce o inhibare a capacității de multiplicare a celulelor de drojdie în mustul de bere fermentat, numărul de celule vii de drojdie fiind aproape nul (valoarea obținută fiind cu 94% mai mică decât cea a probei martor).

De asemenea, Cu^{2+}, sub formă de $CuSO_4 \cdot 5\ H_2O$, la doza de 0,5 mg/l, prezintă acțiune toxică mare, valoarea numărului de celule vii de drojdie fiind cu 68% mai mică decât cea a probei martor. La doza de 10 mg/l, Cu^{2+} produce o inhibare a capacității de multiplicare a celulelor de drojdie în mustul fermentat, numărul de celule vii de drojdie fiind aproape nul (valoarea obținută fiind cu 92% mai mică decât cea a probei martor).

Comparativ cu aceste metale, Mn^{2+} şi Zn^{2+} prezintă un efect toxic mai redus asupra capacității de multiplicare a celulelor de drojdie în mustul de bere fermentat. Astfel, Mn^{2+}, sub formă de $MnSO_4$, la doze de 0,5 şi 10 mg/l, produce o scădere a numărului de celule vii de drojdie, valorile obținute fiind cu 28% şi respectiv 52% mai mici decât cea a probei martor. Zn^{2+}, sub formă de $ZnCl_2$, la doze de 0,5 şi 10 mg/l, produce o scădere a numărului de celule vii de drojdie, valorile obținute fiind cu 12% şi respectiv 36% mai mici decât cea a probei martor.

După cum se poate vedea din Tabelele 1 şi 2, metalele testate prezintă un efect toxic diferențiat asupra metabolismului celular al drojdiei în mustul de bere în fermentație. În funcție de acest efect se poate stabili o anumită ordine a gradului de toxicitate al metalelor asupra dinamicii fermentației mustului de bere de către drojdie şi a dinamicii numărului celulelor vii de drojdie în mustul de bere în fermentație. Astfel, în ordine descrescătoare a gradului de toxicitate, metalele grele testate pot fi aranjate în felul următor: $Cd^{2+}>Pb^{2+}>Cu^{2+}>Mn^{2+}>Zn^{2+}$.

Rezultatele obținute concordă cu unele date din literatură, potrivit cărora Cd^2, Pb^{2+} şi Cu^{2+} fac parte din grupa metalelor intens toxice, iar Mn^{2+} şi Zn^{2+} fac parte din grupa metalelor puțin toxice, atât pentru microorganisme cât şi pentru organismul uman [2,3].

Concluzii. 1. Metalele grele testate prezintă un efect toxic diferențiat asupra metabolismului celular al drojdiei în mustul de bere în fermentație. Efectul toxic poate fi corelat atât cu acțiunea specifică a fiecărui metal, cât şi cu cantitatea de metal prezentă în mediul fermentat.

2. Se poate stabili o anumită ordine a gradului de toxicitate al metalelor grele testate asupra dinamicii fermentației mustului de bere de către drojdie şi a dinamicii numărului celulelor vii de drojdie în mustul de bere în fermentație. Astfel, în ordinea descrescătoare a gradului de toxicitate, metalele grele testate pot fi aranjate în felul următor: $Cd^{2+}>Pb^{2+}> Cu^{2+}>Mn^{2+}>Zn^{2+}$.

3. Se conturează posibilitatea utilizării drojdiei de bere *Saccharomyces carlsbergensis* ca bioindicator simplu şi eficient al prezenței substanțelor poluante în produsele alimentare.

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RECENZII – BOOK REVIEWS

S i l v i a O n a c şi (and) Z o e B u z, **Indice bibliografic tematic al revistei "Studia Universitatis Babeş-Bolyai, Biologia", 1957-1997** (*Bibliographic Subject Index of the Review "Studia Universitatis Babeş-Bolyai, Biologia", 1957-1997*), Editura Presa Universitară Clujeană, Biblioteca Centrală Universitară "Lucian Blaga", Cluj-Napoca, 2000, 253 pages.

This book covers 41 years (1957-1997) of the 44-year history (1957-2000) of the biological review of our University. One can state that this review is one of the oldest European biological periodicals edited by universities.

The thematic index of the book (pp. 19-170) lists 1328 papers that appeared in our biological review in the 1957-1997 period. Most of the papers (1298) are synthesis works, original articles and book reviews; 20 papers are chronicles of the scientific life, and 10 articles were written "In Memoriam" of distinguished scientists.

The 1298 synthesis works, original articles and book reviews were grouped under the following headings: Botany; Zoology; Plant physiology, biochemistry and biophysics; Animal physiology, biochemistry and biophysics; Ecology, Nature protection and conservancy; Genetics; Phytopathology, Plant parasitology, Control of pests and parasites; General and applied entomology, Animal parasitology, Control of pests and parasites; Microbiology, Enzymology, Immunology; Hydrobiology; Pedobiology; Paleontology, Paleobotany, Paleozoology; Agri- and sylvicultural sciences, Animal breeding.

Besides the thematic index, the book also comprises Index of authors (pp. 171-198; 530 authors), Index of scientific terms (pp. 199-241) and Index of geographic names (pp. 243-252).

This book, indexing Studia Universitatis Babeş-Bolyai, Biologia for 41 years, makes it possible, for a broad circle of students and experts in different fields of fundamental and applied life sciences, to easily and efficiently use the information offered by our biological review; this information is available, practically, to everybody, as part of the papers were published in English, German, French or Russian and all synthesis works and original articles are accompanied by summaries written in at least one of the four languages mentioned above.

The authors of this book, the librarians Biologist Zoe Buz, Ph.D. and Biologist Silvia Onac deserve all thanks and congratulations for initiating the elaboration and publication of this book at a high level of quality.

The valuable contribution of the Cluj University Press and of the Lucian Blaga Central University Library to edition of this book is also acknowledged.

STEFAN KISS