NEW RESULTS OF OUR SUPEROXIDE DISMUTASE STUDIES
AND FUTURE PLANS IN THIS FIELDS

Our current studies relating to changes in superoxide dismutase activity can be divided into four groups:

a) In joint work with the Department of Paediatrics of the University Medical School in Szeged, we have studied and compared the superoxide dismutase activities, other enzymes of the oxidative metabolism and the lipid peroxidation in some genetically well-defined diseases. In this group we have examined, and continue to examine, the activities of the antioxidant enzymes in children with cystic fibrosis or Duchenne muscular dystrophy.

b) In other joint work with the Department of Paediatrics of the University Medical School in Debrecen and the Children's Hospital in Szeged, we examine the effects of D-penicillamine and of riboflavin in neonatal hyperbilirubinemia. Here too studies have been made of the effects on the antioxidant enzymes, but more recently we have been investigating the activities of delta-aminolevulinic acid synthetase and haeme oxygenase.

c) We cooperate with the Department of Public Health and Epidemiology of the University Medical School in Szeged in clarifying the mechanism of molecular action of the well-known dipyrindyl herbicide paraquat in various living organisms. An account of some these investigations will be presented in our other lecture.

d) We deal with the ever more Important oxidative metabolism changes in human and experimental diabetes. We shall give a brief survey of our results in this field and of our future ideas.

Materials and methods

Venous blood was taken from cubital or umbilical vein and heparin was used as anticoagulant. The red blood cells (RBC) were separated by centrifugation and washed 2-3 times with iso-
nic saline solution. Following this, the RBC were haemolysed in twice their volume of distilled water by freezing and thawing and were separated from intact RBC and debris by centrifugation. Haemoglobin interferes with enzyme activity measurements and was removed from the haemolysates by chloroform/ethanol treatment. Aliquots of the initially separated plasma and the final supernatants of the haemolysates were used for measurements.

The DMD patients were children of both sexes, aged 6-12 years and had been receiving vitamin E therapy for years. The normal control values are the means for a group of 10 children ranging in age from 4 to 12 years.

The adult control human blood samples were obtained from the Blood Donor Center of the University Medical School of Szeged, while the diabetic human blood samples originated from the Diabetic Station of the University Medical School of Szeged.

About 2000 registered diabetics in a population of about 170,000 at Pécs, 48 individuals of various sexes and ages with diseases of different aetiologies, treated with insulin or others were selected in accordance with the aims of the examinations. In all diabetics the blood sugars were determined in the fasting state. One of the bases of the classification was the blood sugar level. The washed diabetic RBC were haemolysed, and the enzyme activities were determined from the aliquots of the haemolysates.

The blood glucose was always determined by the GOD-PERID (Boehringer, FRG) test. The examined patients were children into the following three groups (1) 3.9-6.0 (n = 10), (2) 6.1-11.0 (n = 18), (3) above 11.0 mEq/l (n = 20) blood glucose.

The cystic fibrosis (CF) children were aged 1-12 years and were of both sexes, and the obligate heterozygous parents were also of both sexes. Some of the CF children had been participating in continuous oral vitamin E treatment since the diagnosis of their disease.

In the animal experiments for diabetes rats of both sexes from the CFY strain were used. Comparisons were generally made between the data on individuals of the same sex, about the same weight and age. Diabetes was induced by i.v. administration of streptozotocin (70 mg/kg) or alloxan (50 mg/kg) in distilled wa-
After the injections the rats were fed standard laboratory diet, with water and libitum. Of the rats treated with diabetogens, only those in which glucose could be detected in the urine were regarded as diabetics, and only these were used in the subsequent experiments. The rats were starved for 12 hours before they were decapitated and exsanguinated. Their tissues were homogenized at 0°C in a glass Potter homogenizer.

In general 1 g or less of wet tissue weight was homogenized in 1:10 ml ratio (or a proportionally chosen amount) of 0.005 M phosphate buffer (pH 7.2) and the supernatant from the centrifuged homogenates were used for determination of enzymatic activities.

For toxicological examinations CFLP mice of both sexes, with weights of 21-36 g were used. The mice were kept on normal feed and received water as the rats.

The RBC superoxide dismutase (SOD, EC 1.15.1.1.) activity was estimated from the extent of the inhibition of the superoxide (O$_2^-$)/dependent epinephrine - adrenochrome transformation. 1 unit of SOD can be regarded as the amount of enzyme that causes a 50% inhibition in the extinction change (min, as compared to the control) Matakovic et al.[11].

Catalase (C-ase, EC 1.11.1.6.) activity was measured by the method of Beers and Sizer modified by Matakovic et al.[11]. The extent of H$_2$O$_2$ consumption, which depends on the amount of enzyme, is measured in a given time under fixed conditions.

Peroxidase (P-ase, EC 1.11.1.7) activity of the haemolysates were measured by the method of Maehly et al. described by Matakovic et al.[11]. The method is based on the spectrophotometric determination of P-ase activity dependent on quaiacol - quaiacol tetra- transformation.

The glutathione peroxidase activity (GP-ase, EC 1.11.1.9) was measured by the combined method of Chiu et al.[3] with cumene hydroperoxide substrate. The reduced glutathione residue was measured by the method of Sedlak et al.[21].

The lipid peroxidation (LP) was determined by the method of Placer et al.[19].

The protein contents were measured spectrophotometrically by the method of Lowry et al.[9].
The reagents used were of the purest quality, and were used without further purification.

The results were subjected to statistical evaluation with the Student t test. All numerical data are given as mean ±SD. In the enzyme activity and LP measurement the difference between duplicate determinations were never in excess of 5%.

Results and discussion

like to make the following additions to section (a) in the first part of the Summary:

Our colleagues with whom we collaborate in the Department of Paediatrics at the University Medical School in Szeged are K. Gyurkovits, A. László and A. Megyéni. Our joint work began about 3 years ago, and is planned to continue in the future too. A few words will be said about this at the end of this section, after the account of the details.

1. We first compared the RBC antioxidant enzyme (AOE) activities and lipid peroxidation values in children with Duchenne muscular dystrophy (DMD) and in healthy volunteers of the same age. The literature background for our studies was as follows:

i) A number of examinations had been performed on muscle biopsy material in DMD cases, the AOE activities being compared with the values for normal muscle biopsy material. The same applied to the LP values. Such measurements were made in a chicken muscular dystrophy model, Perkins et al. [16], and on human material, Kar et al. [6].

ii) The AOE and LP results relating to plasma and RBC haemolysates were fairly contradictory: in one case RBC membrane differences were demonstrated, while in another no differences were found concerning the members of the AOE system, Burr et al. [1] Gomez et al. [22].

Our results are illustrated in the following Tab. 1.

It is clear that the LP values and the AOE activities of the RBC haemolysates in DMD are significantly higher than in healthy children of the same age, Matkovic et al. [13].

The other hereditary disease examined was cystic fibrosis (CF) (also known as mucoviscidosis), which was similarly inve-
New results of our superoxide dismutase studies

Table 1

TBA-reactive products (Lipid Peroxides), catalase and superoxide dismutase activities in the red blood cells of DMD cases and healthy individuals

Produkty TBA-reaktywne (nadtlenki lipidów), aktywność katalezy i dysmutazy ponadtlenkowej w krwinkach czerwonych chorych z dystrofią mięśniową Duchenne’a i osobników zdrowych

<table>
<thead>
<tr>
<th>Assay</th>
<th>Controls (n = 10)</th>
<th>DMD (n = 22)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD U/g prot. mean±SD</td>
<td>1,266.0±37.7</td>
<td>7131.9</td>
<td>p &lt; 0.0005</td>
</tr>
<tr>
<td>C-ase BU/1 haemol. mean±SD</td>
<td>8.04×10³±</td>
<td>22.2×10³</td>
<td>p &lt; 0.0005</td>
</tr>
<tr>
<td>1.078×10³</td>
<td>7.1×10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBA-reactive plasma products (nmol MDA/1 plasma) mean±SD</td>
<td>15.46×10³±</td>
<td>15.45×10³</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>1.50×10³</td>
<td>9.17×10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBA-reactive products from haemolysates (nmol MDA/1 haemol) mean±SD</td>
<td>201.9×10²±</td>
<td>310.7×10³</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td>18.9×10²</td>
<td>115.1×10³</td>
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<td></td>
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</tbody>
</table>

#BU, Bergmeyer units.

igated in collaboration with our pediatric colleagues. Here, fewer literature data were available on the oxygen metabolism change, Feigal et al. [4], Campbell et al. [2].

The first of these authors demonstrated that in CF the Ce²⁺ uptake in the mitochondria is changed, and the O₂ requirement is higher. In the other reference it was shown that the O₂ supply to the tissues is impaired because of the altered fatty acid composition of membranes.

Our own results are outlined in the next Tab. 2. This gives the SOD, C-ase and LP results not only for the ill children, but also for their heterozygous parents. In general, it may be said that the data for the CF children and their parents are comparable, and in both cases are close to the data for healthy adults.
<table>
<thead>
<tr>
<th>Assay</th>
<th>Age group, I (from 4–12 years) (n = 10)</th>
<th>Age group, II (from 20–55 years) (n = 30)</th>
<th>CF homozygotes (n = 8)</th>
<th>Obligate heterozygote parents (from 25–30 years) (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD U/l haemol. mean±SD</td>
<td>293.6 x 10^3 ± 51.3 x 10^3</td>
<td>716.5 x 10^3 ± 92.5 x 10^3</td>
<td>596.67 x 10^3 ± 61.97 x 10^3</td>
<td>599.35 x 10^3 ± 60.80 x 10^3</td>
</tr>
<tr>
<td>SOD U/g prot. mean±SD</td>
<td>1.266 ± 0.05 ± 273.7</td>
<td>2.047 ± 0.05 ± 412.2 (n = 52)</td>
<td>1808.1 ± 0.3 ± 186.8</td>
<td>18 16.2 ± 0.4 ± 182.3</td>
</tr>
<tr>
<td>C-ase BU/l haemol. mean±SD</td>
<td>2.68 ± 0.78 x 10^3</td>
<td>2.05 ± 0.68 x 10^3 (n = 20)</td>
<td>5.2 ± 0.3 x 10^3</td>
<td>5.5 ± 0.4 x 10^3</td>
</tr>
<tr>
<td>TBA-reactive plasma prot. (nmol MDA/l plasma)</td>
<td>5.46 ± 1.5 ± 10^3</td>
<td>10.3 ± 3.9 ± 10^3</td>
<td>7.9 ± 1.5 ± 10^3</td>
<td>7.5 ± 2.0 ± 10^3</td>
</tr>
<tr>
<td>TBA-reactive products from haemol. (nmol MDA/l haemol.) mean±SD</td>
<td>201.9 ± 18.9 ± 10^3</td>
<td>67.4 ± 15.9 ± 10^3</td>
<td>68.8 ± 11.2 ± 10^3</td>
<td>68.3 ± 12.8 ± 10^3</td>
</tr>
</tbody>
</table>
The SOD and C-ase activities of CF children are significantly higher than those of healthy children, whereas the LP is lower. The increased activities of the two enzymes may be a consequence of the partial anoxia, while the LP decrease may be related to the increased viscosity of the blood in this disease.

We consider that the high AOE activities, together with the partial anoxia, may be correlated with the rapid aging of the CF children Matkovic et al. [14, 15].

In a few cases we have examined the RBC AOE system in Down's syndrome, but our results so far are in contradiction with the literature data. We have found lower AOE activities.

We are continuing with examination of the carbonic acid anhydrase activity in CF, and are acquiring new data on Down's disease.

Part (b) is supplemented as follows: From the Department of Paediatrics at the University Medical School in Debrecen, L. Lakatos and his colleagues reported in 1976 that large doses of D-penicillamine (D-PA) (Metalcaptase R, Roche) induce a rapid fall of the high bilirubin level in premature infants and neonates with hyperbilirubinemia. The oxygen treatment of premature (which among others serves to lower the hyperbilirubinemia) may be followed by retrolental fibroplasia, which can result in blindness. This complication is prevented if D-PA is administered in parallel with the oxygenation Lakatos et al. [7, 8].

The question arises of how D-PA acts. It was first believed that it plays a role as a metal-chelating agent in the metabolism. However, this has not yet been conclusively proved by any authors. Our studies to date permit the following remarks about the effect of D-PA:

i) D-PA lowers the LP, primarily as a membrane protector, in the liver of newborn rats. In adult rats it increases the activities of the AOE's in most organs Matkovic et al. [12].

ii) D-PA enhances the activity of haeme oxygenase Groszmann et al. [16].

Here we plan to study the porphyrin metabolism enzymes, and primarily delta-aminolevulinic acid synthase in the near future.

In joint work with the Children's Hospital in Szeged, we have investigated the autocatalytic effect of riboflavin (vita-
min B₂, BeflavinR, Roche) observed in hyperbilirubinaemia. It was reported by L. Paták et al. [17] that exchange transfusion can be avoided in hyperbilirubinaemic preterms and neonates if the "blue light" treatment is combined with the administration of riboflavin. These results are of importance, partly as concerns the curing of ill neonates, and partly for reasons of economy. Here too we have compared the AOE activities and the LP in children treated with exchange transfusion, with blue light, with exchange transfusion + blue light, and with blue light + riboflavin. These examinations are also continuing.

The work in point (c) is common research with K. Barabás. This has been going on for about 5 years, and relates to the effect of GramoxonR (which contains paraquat as active ingredient) on mammals, fish and amphibia.

Table 3

Most important measured parameters of human diabetics and controls blood

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls</th>
<th>Diabetics</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose mg/100 ml blood mean±SD</td>
<td>61.5±10.4</td>
<td>210.2±64.7</td>
<td>-</td>
</tr>
<tr>
<td>Protein mg/ml plasma mean±SD</td>
<td>99.4±4.6</td>
<td>78.3±15.1</td>
<td>-</td>
</tr>
<tr>
<td>Protein kg/ml haemolysates mean±SD</td>
<td>589.8±131.5</td>
<td>501.2±172.9</td>
<td>-</td>
</tr>
<tr>
<td>G-Pase U/ml haemolysates mean±SD</td>
<td>6.43±0.65</td>
<td>16.45±0.58</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>LP nM MDA/ml haemol. mean ± SD</td>
<td>242.8±76.0</td>
<td>615.0±45.0</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>C-ase BU/ml haemolysate mean±SD</td>
<td>2.29±0.78</td>
<td>2.15±0.28</td>
<td>-</td>
</tr>
<tr>
<td>SOD U/ml haemolysates mean±SD</td>
<td>755.4±61.6</td>
<td>23.0±3.3</td>
<td>p &lt; 0.002</td>
</tr>
</tbody>
</table>
Enzyme activities and lipid peroxidations of organ homogenates from diabetic and control rats

<table>
<thead>
<tr>
<th>Organs</th>
<th>SOD U/g w.t.w. mean±S.D.</th>
<th>P-ase U/g w.t.w. mean±S.D.</th>
<th>C-ase BU/g w.t.w. mean±S.D.</th>
<th>LP nM MDA/g w.t.w. mean±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>4000±600</td>
<td>200±19</td>
<td>4.8±0.4</td>
<td>33.2±1.6</td>
</tr>
<tr>
<td>Kidney</td>
<td>200±51</td>
<td>272±36</td>
<td>0.4±0.04</td>
<td>22.4±1.8</td>
</tr>
<tr>
<td>Spleen</td>
<td>960±63</td>
<td>272±36</td>
<td>2.4±0.2</td>
<td>19.5±1.2</td>
</tr>
<tr>
<td>Testses</td>
<td>240±24</td>
<td>120±10</td>
<td>0.04±0.004</td>
<td>44.0±2.9</td>
</tr>
<tr>
<td>Whole brain</td>
<td>210±20</td>
<td>120±10</td>
<td>0.3±0.02</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>310±31</td>
<td>120±10</td>
<td>0.19±0.01</td>
<td>-</td>
</tr>
<tr>
<td>Pancreas</td>
<td>480±47</td>
<td>263±29</td>
<td>0.04±0.02</td>
<td>-</td>
</tr>
<tr>
<td>Heart muscle</td>
<td>300±27</td>
<td>223±30</td>
<td>0.11±0.01</td>
<td>-</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>696±68</td>
<td>525±50</td>
<td>2.67±0.31</td>
<td>-</td>
</tr>
</tbody>
</table>

*U/ml.
The fish AOE investigations were joint work, in part with J. Nemcsök et al., in the Department of Biochemistry of our University, and also with the team of Prof. Layko in the Department of Biophysics at the University of Lodz in Poland. I do not wish to go into details here on this, I presume that similar topics will be discussed in other lectures, or that we shall be able to talk about our results separately.

To turn to section (d), our results to date are presented in Tab. 3 and Tab. 4. They deal with the results of our human blood examinations (Tab. 3). These relate in part to experimental data.

Fig. 1. SOD activities in U/l and U/g protein units (means ±SD). Actual blood glucose levels during treatment: 1) 3.9-6.0 (n = 10); 2) 6.1-11.0 (n = 18); 3) above 11.0 mmol/I (n = 20). Period since diagnosis: A) 0-5 (n = 15); B) 5-10 (n = 17); C) above 10 years (n = 16). Therapy: a) insulin + diet (n = 22); B) oral antidiabetics, mainly of sulphonylurea type (n = 23); c) diet carbohydrate restriction (n = 3). Normal values relate to hemolysates of heparin-containing blood from the Blood Bank in Szeged (accuracy of enzyme determinations: ±5%).
Diabetes material (Tab. 4), these were rat experiments, where alloxan and streptozotocin were used as diabetogenic agents. From these it is quite clear that in both cases the AOE defence is lowered. If we take into account the newer results, which strikingly demonstrate the importance of oxygen radicals in experimental diabetes (alloxan → dialuric acid + O$_2^-$), Houé e-L o-v v i n et al. [5], Robins et al. [20], significance must be attributed to our human results connected with the decreased antioxidant defence. An example is provided here by our joint experiments with J. St r e n g e r [23]. Figure 1 shows the decrease in one of the AOEAs in parallel with the increase in the glucose concentration of the RBC haemolysates, also illustrated is the correlation between the time of diagnosis of the diabetes and the SOD activity of the haemolysate, together with the correlation between the diabetes treatment and the haemolysate SOD. Figure 2 presents the C-ase reactions as a function of the above conditions, and Fig. 3 the P-ase changes.

Figure 4 depicts the SOD decrease in human material as functions of the diagnosis and treatment time.
Fig. 3. P-ase activities. For notes, see Fig. 1
Aktywność P-azy. Objaśnienia jak do rys. 1

Fig. 4. SOD activities as a function of the period since diagnosis and commencement of treatment
Aktywność SOD w funkcji czasu, który upłynął od diagnozy i po- czątku doświadczenia
It should also be mentioned here that, in cooperation with oxygen radicals, the anthracycline antitumour agent adriamycin becomes cardiotoxic, with scavengers (DMSO and ascorbic acid) the antitumour effect remains unchanged, while this toxicity of the drug is diminished Maríán et al. [10].

Finally, I should like to express my thanks to our hosts, and particularly to Professor Krajewski and Leyko, for providing me with the opportunity to attend. My thanks are also due to all those who take part in our joint work, including those who may not have been mentioned by name.

REFERENCES


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NOWE WYNIKI BADAŃ WŁASNYCH DYSMUTAZY PONADTLENKOWEJ
I ZAMIERZENIA BADAWCZE

Prace podsumowują badania aktywności dysmutazy ponadtlenkowej
w przypadkach wybranych chorób dziedzicznych i cukrzycy oraz
molekularnych mechanizmów działania parakwatu w różnych warunkach, a
także D-penicilaminę i ryboflawynę w hiperbilirubinemiach.