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ADVANCES IN SOIL ENZYMIOLOGY (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. ENZYMIOLOGY 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 Maslovetkaya [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 supplemented 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 1 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 1 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 wastewaters 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/m². 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/m² and decreased values at 25 l crude oil/m². At the same time, the other carbohydrate 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 inhibitor 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]

<table>
<thead>
<tr>
<th>Partial oxidative product</th>
<th>Carbohydrase activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Invertase (mg glucose)</td>
</tr>
<tr>
<td></td>
<td>Cellulase (mg glucose)</td>
</tr>
<tr>
<td></td>
<td>Amylase (mg maltose)</td>
</tr>
<tr>
<td></td>
<td>Xylanase (mg xylose)</td>
</tr>
<tr>
<td>1-Hexadecanol</td>
<td>16.6</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>12.4</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>8.3</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>1.4</td>
</tr>
</tbody>
</table>

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 Devoroi), 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 Devoroi 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 Devoroi/m² 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 Devoroi-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 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/m² 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) O₂ (aeration) and nutrient (NH₄⁺) 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 $\text{NH}_4^+$ and formation of $\text{NO}_3^-$ 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 $\text{NH}_4^+$ 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 $\gamma$-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 Hahntenjoch) 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 $5 \times 10^5$ 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, CO₂ 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 CO₂ production. Biodegradation of oil in a peat-like soil did not take place and could not be monitored with CO₂ production, FDA hydrolysis or bacterial numbers, but the absence of bioremediation was confirmed with TTC reduction.

Part II. ENZYMEOLOGY 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 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 nonremediatted sites (sampling period: autumn 1995) and from three nonremediatted sites and four sites remediatted in 1986, 1991, 1993 and 1995, respectively (sampling period: summer 1996). For remediattion, a 2:1 (wet weight) mixture of municipal sewage sludge and power plant fly ash was surface-applied at a single rate of $\sim 448 \text{ t/ha}$. 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 nonremediatted 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 1.6 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.

Remediattion 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-Głogów Copper District were studied by Zwołiński 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/km²/year emitted by the zinc smelter, and 118, 87 and 72 t/km²/year emitted by the copper plant. It was only 32 t/km²/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 CO₂ production and O₂ uptake distinctly declined with the increase of pollution. As the CO₂ production decreased to a larger extent than did the O₂ uptake, the respiratory coefficient (Q=CO₂/O₂) 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 Olkus 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-m² 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 H₂O 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 $\beta$-glucosidase activity decreased significantly and dehydrogenase activity increased significantly in the Cd dust-treated plots; urease and acid phosphatase activities 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 effects of technologies applied for remediation on such soils.

Galilin 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 antimonial lead.

Due to the industrial emissions, the studied soil (a loamy sand; pH in H$_2$O 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$^\circ$C 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]. Kairogodova and Vorobechik [27] and Kairogodova [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.

Mukatano 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 A1F₃. 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.

Expression of enzyme activities: arylsulphatase in µg p-nitrophenol/g Cₘᵢᶜ/hour, and dehydrogenase in µg TPF/mg Cₘᵢᶜ/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 according 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 20
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 SO₂ 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 A₁) 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 Kőlosváry [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 atmosphere 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 (5Z).

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

<table>
<thead>
<tr>
<th>Region</th>
<th>Forest station</th>
<th>Stand number</th>
<th>Stand surface (ha)</th>
<th>Dominant plants</th>
<th>Distance from the factory (km)</th>
<th>Soil depth (cm)</th>
<th>pH in KCl</th>
<th>Total C (%)</th>
<th>Total N (%)</th>
<th>C:N</th>
<th>Enzyme activities*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Pulawy</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td>Dehydrogenase</td>
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<td></td>
<td></td>
<td>10-20</td>
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<td>2.01</td>
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<td>3.1</td>
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<td></td>
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<td>2 Z</td>
<td>3.63</td>
<td>Pine, alder</td>
<td>8.0</td>
<td>5-10</td>
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<td>7.69</td>
<td>2.83</td>
<td>2.7</td>
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<td>10-20</td>
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<td>3.2</td>
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<tr>
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<td>5-10</td>
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<tr>
<td></td>
<td>Wola Osierska</td>
<td>4 Z</td>
<td>3.90</td>
<td>Oak, maple, larch, spruce, alder</td>
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<td>5-10</td>
<td>3.4</td>
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<td>10-20</td>
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<td>10-20</td>
<td>3.9</td>
<td>1.40</td>
<td>0.74</td>
<td>1.9</td>
<td>0.63</td>
</tr>
</tbody>
</table>

* Expression of enzyme activities: dehydrogenase in μg TPF/g dry soil/24 hours; urease in μg NH₄⁺-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 Naprasnıkova 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 Carrheiro 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 SO₂, NOₓ 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 determined 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 emissions. 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 hydrolysat, 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 (CO₂ 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 cellulolyltic 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 Vorobechik [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 study of soils in the city of Dorsten (Northwest-Germany), Bröll 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.

Macuilla [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
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

<table>
<thead>
<tr>
<th>Soil property</th>
<th>Parent material and number of analysed samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rubbish n=60                          Spoil banks n=84</td>
</tr>
<tr>
<td>Number of bacteria</td>
<td>0.72*                                    0.62*                  0.46*                  0.84*</td>
</tr>
<tr>
<td>Number of fungi</td>
<td>0.54*                                    0.28                    0.35                    0.26</td>
</tr>
<tr>
<td>Microbial biomass C content</td>
<td>0.43</td>
</tr>
<tr>
<td>Total organic C content</td>
<td>0.55*                                    0.02                    -0.04                    0.33</td>
</tr>
<tr>
<td>Total N content</td>
<td>0.67*                                    0.46*                  0.17                    0.66*</td>
</tr>
<tr>
<td>pH</td>
<td>-0.44                                    0.18                    -0.40*                  0.33</td>
</tr>
<tr>
<td>Clay content</td>
<td>0.54*                                    0.15                    -0.12                   0.69*</td>
</tr>
<tr>
<td>P content</td>
<td>0.29                                    0.42*                  -0.15                   -0.24</td>
</tr>
<tr>
<td>K content</td>
<td>0.51*                                    0.40*                  0.24                    0.05</td>
</tr>
</tbody>
</table>

* 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 O₂/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 (CO₂ 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. ENZYMEOLOGY 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-m² 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]

<table>
<thead>
<tr>
<th>Soil</th>
<th>Dominant tree</th>
<th>Depth (cm)</th>
<th>pH in CaCl₂</th>
<th>Organic C (%)</th>
<th>Total N (%)</th>
<th>C:N</th>
<th>Respiration*</th>
<th>DMSO reduction*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-15</td>
<td>7.51</td>
<td>3.34</td>
<td>0.27</td>
<td>13</td>
<td>3.2</td>
<td>58.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15-30</td>
<td>7.52</td>
<td>1.20</td>
<td>0.10</td>
<td>12</td>
<td>0.9</td>
<td>15.1</td>
</tr>
<tr>
<td>Forest</td>
<td>Poplar</td>
<td>0-15</td>
<td>7.44</td>
<td>2.35</td>
<td>0.24</td>
<td>10</td>
<td>2.0</td>
<td>37.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15-30</td>
<td>7.43</td>
<td>1.28</td>
<td>0.13</td>
<td>10</td>
<td>0.5</td>
<td>13.6</td>
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<tr>
<td></td>
<td>Alder</td>
<td>0-15</td>
<td>7.39</td>
<td>2.73</td>
<td>0.21</td>
<td>13</td>
<td>2.1</td>
<td>39.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15-30</td>
<td>7.52</td>
<td>1.05</td>
<td>0.11</td>
<td>10</td>
<td>0.6</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>Larch</td>
<td>0-15</td>
<td>7.60</td>
<td>1.05</td>
<td>0.11</td>
<td>10</td>
<td>1.3</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15-30</td>
<td>7.60</td>
<td>1.05</td>
<td>0.11</td>
<td>10</td>
<td>1.3</td>
<td>15.0</td>
</tr>
</tbody>
</table>

* Respiration is expressed in µg CO₂/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 Hützl 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);
- C1 - mature biocompost, obtained mainly from household biowaste;
- C2 - 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 m² and ~1 m depth) were filled with TLS-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 C2 and 500 C2. 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 m² and 3 m in depth.

In the lysimeter trial "sewage sludge", the effects of sludge and composted sludge on TS and TIS were compared. TS and TIS 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 TIS. 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, TIS, 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°C 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 QIS.

* * *

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-m² plots, the earthworm input being of almost 70 individuals/m². 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 Šiša 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 Šiša et al. in 1996 and 1997 and the results obtained were published in 2000 [72]. Four technogenic 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

<table>
<thead>
<tr>
<th>No.</th>
<th>Locality</th>
<th>Soil Description</th>
<th>Organic C (‰)</th>
<th>Total N (‰)</th>
<th>Total P (mg/kg dry soil)</th>
<th>C:N</th>
<th>pH</th>
<th>Plants 1995</th>
<th>Plants 1996</th>
<th>Plants 1997</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Úžin</td>
<td>Technogenic soil: overburdens not covered with topsoil</td>
<td>2.80</td>
<td>0.26</td>
<td>1287.78</td>
<td>10.67</td>
<td>6.75</td>
<td>Winter wheat</td>
<td>Fallow</td>
<td>Rape</td>
</tr>
<tr>
<td>2</td>
<td>Úžin</td>
<td>Technogenic soil: overburdens covered with topsoil (50 cm)</td>
<td>2.77</td>
<td>0.26</td>
<td>1667.86</td>
<td>10.55</td>
<td>6.92</td>
<td>Winter wheat</td>
<td>Fallow</td>
<td>Rape</td>
</tr>
<tr>
<td>3</td>
<td>Svoboda</td>
<td>Technogenic soil: toxic overburdens covered with bentonite (20 cm) and topsoil (50 cm)</td>
<td>1.65</td>
<td>0.15</td>
<td>955.86</td>
<td>10.75</td>
<td>6.82</td>
<td>Spring barley</td>
<td>Legume-grass</td>
<td>Clover-grass</td>
</tr>
<tr>
<td>4</td>
<td>Brašany</td>
<td>Undisturbed soil</td>
<td>2.11</td>
<td>0.20</td>
<td>1470.76</td>
<td>10.37</td>
<td>6.11</td>
<td>Alfalfa</td>
<td>Winter wheat</td>
<td>Winter wheat</td>
</tr>
<tr>
<td>5</td>
<td>Rudolice</td>
<td>Undisturbed soil</td>
<td>1.36</td>
<td>0.12</td>
<td>1520.01</td>
<td>11.24</td>
<td>8.33</td>
<td>Grapevine</td>
<td>Grapevine</td>
<td>Grapevine</td>
</tr>
<tr>
<td>6</td>
<td>Čepírkoš</td>
<td>Recultivated loessoid soil</td>
<td>1.08</td>
<td>0.09</td>
<td>630.57</td>
<td>12.20</td>
<td>8.58</td>
<td>Grapevine</td>
<td>Grapevine</td>
<td>Grapevine</td>
</tr>
<tr>
<td>7</td>
<td>Triskolupy</td>
<td>Technogenic soil: overburdens covered with topsoil (50 cm)</td>
<td>1.25</td>
<td>0.12</td>
<td>688.36</td>
<td>10.55</td>
<td>8.52</td>
<td>Spring barley</td>
<td>Rape</td>
<td>Winter wheat</td>
</tr>
<tr>
<td>8</td>
<td>Havraň</td>
<td>Undisturbed soil</td>
<td>1.52</td>
<td>0.17</td>
<td>769.11</td>
<td>9.21</td>
<td>7.19</td>
<td>Sunflower</td>
<td>Ryegrass</td>
<td>Winter wheat</td>
</tr>
</tbody>
</table>

Characterisation of technogenic and undisturbed soils cultivated with agricultural plants, in 3-year experiments [72]
Table 6

Enzyme activities and microbial parameters in technogenic and undisturbed soils, in 3-year experiments [72]

<table>
<thead>
<tr>
<th>Enzyme activity or microbial parameter*</th>
<th>Number of locality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Invertase activity</td>
<td>5.11</td>
</tr>
<tr>
<td>Phosphatase activity</td>
<td>28.57</td>
</tr>
<tr>
<td>Catalase activity</td>
<td>0.80</td>
</tr>
<tr>
<td>Basal respiration (BR)</td>
<td>0.41</td>
</tr>
<tr>
<td>N-induced respiration (NR)</td>
<td>0.58</td>
</tr>
<tr>
<td>Glucose-induced respiration (GR)</td>
<td>4.29</td>
</tr>
<tr>
<td>N+glucose-induced respiration (NGR)</td>
<td>13.30</td>
</tr>
<tr>
<td>NR:BR</td>
<td>1.53</td>
</tr>
<tr>
<td>GR:BR</td>
<td>10.54</td>
</tr>
<tr>
<td>NGR:BR</td>
<td>35.51</td>
</tr>
<tr>
<td>Microbial biomass C</td>
<td>439.8</td>
</tr>
</tbody>
</table>

*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 µg/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-m² 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 Pașca *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 m² = 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 m² = 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 $H_{2}O_{2}$-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\%), by increase in field moisture (from 9.2 to 40.5\%), by increase in field moisture (from 9.2 to 40.5\%), 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, Bonmati 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 experimental 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

OCTAVIAN POPESCU*, LAZAR T. ŠUMANOVSKI**, MIHAI S. IONESCU*** and GRADIMIR N. MISEVIC****

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].
INVESTIGATION OF GLYCONECTIN-GLYCONECTIN INTERACTIONS

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\(_2\)SO\(_4\) 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'-dithiobis-(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 Heps buffer (pH 7.4) (seawater Heps, 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).
Fig. 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-phosphatidylinositol-linked glypic an 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 \( \text{D} \)-configuration, except fucose, which exists in the \( \text{L} \)-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 C₂-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 Ca²⁺-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 clofibrlic 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 (clofibrlic 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 clofibrlic acid. The present article deals with the most important functional and ultrastructural effects observed after the direct addition of clofibrlic 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 Johnson and Lardy [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 °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 MgSO₄. 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 \(\mu\)M diS-C\(_2\)-(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 \(\beta\)-hydroxybutyrate (\(\beta\)-OH-B) were determined from the effluent by an enzymatic method, using \(\beta\)-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 \(\mu\)M, the effects are significant for mitochondria of both animals. For example, in the case of guinea pig mitochondria, the addition of 200 \(\mu\)M 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 clofibrate acid (CA) in the spectro-fluorometer 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).

![Figure 1](image.png)

**Fig. 1.** Effect of clofibrate acid (CA) on rat liver mitochondria respiring with glutamate + malate (see details in "Materials and methods").
**Fig. 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.
Fig. 3. Gluconeogenesis in the rat liver perfused with clofibrate acid (CA).

Fig. 4. Gluconeogenesis in the rat liver perfused with ethanol (EtOH).
Fig. 5. Gluconeogenesis in the guinea pig liver perfused with clofibric acid (CA).

Fig. 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.

![Diagram of ketogenesis in the guinea pig liver perfused with clofibric acid (CA).](image-url)
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 (antilipemic effect of CA) but produces some effects of its own (nuclei with an irregular shape, mitochondrial matrix rarefaction etc.).
Fig. 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).
Fig. 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.
**Figure 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.

REFERENCES


METABOLIC AND ULTRASTRUCTURAL EFFECTS RECORDED IN ISOLATED MITOCHONDRIA AND PERFUSED LIVER FOLLOWING THE SUBCHRONIC TREATMENT OF RATS AND GUINEA PIGS WITH CLOFIBRIC ACID

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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 Johnson and Lardy [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 °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 MgSO₄, 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-C₂-(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 \textit{in situ} with Krebs-Henseleit-bicarbonate buffer (KHB), as previously described \cite{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 described \cite{13, 14}. Ketogenesis was assayed by using the methods specified in \cite{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 \cite{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>
<thead>
<tr>
<th>Animal</th>
<th>Parameter Group</th>
<th>Mean body weight (b.w.)</th>
<th>Difference (g)</th>
<th>Differential average change/animal (g)</th>
<th>Mean relative liver weight (% of b.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Control</td>
<td>188.7</td>
<td>201.0</td>
<td>+ 12.3</td>
<td>2.99</td>
</tr>
<tr>
<td>Rat</td>
<td>CA-treated</td>
<td>190.0</td>
<td>184.5</td>
<td>- 6.5</td>
<td>18.8</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>Control</td>
<td>201.8</td>
<td>208.2</td>
<td>+ 6.4</td>
<td>2.65</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>CA-treated</td>
<td>189.3</td>
<td>178.7</td>
<td>- 10.6</td>
<td>17.0</td>
</tr>
</tbody>
</table>

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 \( \mu \)moles/100 g body weight/h, which is lower than in the control by about 30 \( \mu \)moles/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 \( \mu \)moles/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 (Aca) formation in the perfused liver of CA-treated rats reaches about 110 \( \mu \)moles/100 g body weight/h (Fig. 3), the steady state level barely exceeds 45 \( \mu \)moles/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 \( \mu \)moles/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).
EFFECTS OF THE TREATMENT WITH CLOFIBRIC ACID ON LIVER STRUCTURE AND FUNCTIONS

Fig. 1. Gluconeogenesis in the perfused liver of CA-treated rats (see details in text).

Fig. 2. Gluconeogenesis in the perfused liver of CA-treated guinea pigs.
**Fig. 3.** Ketogenesis in the perfused liver of CA-treated rats.

**Fig. 4.** Ketogenesis in the perfused liver of CA-treated guinea pigs.
Fig. 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.
Fig. 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).
Fig. 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 $\beta$-oxidation of fatty acids differs from that of the mitochondrial $\beta$-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 $\beta$-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 $\beta$-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.
REFERENCES


COMPARATIVE STUDIES OF THE ADRENAL CORTEX STRUCTURE AND ULTRASTRUCTURE IN MATURE RATS TREATED WITH TOPICAL DERMOCORTICOSTEROIDS

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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 methods.** 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 Reicht-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.
ADRENAL CORTEX AS AFFECTED BY DERMOCORTICOSTEROIDS

Fig. 1. Adrenal cortex in L-group (x 200).

Fig. 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.

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.
Fig. 4. Zona fasciculata with moderate secretory activity in L-group (x 6,510).

Fig. 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.

Fig. 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 feedback 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 hypothalomo-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.

REFERENCES


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 immunosuppressive 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 standardized 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 cm², 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 Fluocinolone-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.
Fig. 1. Somatotrope cell ultrastructure in C-group (x 12,000).

Fig. 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).

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.
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.
Fig. 5. Gonadotrope cell ultrastructure in D-group (x 8,000).

Fig. 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 cisterna 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.
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 adrenocorticotropic 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.

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 paraventricular neurons, which determined the blockage of the adrenocorticotropic 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 agrees with the clinical report showing that spontaneous and GRH-induced GH secretion is suppressed in patients with Cushing’s disease [7]. Fernández 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.

**REFERENCES**


HISTOLOGICAL AND ULTRASTRUCTURAL ASPECTS OF THE MYOCARDIUM OF RATS TREATED WITH AN ANTHRACYCLINE ANTIBIOTIC - EPIRUBICIN

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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 cardiomypathy 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/m² body surface) administrated i.v. on the rat myocardium in concordance with the moment of sacrifice.

Material and methods. Our experiments were carried out with the following four groups of healthy adult male Wistar rats, weighing 190 ± 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 E₁, E₂ and E₃ - treated i.v. with 89 mg Epirubicin/m² 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 sacrifice. 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 sacrifice 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 sacrifice.
The first histological modifications appeared 24 hours after the treatment (group E₁). 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.

Fig. 1. Congestion and perivascular and interfascicular oedema in the myocardium (x 512).

All these modifications were still present after 4 days (group E₂), 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.

Fig. 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 E3 sacrificed 6 days after the treatment, they being a little more obvious, affecting wide areas (both isolated and grouped myocytes).

Fig. 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.

Fig. 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].

**Fig. 5.** Thickened Eberth junction, 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].

**Fig. 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, Hibbs et al. [9] and Schmalarbuck and Dumee [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.

REFERENCES


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 distortions, 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 ± 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 T₁, T₂, T₃ and T₄ 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 sacrifice. 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 sacrifice.

**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 condensed 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.
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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 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).

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
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 crosslinks 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].

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.
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[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 alternative 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.
REFERENCES

INFLUENȚA UNOR METALE GRELE ASUPRA METABOLISMULUI CELULAR AL DROJDII\LOR

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 și a produselor alimentare cu o gamă tot mai largă de substanțe chimice reprezintă un fenomen în progresivă amplificare la scără mondială, ca rezultat al dezvoltării industriale, măririi traficului rutier, chimizării agriculturii etc.

O sursă importantă de poluanții este industria de prelucrare a minereurilor din care rezultă zgară și pulbere 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 și 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 și 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 și rapide de depistare a prezenței substanțelor poluanțe 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 și apoi interesează natura agentului toxic [7].

Pornind de la ideea că substanțele toxice acționează la nivelul celulei prin declanșarea 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 și efficient al prezenței substanțelor poluanțe în produsele alimentare.

În lucrarea de față descriem cercetări comparative privind influența unor metale grele (Cd\(^{2+}\), Pb\(^{2+}\), Cu\(^{2+}\), Mn\(^{2+}\) și Zn\(^{2+}\)) asupra metabolismului celular al drojdiei de bere Saccharomyces carlsbergensis, în mustul de bere în fermentație.

**Materiale și metode.** Pentru cultivarea drojdiei de bere Saccharomyces carlsbergensis a servit, drept mediu nutritiv, mustul de bere industrial preparat din malț, sterilizat în prealabil, nehămat și cu un extract real de 13%. Mustul de bere a fost distribuit în cantitate de 150 ml în baloane cu fund plat de 250 ml, sterilizate și închise cu ventile de fermentație cu acid sulfuric concentrat.

În experiment s-au utilizat următoarele metale grele sub formă de săruri, în cantități diferite, similare celor întâlnite adeseori în produsele alimentare:
- Cd\(^{2+}\) sub formă de CdCl\(_2\) (în doze de 0,005, 0,01 și 0,1 mg/l);
- Pb\(^{2+}\) sub formă de Pb(NO\(_3\))\(_2\) (în doze de 0,01, 0,05 și 1 mg/l);
- Cu\(^{2+}\) sub formă de CuSO\(_4\) · 5 H\(_2\)O (în doze de 0,5, 5 și 10 mg/l);
- Mn\(^{2+}\) sub formă de MnSO\(_4\) (în doze de 0,5, 5 și 10 mg/l);
- Zn\(^{2+}\) sub formă de ZnCl\(_2\) (în doze de 0,5, 5 și 10 mg/l).

Determinările s-au efectuat în serii paralele de probe și în prezența unei probe martor, în care adăosul 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. Fermentația mustului de bere a fost condusă la temperatura camerei (20\(^{0}\)C), timp de 9 zile (192 ore).

În cursul fermentației mustului de bere în prezența metalelor grele, am urmărit dinamica degajării CO\(_2\) de către drojdie și 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 și exprimată în % masice (CO\(_2\)/100 g) și dinamica numărului de celule vii de drojdie prin numărarea cu camera Thoma, după colorare cu soluție 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 sârurilor metalelor grele testate sunt trecute în Tabelul 1.

Dinamica fermentației mustului de bere de către drojdia de bere Saccharomyces carlsbergensis, în prezența unor metale grele

<table>
<thead>
<tr>
<th>Metale grele</th>
<th>Doze (mg/l)</th>
<th>Masa CO₂ degajată în 24 ore (% masice)</th>
<th>Durata fermentației (ore)</th>
<th>CO₂ total degajat</th>
<th>Scădere % față de martor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd⁺²</td>
<td>0,005</td>
<td>0,26 0,83 0,90 1,00 1,02 1,02 1,02 1,02 1,02</td>
<td>1,02</td>
<td>68,12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0,01</td>
<td>0,14 0,35 0,40 0,45 0,50 0,50 0,50 0,50 0,50</td>
<td>0,50</td>
<td>84,37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0,10</td>
<td>0,10 0,10 0,10 0,10 0,10 0,10 0,10 0,10 0,10</td>
<td>0,10</td>
<td>96,88</td>
<td></td>
</tr>
<tr>
<td>Pb⁺²</td>
<td>0,01</td>
<td>0,25 0,90 1,00 1,10 1,20 1,20 1,20 1,20 1,20</td>
<td>1,20</td>
<td>62,50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0,05</td>
<td>0,20 0,55 0,66 0,66 0,66 0,66 0,66 0,66 0,66</td>
<td>0,66</td>
<td>79,37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,00</td>
<td>0,10 0,20 0,20 0,20 0,20 0,20 0,20 0,20 0,20</td>
<td>0,20</td>
<td>93,75</td>
<td></td>
</tr>
<tr>
<td>Cu⁺²</td>
<td>0,50</td>
<td>0,40 0,91 1,25 1,30 1,35 1,45 1,45 1,45 1,45</td>
<td>1,45</td>
<td>54,69</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5,00</td>
<td>0,30 0,30 0,75 1,10 1,15 1,25 1,25 1,25 1,25</td>
<td>1,25</td>
<td>60,94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10,00</td>
<td>0,15 0,15 0,25 0,25 0,25 0,25 0,25 0,25 0,25</td>
<td>0,25</td>
<td>92,19</td>
<td></td>
</tr>
<tr>
<td>Mn⁺²</td>
<td>0,50</td>
<td>0,45 1,40 1,80 1,90 2,20 2,20 2,20 2,20 2,20</td>
<td>2,20</td>
<td>31,25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5,00</td>
<td>0,30 1,25 1,60 1,70 1,75 1,75 1,75 1,75 1,75</td>
<td>1,75</td>
<td>43,75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10,00</td>
<td>0,20 0,65 1,00 1,05 1,10 1,10 1,10 1,10 1,10</td>
<td>1,10</td>
<td>65,27</td>
<td></td>
</tr>
<tr>
<td>Zn⁺²</td>
<td>0,50</td>
<td>0,50 1,60 2,25 2,50 2,60 2,60 2,60 2,60 2,60</td>
<td>2,60</td>
<td>16,90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5,00</td>
<td>0,40 1,45 1,95 2,05 2,10 2,10 2,10 2,10 2,10</td>
<td>2,10</td>
<td>34,38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10,00</td>
<td>0,35 0,80 1,43 1,55 1,60 1,60 1,60 1,60 1,60</td>
<td>1,60</td>
<td>43,80</td>
<td></td>
</tr>
<tr>
<td>Martor (fără metal)</td>
<td></td>
<td>0,65 1,70 2,85 3,10 3,15 3,20 3,20 3,20 3,20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sârurile 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 și cu cantitatea de sare a metalului prezentă în mediul testat. Ca metale intens toxice se remarcă în ordine descrescătoare Cd⁺², Pb⁺² și Cu⁺². Cd⁺², sub formă de CdCl₂, la doza de 0,005 mg/l, produce o scădere a masei de CO₂ degajată de drojdie, valoarea obținută fiind cu 68,12% mai mică decât cea a probei martor. Același metal, la doza de 0,01 mg/l, produce o scădere și mai mare a masei de CO₂ 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 fermentației mustului de bere de către drojdie, masa de CO₂ degajată fiind aproape nulă (valoarea obținută fiind cu 96,88% mai mică decât cea a probei martor).
Toxicitate mare prezintă și Pb\(^{2+}\), care sub formă de Pb(NO\(_3\))\(_2\), la doze de 0,01 și 0,05 mg/l, produce scăderi mari ale masei de CO\(_2\) degajată de drojdie, valorile obținute fiind cu 62,5% și respectiv 79,37% mai mici decât cea a probei mavor. 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 mavor).

De asemenea, Cu\(^{2+}\), sub formă de CuSO\(_4\)\(\cdot\)5 H\(_2\)O, la doze de 0,5 și 10 mg/l, prezintă acțiune toxică mare, valorile masei de CO\(_2\) degajată de drojdie fiind cu 54,69% și respectiv 60,94% mai mici decât cea a probei mavor. 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 mavor).

Comparativ cu aceste metale, Mn\(^{2+}\) și 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 și 10 mg/l, produce o scădere a masei de CO\(_2\) degajată de drojdie, valorile obținute fiind cu 31,25, 43,75 și respectiv 65,27% mai mici decât cea a probei mavor. De asemenea, Zn\(^{2+}\), sub formă de ZnCl\(_2\), la doze de 0,5, 5 și 10 mg/l, produce o scădere a masei de CO\(_2\) degajată de drojdie, valorile obținute fiind cu 16,9, 34,38 și 43,8% mai mici decât cea a probei mavor.

Rezultatele obținute în studierea dinamicii numărului celulelor vii de drojdie în mustul de bere în fermentație în prezența sârurilor metalelor grele testate sunt trecute în Tabelul 2.

**Variația numărului celulelor vii de drojdie în mustul de bere în prezența unor metale grele**

<table>
<thead>
<tr>
<th>Metale</th>
<th>Doze (mg/l)</th>
<th>Număr celule vii de drojdie (\times) 10(^6)/ml must</th>
<th>Durata fermentației (ore)</th>
<th>Scădere % față de mavor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24  48  72  96  120  144  168  192</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cd(^{2+})</td>
<td>0,005</td>
<td>1,80  30,40  56,20  15,30  7,25  3,80  1,80  0,70</td>
<td>72,00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0,10</td>
<td>1,65  1,75  1,10  0,20  0,10  0,10  0,10  0,10</td>
<td>96,00</td>
<td></td>
</tr>
<tr>
<td>Pb(^{2+})</td>
<td>0,01</td>
<td>1,95  31,35  58,30  11,70  7,20  3,35  1,24  0,75</td>
<td>70,00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,00</td>
<td>1,70  1,80  1,25  1,15  0,15  0,15  0,15  0,15</td>
<td>94,00</td>
<td></td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>0,50</td>
<td>1,90  23,85  60,35  12,00  7,90  3,40  1,10  0,80</td>
<td>68,00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10,00</td>
<td>1,75  2,10  1,50  0,40  0,20  0,20  0,20  0,20</td>
<td>92,00</td>
<td></td>
</tr>
<tr>
<td>Mn(^{2+})</td>
<td>0,50</td>
<td>3,40  80,20  100,4  35,36  15,30  7,25  2,00  1,80</td>
<td>28,00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10,00</td>
<td>2,50  55,80  80,50  20,40  10,60  5,10  1,60  1,20</td>
<td>52,00</td>
<td></td>
</tr>
<tr>
<td>Zn(^{2+})</td>
<td>0,50</td>
<td>4,50  100,5  132,1  50,10  19,45  11,10  2,25  2,20</td>
<td>12,00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10,00</td>
<td>2,80  71,00  100,6  30,65  15,20  8,25  1,90  1,60</td>
<td>36,00</td>
<td></td>
</tr>
<tr>
<td>Martor (fără metal)</td>
<td>-</td>
<td>6,35  148,2  170,2  86,70  31,75  15,60  3,80  2,50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 ×10^6 celule/ml must).

Prezența metalelor grele în mustul de bere are o acțiune toxică asupra dinamicii multiplicării și viabilității celulelor de drojdie. În toate probele cu metale grele testate, multiplicarea celulelor de drojdie este mult mai lentă. Se constată că acțiunea de frânerie a procesului de multiplicare al celulelor de drojdie în mustul de bere în fermentație este mai intensă în probele cu Cd^{2+}, Pb^{2+} și 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. Toxicitatea mare prezintă Cd^{2+}, care sub formă de CdCl₂ la doze de 0,005 și 0,1 mg/l produce o scădere a numărului de celule vii de drojdie, valorile obținute fiind cu 66,99% și respectiv 99,36% mai mici decât cea a probei martor. De asemenea, Pb^{2+}, sub formă de Pb(NO₃)₂ la doze de 0,01 și 1 mg/l, produce o scădere a numărului de celule vii de drojdie, valorile obținute fiind cu 65,75% și respectiv 99,27% mai mici decât cea a probei martor. Cu^{2+}, sub formă de CuSO₄:5H₂O, la doze de 0,5 și 10 mg/l, produce o scădere a numărului de celule vii de drojdie, valorile obținute fiind cu 64,55% și respectiv 99,22% mai mici decât cea a probei martor. Se constată că sărurile de Cd^{2+}, Pb^{2+} și 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+} și 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 MnSO₄ la doze de 0,5 și 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% și respectiv 30,42% mai mici decât cea a probei martor. Zn^{2+}, sub formă de ZnCl₂ în doze de 0,5 și 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% și respectiv 50% mai mici decât cea a probei martor.

La sfârșitul 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+}>Zn²⁺.

Cd^{2+} sub formă de CdCl₂, 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 probelor, 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 probelor).

Toxicitatea mare prezintă și Pb^{2+}, care sub formă de Pb(NO₃)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 probelor. 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 probelor).
De asemenea, Cu$^{2+}$, sub formă de CuSO$_4$·5 H$_2$O, 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$^{2+}$ și 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 și 10 mg/l, produce o scădere a numărului de celule vii de drojdie, valorile obținute fiind cu 28% și respectiv 52% mai mici decât cea a probei martor. Zn$^{2+}$, sub formă de ZnCl$_2$, la doze de 0,5 și 10 mg/l, produce o scădere a numărului de celule vii de drojdie, valorile obținute fiind cu 12% și respectiv 36% mai mici decât cea a probei martor.

După cum se poate vedea din Tabelele 1 și 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 și 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$^{2+}$ și Cu$^{2+}$ fac parte din grupa metalelor intens toxice, iar Mn$^{2+}$ și Zn$^{2+}$ fac parte din grupa metalelor puțin toxice, atât pentru microorganisme cât și 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 și 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 și 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 și eficient al prezenței substanțelor poluante în produsele alimentare.
INFLUENȚA UNOR METALE GRELE ASUPRA DROJIILOR

BIBLIOGRAFIE


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 Babeș-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.

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