
RADICALS, IONS AND TISSUE DAMAGE

Editors: B. Matkovics, L. Karmazsin, H. Kalász

Akadémiai Kiadó, Budapest

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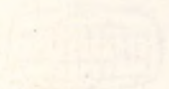
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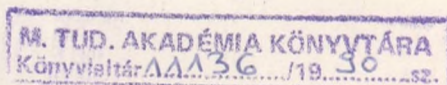
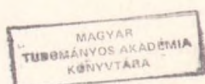
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PREFACE

On 12th-14th January 1989, we organized the Third Oxygen Radical Conference in Szeged, with the generous support of the Hungarian Chemical Society and its local Branch. 10 plenary lectures, 39 lectures and 12 posters were presented at the meeting. More than 100 Hungarian scientists participated, together with some invited guests and speakers from the German Democratic Republic. The participants came from different Medical and Veterinary Universities and Clinics, University Institutes, the Hungarian Academy of Sciences and other Research Groups. The main topics at the conference were the physiological and pathophysiological aspects of the reactions of oxygen radicals. Lectures were also presented on the importance of ions and trace metals. Another important topic related to the antioxidants (synthetic and natural, with low and high molecular weights). We also discussed the mechanism, measurement and pathologic role of lipid peroxidation.

The material of the conference provided appreciable new information at a high level, and gave added stimulus for further research on oxygen radicals. Such investigations demand close collaboration between physicians, biologists, biochemists, physicists, biophysicists, chemists, pharmacologists and other specialists.

Budapest, February 1990

The Editors

ACKNOWLEDGEMENTS

The conference in question was supported by the Hungarian Chemical Society (Budapest, Hungary), and assistance with this edition was provided by the Biogal Pharmaceutical Works (Debrecen, Hungary). We are grateful for their generous help.

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ROLE OF OXIDATIVE STRESS IN PLANTS ON THE DEVELOPMENT OF NECROSIS INDUCED BY PATHOGENS

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Plants often express resistance to pathogenic attack by a mechanism which involves dramatic metabolic changes in the cells at the site of challenge. It has been established that this hypersensitive response (HR) is accompanied by the necrotization of cells and concomitantly by the accumulation of low molecular mass compounds such as phenolics and phytoalexins, i.e. compounds with antibiotic activity (Király, 1986). Although the biochemical, physiological and cytological changes involved in the development of the necrotic lesions and also their connection with resistance have been intensively studied, the early key biochemical events triggering a cascade of subsequent reactions still remain obscure.

Consequently, these studies were initiated to determine whether oxygen free radical production is involved in the early responses that characterize hypersensitive tissue necrosis.

MATERIALS AND METHODS

Plants and pathogens

Plants (*Nicotiana tabacum* cv. Burley) were cultivated under normal greenhouse conditions. Half of the leaves were infiltrated, using a hypodermic syringe, with a bacterial suspension (10^8 cells ml^{-1}) of *Pseudomonas syringae* pv. *syringae* (Hri⁺) or Tn5 transposon mutant of *Pseudomonas syringae* pv. *phaseolicola*; the other half were infiltrated with water. The latter bacteria failed to induce hypersensitive necrosis (Hri⁻) (Somlyai et al., 1986). Later, every hour after bacterial infiltration, the leaf discs were vacuum infiltrated with one of the following solutions:

- (1) 0.01 M potassium phosphate buffer (pH 7.8);
- (2) 300 μM p-nitroblue tetrazolium chloride (NBT, Ega-Chemie, W-Germany, 10 mM NaN_3 and 0.1 mM EDTA dissolved in the same buffer;
- (3) 300 μM NBT 100 μg ml^{-1} SOD (Sigma, USA) and 0.1 mM EDTA. Fifteen minutes after treatment with solutions 1, 2 or 3, the discs were examined by light microscopy (Doke, 1983).

In order to detect necrosis at the cellular level, leaf discs were also vacuum infiltrated with a 0.25 % (w/v) solution of Evans blue at various intervals after bacterial infiltration, and then examined by light microscopy (Turner and Novacky, 1974). In some experiments, half of the leaves were treated prior to bacterial infiltration with α -tocopherol acetate, reduced glutathione (GSH), bovine serum albumin (BSA) or allopurinol (4-hydroxypyrazolo(3,4-d)pyrimidine). For further details of the experimental conditions, see the legends to the Figures.

The Ul strain of tobacco mosaic virus (TMV) was maintained in N. tabacum cv. Samsun plants. N. tabacum cv. Xanthi nc plants treated with different amounts of nitrogen in Hoagland's solution (Cheo et al., 1952) were mechanically inoculated with TMV. The number of local lesions was determined five days after inoculation. Eight days after TMV infection, inoculated and non-inoculated leaves were harvested for biochemical assays.

The potato tubers (Solanum tuberosum cv. Magyar Rózsa) used in the experiments were stored for 7 months in a cold room at 4 °C after harvesting. A core of tuber tissue (1.0 cm in diameter) was removed with a cylindrical stainless steel cutter, and slices of uniform thickness (2 mm) were cut. Slices were washed several times in distilled water and aged for 3 hrs at room temperature before treatment with a crude elicitor preparation. The elicitor preparation was obtained from the sonicated mycelium of the late blight fungus, Phytophthora infestans race 2.3.4. (Érsek et al., 1973), and carefully layered on the surface of slices (30 μ l per slice).

Extraction and quantitative determination of phospholipids

Lipids were extracted according to Folch et al. (1957) after boiling in isopropanol for 5 min. Separation by TLC on silica gel G plates (Merck, W-Germany) and quantitative determination of phospholipid molecular species by photodensitometry was performed as described earlier (Ádám and Nagy, 1989). The electrolyte leakage of leaf discs was determined by measuring the conductivity of the bathing solution (Ádám et al., 1987).

Measurement of lipid peroxidation

Thiobarbituric acid-reactive substrates (TBA-RS) were determined in 1 g samples homogenized in 2 ml ice-cold 0.01 M sodium phosphate buffer (pH 7.0) according to Lee and Csollany (1987). The malondialdehyde standard

was prepared by acidic hydrolysis of 1,1,3,3-tetraethoxypropane (Aldrich Chemie, W-Germany).

Determination of rishitin content of potato tuber tissue

Rishitin, the sesquiterpenoid stress metabolite, was extracted from potato slices according to the method of Henfling and Kuč^Y (1979) and quantified by gas chromatography (GC). GC was performed on a Packard 846 instrument fitted with a flame ionization detector. A 220 cm long glass column (3 mm inner diameter) packed with 3% OV 1 + 1.5% OV 210 + 1.5% OV 225 coated onto 80-100 mesh Supelcoport (Supelco, USA) was used. The internal standard was methyl stearate. The rishitin standard was kindly supplied by Dr. N. Doke (Nagoya University, Nagoya, Japan).

Preparation of microsomal membrane fraction from potato tubers

Discs from potato tubers (30-40 g) were homogenized with a commercial blender for 2 min in 100 ml of ice-cold 20 mM N-2-hydroxyethylpiperazine propane sulfonic acid (EPPS, Sigma, USA), pH 8.0, containing 0.5 M sucrose (Sigma, USA), 2 mM dithiothreitol and 1 mM EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged at 16,000 g for 20 min. The resulting supernatant was centrifuged at 105,000 g for one and a half hours with a Beckman L5-50B ultracentrifuge to obtain the microsomal membrane pellet. The pellet was resuspended in 2 mM EPPS, pH 8.5, to a protein concentration of 2 mg ml⁻¹ and was left to stand overnight at 4 °C. Protein was measured as described by Bradford (1976) with BSA as standard.

Measurement of free radical production by electron spin resonance spectroscopy (ESR)

Lyophilized potato slices were ground and packed into a sample tube. All samples (about 100 mg) were compressed with the same strength. The content of organic free radicals was determined with a JEOL JES FE 3X (x-band) ESR spectrometer. Spectra were recorded at 9.42 GHz and 1 mW power setting with a modulation amplitude of 4 gauss. The field set was 3350 gauss and the scan range 100 gauss. The amplitude of the ESR signal was used to express the organic free radical content of the sample.

To measure the amount of superoxide anions ($O_2^{\cdot-}$) produced by the microsomal membrane fraction, Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid, Sigma, USA), freshly prepared prior to each test, was brought to a final concentration of 10 mM in an incubation mixture containing 100 μ g microsomal membrane protein ml^{-1} in 85 mM EPPS, pH 8.5, in a final volume of 2 ml. Before ESR measurements, the samples were incubated for 10 min (Droillard et al., 1987). Tiron, a disulfonated catechol that is readily oxidized to the corresponding semiquinone (a more stable free radical) by $O_2^{\cdot-}$, has been proposed as a specific test for the presence of superoxide radical above pH 7.0 (McRae and Thompson, 1983; Ledenev et al., 1986). The tiron radical produced a 4-line ESR spectrum (Fig. 9), attributable to the interaction of the free electron derived from $O_2^{\cdot-}$ with 2 non-equivalent protons. The g value was 2.0052.

Determination of superoxide dismutase (SOD) activity: photochemical method

Leaf and tuber extracts were prepared by grinding 1 g tissues with a pestle and mortar in 5 ml 0.05 M K-phosphate buffer (pH 7.8), containing 0.1 mM EDTA and 1% (w/v) water-insoluble polyvinylpyrrolidone (PVP, Polyclar AT, Serva, W-Germany). After a 20-min centrifugation at 16,000 g at 4 °C, the supernatant was used for the determination of soluble SOD activity.

Measurement of SOD activity was carried out according to Dhindsa et al., (1981). To 1.5 ml 0.05 M potassium phosphate buffer (pH 7.8) containing 150 μ M NBT, 0.1 mM EDTA and 26 mM methionine, 1.5 ml of 8 μ M riboflavin (dissolved in the same buffer) and 5-100 μ l enzyme extract were added. The tubes containing the mixture were placed 15 cm below a 25 W fluorescent lamp for 6 min, during which time the reaction was found to be linear. The photochemical reduction of NBT was measured at 560 nm against a reagent blank kept in the dark. One SOD unit was defined as 50% inhibition of the basic rate of reaction (V) (McCord and Fridovich, 1969). However, to get a linear relationship between SOD activity (SOD units) and % inhibition of basic reaction rate, a V/v transformation (V = basic reaction rate without enzyme extract; v = reaction rate with extract) was performed (Monk et al., 1987).

Aliquots (50 μ l) of supernatants were used for the separation of SOD isoenzymes by gradient slab gel electrophoresis. The electrophoresis was

performed on a 4-20% acrylamide gel, using the buffer system of Davis (1964). The gels were then stained for SOD activity according to Beauchamp and Fridovich (1971). After colour development, the SOD activity was detected by photodensitometry at 550 nm (Shimadzu CS-930, Japan).

RESULTS AND DISCUSSION

Bacterially induced HR

To investigate the possibility that $O_2^{\cdot-}$ formation is involved in early responses that characterize hypersensitivity, the $O_2^{\cdot-}$ level (i.e. the number of cells showing NBT-reducing activity) was monitored during HR development (Fig. 1/a). A detectable increase in NBT reduction was observed about 3 h after infiltration of *P. syringae* pv. *syringae*. Infiltration with a transposon mutant of *P. syringae* pv. *phaseolicola*, which had lost the ability to induce HR (HRI^-) (Somlyai et al., 1986), did not result in an increase of NBT reduction. Treatment with SOD considerably decreased the accumulation of diformazan. Thus, the comparison of HRI^+ bacteria with HRI^- bacteria suggests that the loss of the HR-inducing capacity of the latter is accompanied by the loss of their ability to induce $O_2^{\cdot-}$ generation in plant cells. According to our results, the time course of the increase in number of dead cells was similar to that showing the NBT-reducing activity (Figs 1/a and 1/b). From this similarity, one can conclude that cells with an increased NBT-reducing activity (i.e. an increased $O_2^{\cdot-}$ generation) will die within a few hours. This suggests a direct or indirect involvement of $O_2^{\cdot-}$ in host cell death during bacterially induced HR. However, the biological sources of $O_2^{\cdot-}$ generation are only partially defined (Elstner, 1982). Recently, it has been hypothesized that xanthine oxidase-generated $O_2^{\cdot-}$ production is involved in the HR of French bean to rust infection (Montalbini and Buonauro, 1989). Application of allopurinol, a potent competitive inhibitor of xanthine oxidase in plants (Sandalio et al., 1988), did not delay the increase in membrane permeability during bacterially induced HR (Fig. 2). Since the increase in membrane permeability is due to increased oxyradical production and lipid peroxidation of membranes (Keppler and Novacky, 1987; Ádám et al., 1989), it is supposed that the xanthine/xanthine oxidase system does not play a role in enhanced $O_2^{\cdot-}$ generation during bacterially induced HR.

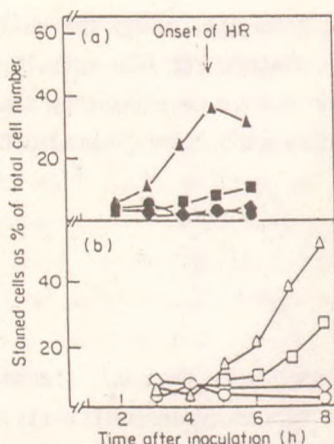


Fig. 1. Leaf discs (4 per treatment, 1 cm in diameter) of tobacco, cv. Burley 21, were stained with (a) 300 μM nitroblue tetrazolium (NBT) (dissolved in 0.01 M potassium phosphate buffer, pH 7.8) solution or (b) 0.25% (w/v) Evans blue solution. The leaf discs had earlier been infiltrated with water (●, ○), *Pseudomonas syringae* pv. *syringae* (10^8 cells ml^{-1}) (▲, △, ■), *Pseudomonas syringae* pv. *syringae* (10^8 cells ml^{-1}) plus SOD ($100 \mu\text{g ml}^{-1}$) (□), or the In5 transposon mutant of *Pseudomonas syringae* pv. *phaseolicola* (10^8 cells ml^{-1}) (◆, ◇) which had lost the capacity to induce HR (Somlyai et al., 1986). (▲, ◆, ●) NBT solution contained 0.1 mM EDTA and 10 mM $\text{Na}_2\text{S}_2\text{O}_3$. (■) NBT solution contained $100 \mu\text{g ml}^{-1}$ SOD, but without $\text{Na}_2\text{S}_2\text{O}_3$. Each value is a mean of two experiments. From Ádám et al., 1989.

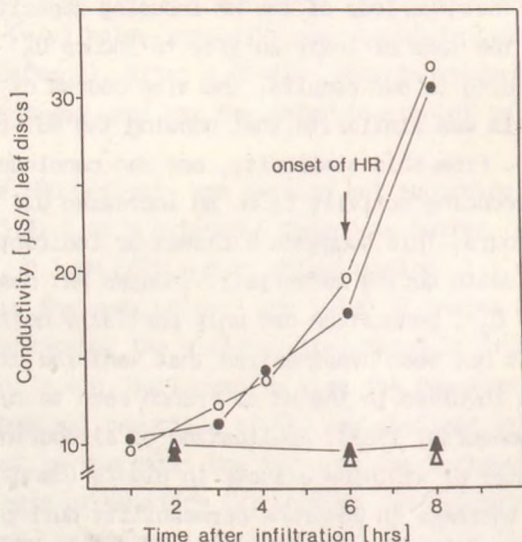


Fig. 2. The effect of treatment with 1 mM allopurinol on the electrolyte leakage of discs from tobacco leaves inoculated with *Pseudomonas syringae* pv. *syringae* (10^7 cells ml^{-1}). Half of the leaves were treated with allopurinol (open symbols), and the other half with K-phosphate buffer (0.01 M, pH 7.8) (closed symbols). One hour after treatment, the whole leaves were infiltrated with a bacterium suspension (○, ●) or with water (△, ▲).

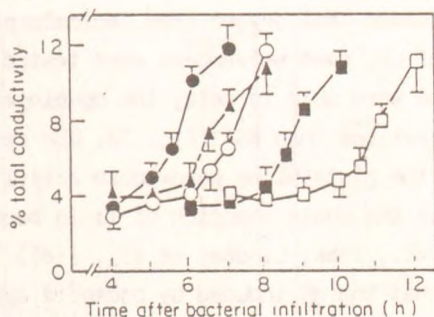


Fig. 3. The effects of GSH (100 mM), α -tocopherol acetate (0.75 mM) and BSA (0.5–1.0% w/v) treatments on the development of *Pseudomonas syringae* pv. *syringae*-induced HR. The time course of electrolyte leakage (6 discs per treatment, 1 cm in diameter) was measured after bacterial infiltration. In the case of BSA treatments, the bacterial concentration was 10^7 cells ml^{-1} ; otherwise, $5 \cdot 10^6$ cells ml^{-1} . Before bacterial infiltration, the leaves of tobacco, cv. Burley 21 had been treated with 0.05 M phosphate buffer, pH 7.8 (●); GSH (▲); α -tocopherol acetate (○): 0.5% BSA (■) or 1.0% BSA (□). The results are expressed as percentage total ion content (23.1 ± 1.4 mS g^{-1} d.w.). Values are the means of four replicates; vertical bars are means $\pm$ SE. From Adam et al., 1989.

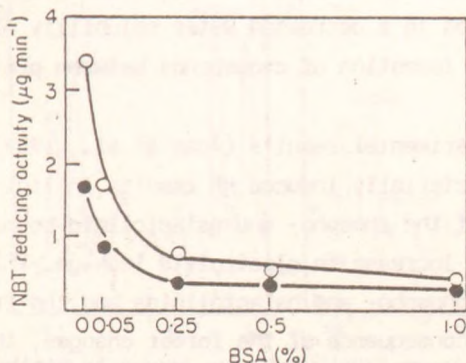


Fig. 4. The effects of different concentrations of BSA on the reducing activity of NBT in a non-biological $\text{O}_2^{\cdot-}$ -generating system. One and a half ml of 150 μM NBT in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA and 26 mM methionine, was mixed with 1.5 ml of 4 (●) or 8 (○) μM riboflavin in the same buffer containing various concentrations of albumin. NBT-reducing activity (i.e. formation of diformazan) was measured spectrophotometrically at 560 nm and expressed in $\mu\text{g min}^{-1}$ ($E_{560} = 28,600 \text{ M}^{-1}\text{cm}^{-1}$; Bors et al., 1982). Each value is a mean of four measurements.

To obtain more evidence that oxygen free radicals play an important role in the induction of HR, some scavengers were tested. We found that GSH and α -tocopherol acetate were able to delay the development of HR, but at lower bacterial concentrations than BSA (Fig. 3). GSH reacts mainly in an enzymatic way, through the glutathione peroxidase activity, while α -tocopherol directly inhibits the chain reaction of lipid peroxidation (Halliwell, 1982; Lielder et al., 1986; Lindsay et al., 1985). Király et al. (1977) earlier showed that the HR induced by bacteria appeared later, if the leaves had previously been treated with BSA. However, the possible mechanism was not explained. BSA treatment had no effect on the multiplication of bacteria. As albumin is known to inhibit various radical reactions in mammalian blood by sequestering metals (Halliwell and Gutteridge, 1986), we suggest that BSA may play a similar role in the inhibition of HR caused by bacteria (Fig. 3). The results presented in Fig. 4 provide evidence for the direct $O_2^{\cdot-}$ -scavenging effect of BSA in vitro. Experiments with the same $O_2^{\cdot-}$ -generating system (riboflavin-EDTA) in vivo (Ádám et al., 1989) support this conclusion. In addition, it was found that treatment of BSA with oxyradicals leads to a decreased water solubility of BSA, probably as a consequence of the formation of crossbonds between protein chains (Zs.-Nagy et al., 1981).

Our earlier experimental results (Ádám et al., 1989) suggest that $O_2^{\cdot-}$ generation during bacterially induced HR results in lipid peroxidation, increased saturation of the phospho- and galactolipid-bound fatty acids occurring before the increase in electrolyte leakage. This, in turn, leads to decreases in the phospho- and galactolipids and the free sterol level of the membranes. As a consequence of the former changes, the loss of membrane integrity results in an increased membrane permeability and finally in the development of tissue necrosis.

Virus-induced HR

Treatment of tobacco plants with a high amount of nitrogen resulted in a massive accumulation of phospho- and galactolipids and in the increased activity of SOD (Fig. 5 and Table 1). The number of local lesions developing after TMV infection decreased with increasing nitrogen supply (Table 1). The appearance of local lesions after TMV infection caused a much higher lipid decomposition in nitrogen-deficient plants (70 mg kg^{-1}) than in plants treated with a high dose of nitrogen (630 mg kg^{-1}) (Fig. 6). Near

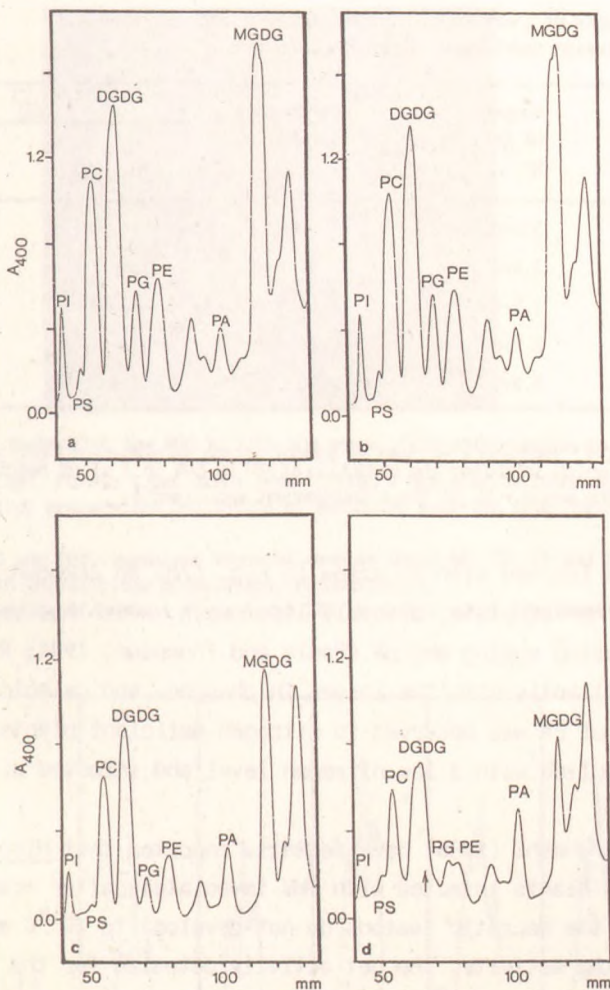


Fig. 5. Densitometric patterns of phospho- and galactolipid composition of *Nicotiana tabacum* cv. Xanthi nc leaves in relation to TMV infection and nitrogen supply. Plants were supplied with Hoagland's nutrient solution containing either 630 mg kg^{-1} (a and b) or 70 mg kg^{-1} (c and d) nitrogen throughout the experiment, and infected with TMV (b and d) or sham-inoculated with buffer (a and c) 8 days before harvest.

PI, phosphatidylinositol;

PC, phosphatidylcholine;

DGDG, digalactosyldiglyceride;

PG, phosphatidylglycerol;

PE, phosphatidylethanolamine;

PA, phosphatidic acid;

MGDG, monogalactosyldiglyceride.

Table 1 The effects of increasing nitrogen supply on SOD activity and on the lesion development after TMV infection

Nitrogen level ⁻¹ mg kg	Number of lesions ⁻² cm	Percentage inhibition	SOD ¹	
			EU g ⁻¹ fresh wt	EU mg ⁻¹ protein
70	5.6±0.3	0.0	141±18	19.0±2.8
284	3.5±0.2	37.5	208±21	17.2±1.4
630	2.5±0.2	55.4	246±16	25.4±1.6
1050	2.2±0.3	60.8	340±12	28.1±1.9
1350	2.8±0.4	50.3	423±22	29.3±1.8

¹Data expressed as enzyme units (EU), where one unit of SOD was defined as the amount of enzyme extract which inhibited the reduction of NBT by 50% in 3 ml of reaction mixture. Data presented are the means + SE of three independent experiments.

the DGDG peak (marked with an arrow in Fig. 6/d) an unknown reddish material appeared on the TLC plate, probably lipofuscin, which has been reported to accumulate during ageing and HR (Pauls and Thompson, 1984; Rogers et al., 1988). Concomitantly with the losses in phospho- and galactolipids, the accumulation of PA was observed in nitrogen-deficient plants (Fig. 6/c) and in plants supplied with a low nitrogen level and infected with TMV (Fig. 6/d).

Doke and Ohashi (1988) have recently reported that Nicotiana tabacum cv. Samsun NN plants infected with TMV immediately after transfer from 30 °C (where the necrotic lesions do not develop) to 20 °C showed a marked O₂^{•-}-generating activity. The net activity detected for the initial 10 min after the transfer to 20 °C correlated significantly with the number of later occurring necrotic lesions. The infiltration of TMV-infected Samsun NN leaves with SOD or catalase caused a reduction in necrotic lesions following transfer from 30 to 20 °C. These results support our proposal that the SOD activity changes in plants supplied with various amounts of nitrogen could influence the number of local lesions formed after TMV infection (Table 1). The role of catalase in lesion reduction could also be hypothesized, since a decrease in catalase activity coincided with a decrease in SOD activity during the natural ageing of tobacco plants (Dhindsa et al., 1981).

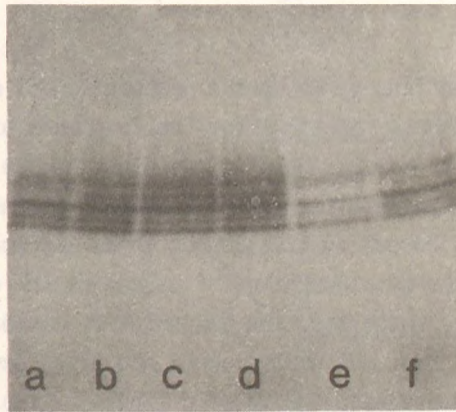


Fig. 6. Separation of SOD isoenzymes of potato tubers by PAGE on a gradient (4-20%) slab gel stained with NBT. Potato tuber discs were treated 3 hrs after slicing with water or with an elicitor preparation consisting of sonicated mycelium from *Phytophthora infestans*.
 (a), (b), (c) and (d), isoenzyme bands of samples taken 48, 72, 96 and 120 hrs after treatment with the elicitor preparation, respectively;
 (e) and (f), bands of control discs 0 and 120 hrs after water treatment, respectively.

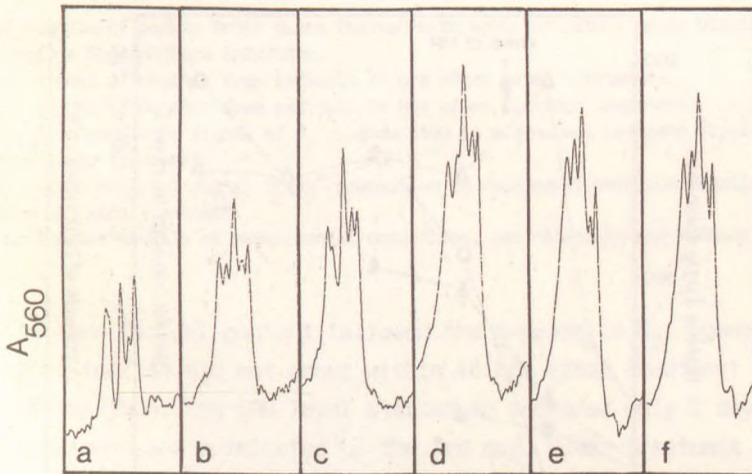


Fig. 7. Desitometric patterns of SOD isoenzymes separated by gel electrophoresis (See Fig. 6).
 (a) and (b) are samples from control discs, 0 and 120 hrs after treatment with water, respectively;
 (c), (d), (e) and (f), samples from discs 48, 72, 96 and 120 hrs after treatment with elicitor preparation, respectively.

Instead of a live fungus, we used a preparation of mycelial homogenate (elicitor) of *Phytophthora infestans* for the treatment of potato tuber slices for two reasons. First, the elicitor gives a more consistent and uniform response than inoculation with a zoospore suspension; second, it mimics the natural HR caused by live fungus in an incompatible host-parasite interaction. During the development of HR caused by the elicitor preparation from *Phytophthora infestans*, the accumulation of TBA-reactive substrates (expressed in nmol MDA equivalents) and of a sesquiterpenoid stress metabolite, rishitin, and also the level of organic free radicals were monitored as a function of time (Figs 8 and 9). The $O_2^{\cdot-}$ -generating activity of the microsomal membrane fraction and the level of SOD activity were also measured (Figs 6, 7 and 8). As early as 12 hrs after treatment with the elicitor preparation, an increase in $O_2^{\cdot-}$ -generation was found, which coincided with a burst of lipid peroxidation (Fig. 8). An enhancement of

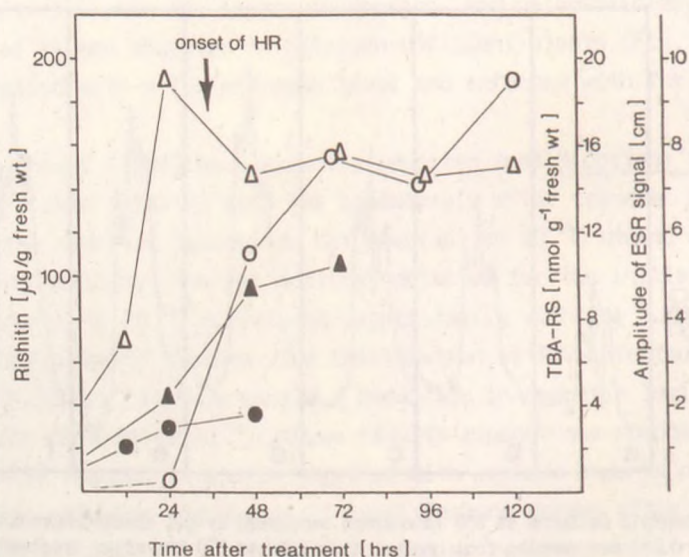


Fig. 8. The effects of treatment of tuber slices of potato cv. Magyar Rózsa with a crude elicitor preparation of *Phytophthora infestans* race 2.3.4 on the accumulation of rishitin (○) of TBA-reactive substrate (TBA-RS) (Δ) and of organic free radicals (▲) and on the $O_2^{\cdot-}$ -generating activity of the microsomal membrane fraction (●). The free radical contents of samples were characterized by the amplitudes of the ESR signals. (For further details, see Materials and Methods.)

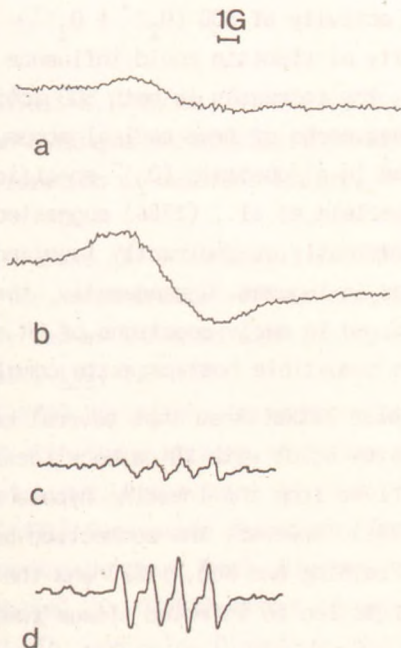


Fig. 9. ESR signals of potato tuber discs treated with water or with a crude elicitor preparation from *Phytophthora infestans*.

- (a), signal of organic free radicals 24 hrs after water treatment:
 - (b), signal of organic free radicals 24 hrs after elicitor treatment:
 - (c), Tiron-mediated signal of $O_2^{\cdot-}$ production in microsomal membrane fraction, 24 hrs after water treatment:
 - (d), Tiron-mediated signal of $O_2^{\cdot-}$ production in microsomal membrane fraction 24 hrs after elicitor treatment.
- (For further details of experimental conditions, see Materials and Methods.)

the organic free radical content followed the increase in $O_2^{\cdot-}$ generation, but a further increase did not occur within 48 hrs after treatment with the elicitor preparation. The SOD level started to increase only 2 days after elicitor treatment and culminated by the 3rd day. Water treatment (i.e. naturally occurring ageing) caused little increase in SOD activity by the 5th day (Figs 6 and 7). The first signs of the HR became visible to the eye about 30-36 hrs after treatment (Fig. 8). Phytoalexin (rishitin) accumulation started only 24 hrs after elicitor treatment and showed an increasing tendency throughout the experiment (Fig. 8).

These results suggest that rishitin accumulation and the increase in SOD activity take place only after the free radical generation and after the visual onset of HR (i.e. necrotization). Thus, we suppose that neither

the $O_2^{\cdot-}$ -decomposing activity of SOD ($O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$) nor the antibiotic activity of rishitin could influence the early steps of HR elicitation. Even so, the increases in both SOD activity and rishitin content could be consequences of free radical accumulation or/and damage. SOD could be activated in a substrate ($O_2^{\cdot-}$ -specific manner (McCord and Fridovich, 1969). Epperleim et al., (1986) suggested that hydroxyl ($OH^{\cdot}$) radical formation is directly or indirectly involved in the abiotic elicitation of phytoalexins in legumes. Consequently, the increased $O_2^{\cdot-}$ generation seems to be involved in early reactions of HR elicitation, which was found to be absent in compatible host-parasite complexes of Phytophthora (Doke, 1983). It was also established that several compounds, i.e. glucans and polyunsaturated fatty acids with 20 carbons (arachidonic and eicosapentaenoic acids) derived from the invading hyphae of Phytophthora, elicit HR (Bostock et al., 1981). However, the connection between the metabolism of arachidonic acid (Preisling and Kuč, 1988) and the $O_2^{\cdot-}$ -generating activity of the microsomal fraction in infected tissue remains to be elucidated in the future.

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ROLE OF FREE RADICALS IN THE DEVELOPMENT OF PNEUMOCYTE PROLIFERATION

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Following the administration of paraquat (PQ) and phytohaemagglutinin (PHA), the histological picture of characteristic "paraquat lung" changed and marked perivascular and peribronchial cell proliferation could be observed. To decide whether or not the presence of oxygen free radicals is necessary in the induction of the above process, two compounds were examined. One of them, CdCl₂ has a pulmonary fibrosis-inducing effect independent of free radical generation, while the other compound, Bleomycin (BLEO), exerts its effects through the production of free radicals. The pulmonotoxic effect of CdCl₂ was only slightly influenced by PHA, while the combined administration of BLEO and PHA resulted in perivascular and peribronchial cell infiltration, though this was less than the reaction caused by PQ + PHA.

During studies of the pulmonary fibrosis-inducing effect of xenobiotics, it was found that the pathological picture of pulmonary fibrosis

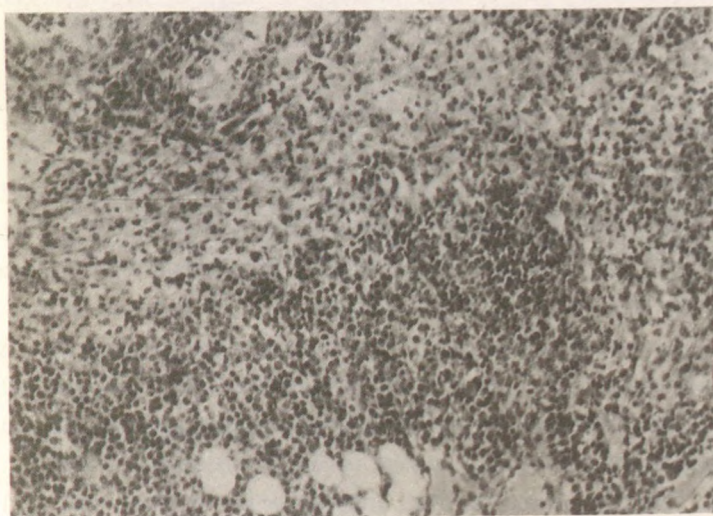


Photo 1. Light micrograph of lung of PQ and PHA-treated mouse. (Original magnification x 170)

caused by Gramoxone (a herbicide with an oxygen radical-generating ingredient paraquat (PQ) can be changed by the administration of phytohaemagglutinin (PHA) (Photo 1)^{1,2,8,9}.

This observation raised the question of whether or not the presence of oxygen radicals is necessary in the induction of the above process, or whether the pathomechanism comes about without the triggering effect of oxygen radicals. In order to settle this question, a compound (CdCl_2) toxic to the lung by inducing pulmonary change not via the production of free radicals, and an oxygen radical-generating compound, Bleomycin (BLEO) were chosen^{4,5,7,8}.

In the study, the additive effect of PHA was used as a differentiating factor.

In the pulmonary process is independent of oxygen radical production, CdCl_2 and PHA can also induce the atypical cell proliferation described in the case of PQ + PHA. To confirm this assumption, the effect of combined treatment with BLEO + PHA was studied as a positive control.

MATERIAL AND METHODS

Adult male and female CFLP mice (28.6 ± 1.2 g) free of any respiratory infection were used. They were maintained on standard laboratory chow and water ad libitum. The mice were injected once i.p. with 5 mg/kg of CdCl_2 . BLEO ("Bleocin", Nipon Kayoku Co.) was administered i.p. in a dose of 0.004 mg/mouse, that is 2.7×10^{-3} mol/mouse, as suggested by Hakkinen³.

On the basis of the results of Dubaybo², PHA was administered on the 7th day, in a dose of 30 mg/kg. On the 21st day, after cervical dislocation, the lung weight was measured and the relative weight of the lungs was calculated. Tissues for histological analyses were paraffin-embedded and H-E-stained.

RESULTS

The doses of the compounds administered did not cause mortality. Histological sections prepared after dissection on the 21st day proved that CdCl_2 had a toxic effect on the lungs. Oedema and haemorrhage could be observed three days after the administration of 5 mg/kg CdCl_2 . The development of a pulmonary process was indicated by the increase in the lung mass/body mass ratio. Upon combined treatment with PHA + CdCl_2 , there was a significant increase in the relative lung weight as well (Fig. 1), though no serious histological change could be observed (Photo 2). The histological section showed dilated interalveolar regions and cell proliferation.

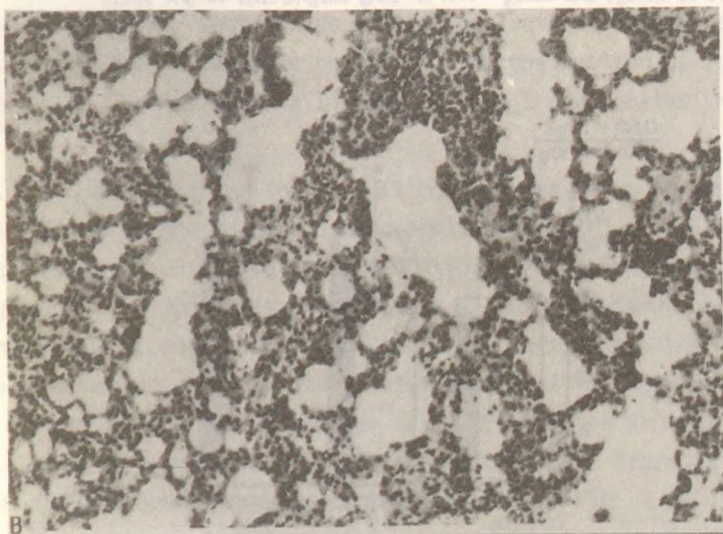
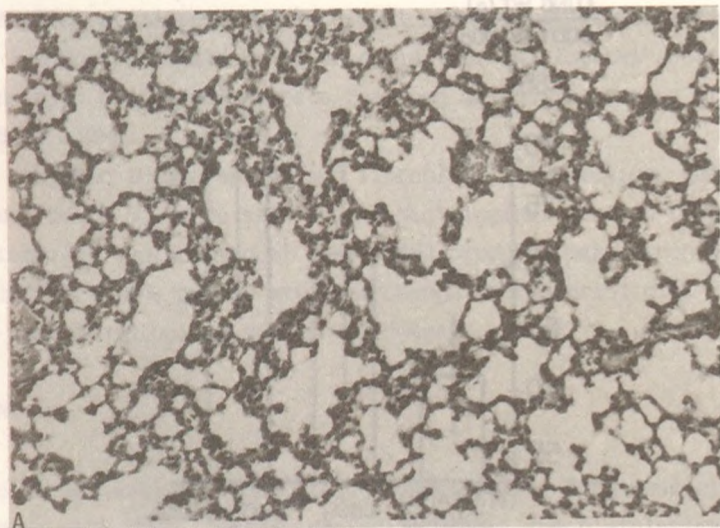


Photo 2. Light micrographs of lungs of

(a) CdCl_2 and

(b) CdCl_2 + PHA treated mice. $\times 170$

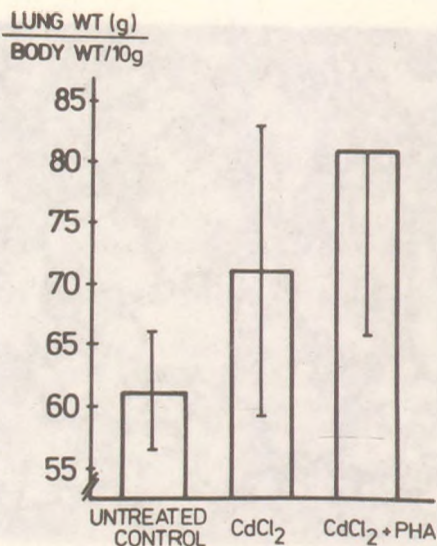


Fig. 1. Effect of CdCl₂; and CdCl₂ + PHA on lung weight/body weight ratio

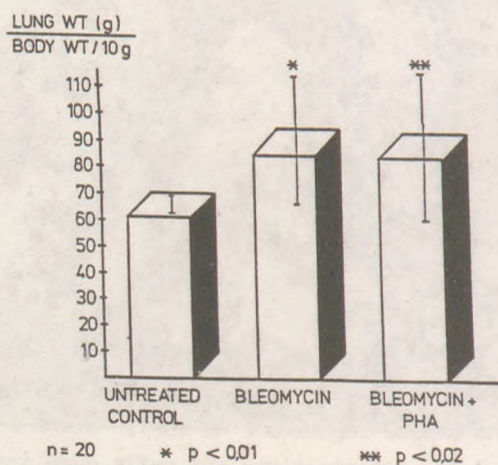


Fig. 2. Effect of Bleomycin; and BLEO + PHA on lung weight/body weight ratio

PHA applied alone resulted in slight changes: scattered inflammatory cells and lymphocytes could be observed in the histological section (Photo 3).

The data in Fig. 2 demonstrate the lung weight-increasing effects of BLEO nad BLEO + PHA.

BLEO resulted in macrophage and fibroblast accumulation, and scattered inflammatory cells could be seen in the histological section (Photo 4).

The combined application of BLEO + PHA produced peribronchial and perivascular infiltration, but no atypical change characteristic of PQ + PHA developed (cf. histological section) (Photos 5 and 6).

DISCUSSION

It could be concluded that, though the relative lung mass of the mouse group treated with CdCl_2 increased significantly, the histological picture proved that PHA did not aggravate the toxic effect of CdCl_2 , as had been observed in combined treatment with PQ. It may be presumed that the presence of $\text{O}_2^{\cdot-}$ and $\text{OH}^{\cdot-}$ radicals as initiative factors is necessary. The production of these free radicals results in the destruction of type II pneumocytes in the case of PQ and in that of type I pneumocytes in the case of BLEO^{5,9}.

After the destruction of the type II pneumocytes, a macrophage-T lymphocyte activation and an unregulated proliferation of alveolar epithelium developed, which became even more atypical upon PHA treatment by activating helper T lymphocytes. In the case of BLEO treatment, the destruction of the mature alveolar epithelium and macrophage activation was followed by a compensated and regulated proliferation of the type II pneumocytes and a lower T cell and macrophage activation, which finally led to fibroblast - fibrocyte conversion and the development of fibrosis (Fig. 3).

Though the presumed mechanism of action shown in Fig. 3 is a mechanism which triggers a process identical with oxygen radical production in the cases of BLEO and PQ, since the result was fibrosis in both cases, there were some differences in the pathomechanism process. A better understanding of this process will be based upon clarification of the impact of oxygen radicals on the immune function of the lungs.

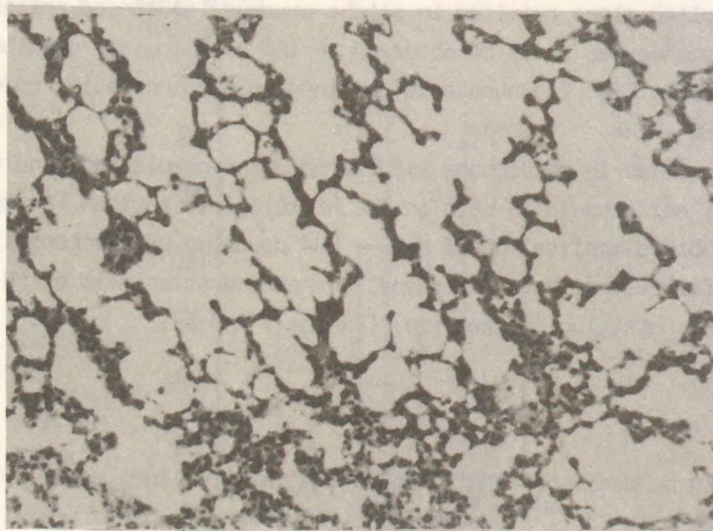


Photo 3. Light micrograph of lung of PHA-treated mouse. x 170

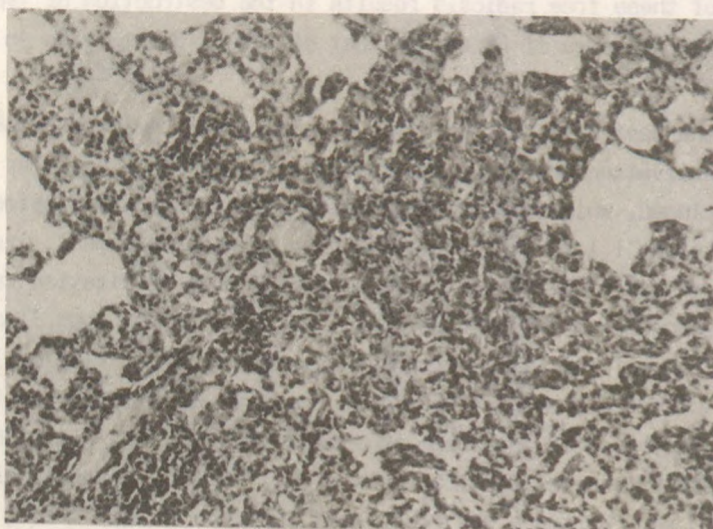


Photo 4. Light micrograph of lung of BLEO-treated mouse. x 170

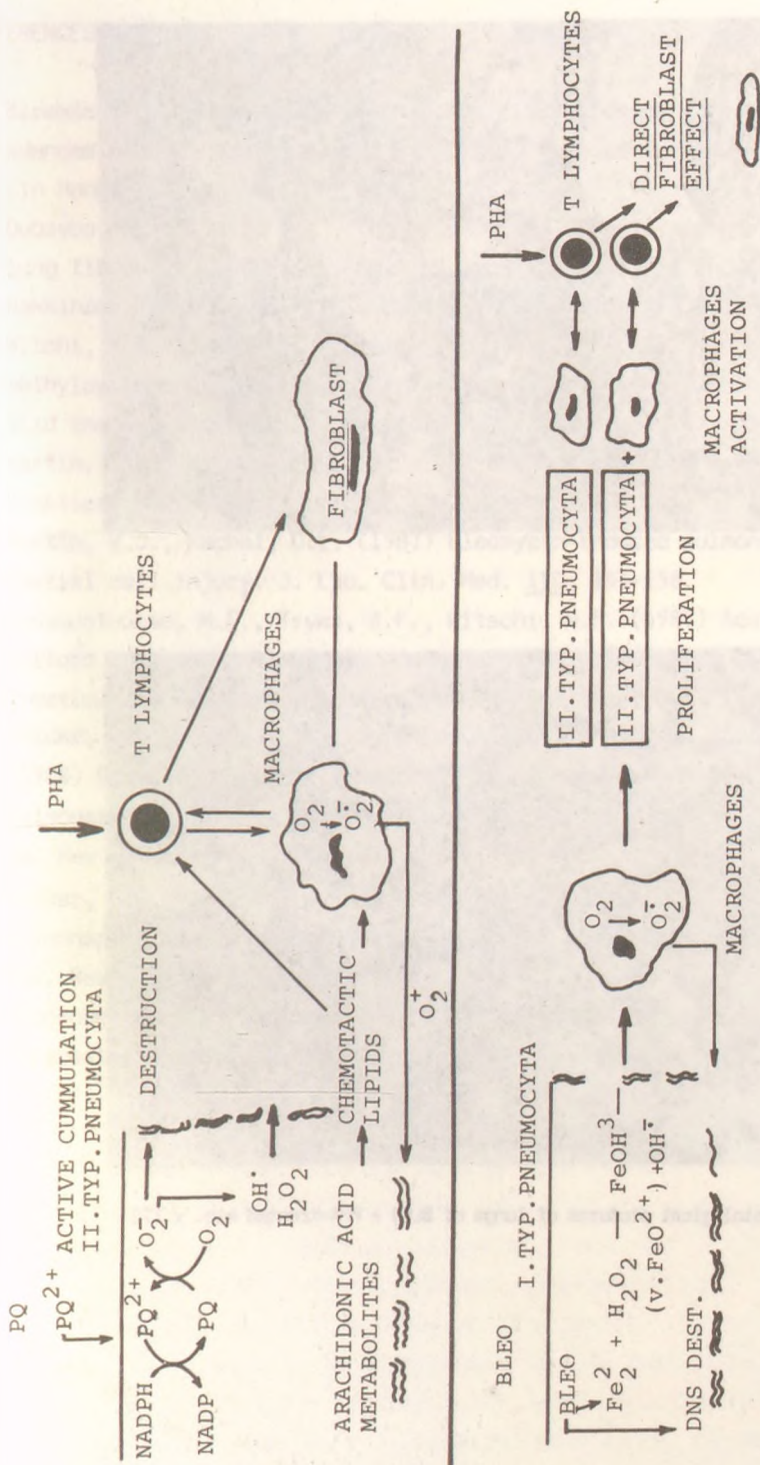


Fig. 3. Hypothetical pathways of fibrosis

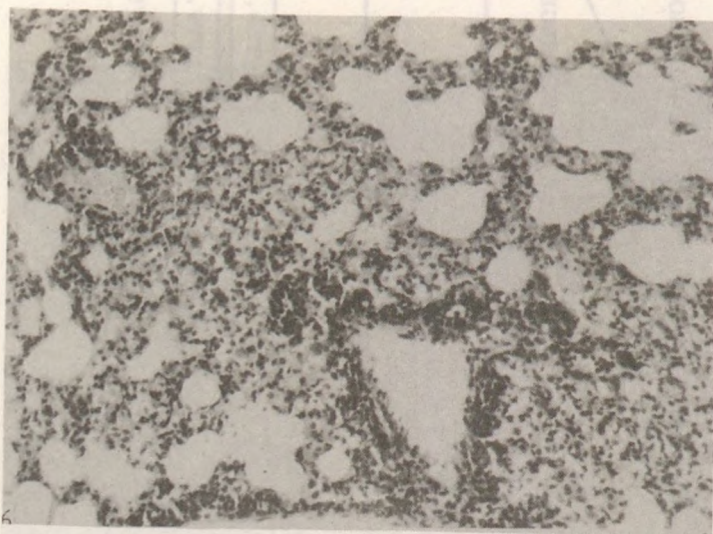
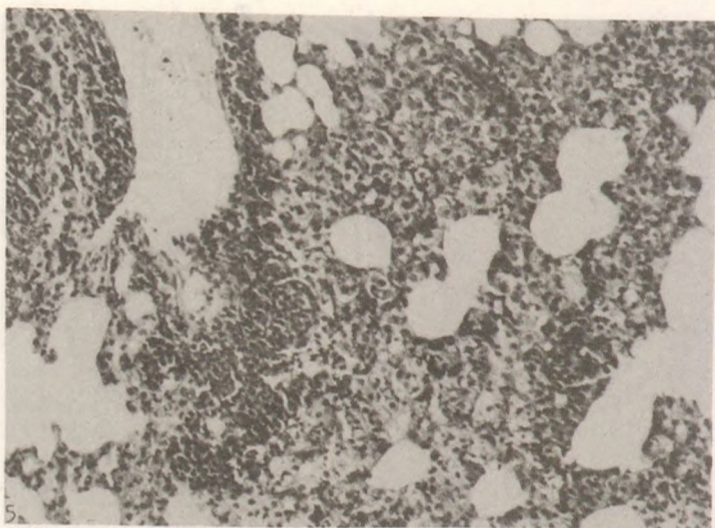


Photo 5 and 6. Histological pictures of lungs of BLEO + PHA-treated mic. x 170

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MODIFIED LIPOPROTEINS

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THE SIGNIFICANCE OF MODIFIED LIPOPROTEINS

Modified lipoproteins are relevant to the early stages of atherogenesis. It is well known that the fatty streak lesions of atherosclerosis contain foam cells. These cells are rich in cholesteryl ester and are thought to be formed from tissue macrophages (derived from circulating monocytes) by uptake of the major carrier of cholesteryl ester in the plasma low density lipoprotein (LDL). However, there are two major lines of evidence indicating that this uptake does not occur via the LDL receptor:

1. Patients with homozygous familial hypercholesterolaemia (FH) and Watanabe heritable hyperlipidaemic (WHHL) rabbits, although both lack LDL receptors, still develop lesions containing foam cells.
2. Incubation of monocytes or macrophages with native LDL in vitro does not result in foam cell formation.

It has therefore been suggested that some form of modified LDL may be responsible for the cellular accumulation of cholesteryl ester via a route other than the native LDL (B/E) receptor. Two such modifications are oxidised LDL and acetyl LDL, which are taken up by specific "scavenger" receptors on macrophages, leading to foam cell formation in vitro.

Other forms of modification which have been found to increase LDL uptake into macrophages include glycolysation, complexes of LDL with macromolecules such as proteoglycans, and self-aggregates of LDL.

Interestingly, unmodified beta-very low density lipoprotein (beta-VLDL) can also be taken up by macrophages to form foam cells, and this occurs via the B/E receptor. Beta-VLDL is found physiologically in cholesterol-fed animals, but the only modified lipoprotein for which there is

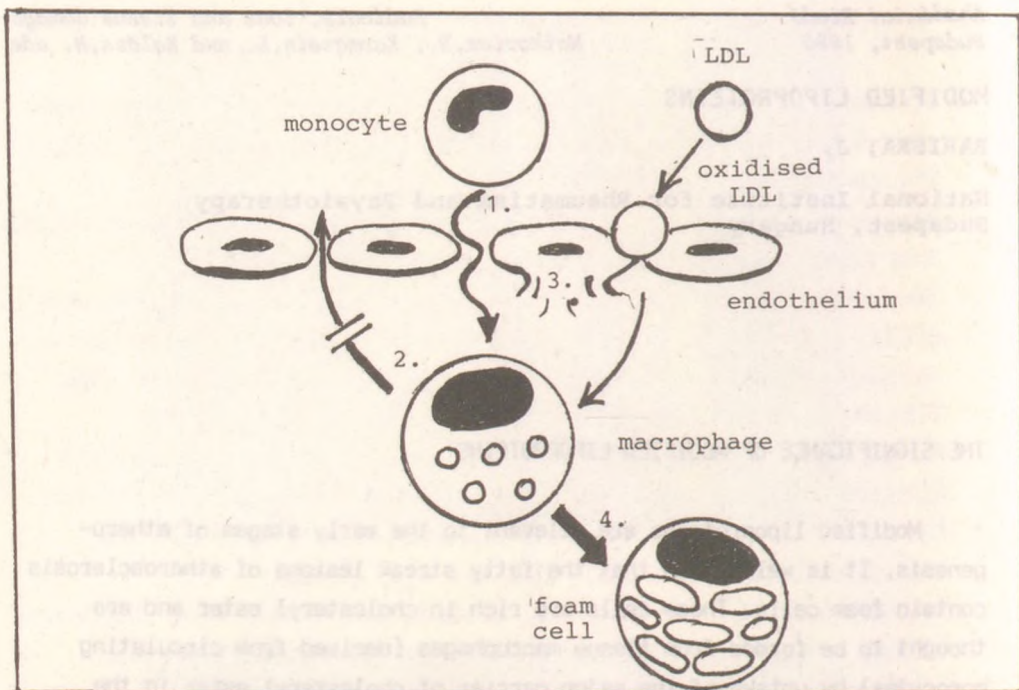


Fig. 1. Possible effects of oxidised LDL as related to atherogenesis.

1. Stimulation of monocyte chemotaxis.
2. Inhibition of macrophage mobility.
3. Endothelial cell toxicity.
4. Foam cell formation.

evidence of natural occurrence is oxidised LDL. This fact, together with other features which would favour atherogenesis (Fig. 1), suggests that oxidised LDL is certainly important in the initiation and progression of atherosclerosis.

MECHANISMS OF OXIDATIVE MODIFICATION

In the opinion of Parthasarathy, lipoxygenase may be important in the oxidative modification of LDL by cells.

A number of cells, including endothelial and smooth muscle cells, monocytes, macrophages and even fibroblasts, have been shown to oxidise LDL in vitro. It has been suggested that this effect is mediated by superoxide radicals, but Parthasarathy has found that superoxide dismutase, which engulfs these radicals, does not inhibit the effect. In addition, when

superoxide radicals are generated by way of xanthine oxidase, this does not increase oxidation of the lipoprotein. Furthermore, the action of the superoxide radicals would be expected to be non-selective and yet high density lipoprotein (HDL) is not oxidised by endothelial cells.

However, inhibitors of lipoxxygenase such as piriprost eicosatetraenoic acid have been found to inhibit the cell-mediated oxidation of LDL. Lipoxxygenase is thought to generate lecithin, which is converted to lysolecithin and oxidised fatty acids by phospholipase A₂. It is these oxidised fatty acids which are believed to cause oxidative modification of LDL. Despite these findings, Parthasarathy warns that this may not be the end of the superoxide story, since oxygen radicals are thought to be generated during the lipoxxygenase reaction itself.

AUTO-OXIDATION AND CELL INJURY

Sevanian presented what he claimed was a "slightly different view" of the mechanism of atherogenesis, focusing on the cytotoxicity of cholesterol oxides and the mechanisms by which cholesterol becomes oxidised via free radicals.

Cholesterol is often regarded as a chemically inert material, but while this may be so in vitro, it appears not to be the case in vivo. Cholesterol oxides are known to exhibit a variety of effects on cell membranes and to interfere with cholesterol synthesis. Sevanian's evidence of their cytotoxic effects suggests that it is the level of cholesterol oxidation rather than cholesterol itself which is important in atherogenesis, particularly α - and β -cholesterol epoxides and cholestanetriol.

Free radical formation produces cholesterol epoxides and a series of ketone and alcohol derivatives based around carbon-7. Cholestanetriol appears to be particularly toxic. At levels as low as 10 μ M, it can cause retraction of confluent endothelial cells with loss of their communication and metabolic cooperation.

Experiments on New Zealand white rabbits made either hypertensive, hyperlipidaemic (by cholesterol feeding) or both, show that the severity of hypertension and hyperlipidaemia in rabbits exposed to these conditions was better correlated with the proportion of cholesterol oxides in serum cholesterol than with cholesterol itself, and that even in hyperlipidaemic/normotensive rabbits there was a marked increase in levels of cholesterol oxides in the serum and in the aortic arch lipid, while the cholesterol

levels stayed within normal limits. Cholesterol, epoxides and cholestane-triol were particularly elevated.

LDL MODIFICATION BY MALONDIALDEHYDE

There is evidence that LDL modification by the lipid peroxidation product malondialdehyde occurs in vivo.

Haberland and her colleagues, using monoclonal antibodies raised against both the LDL-receptor binding site on apolipoprotein B-100 (ApoB-100) and the malondialdehyde-linked lysines on modified LDL, have demonstrated that there is focal deposition of malondialdehyde-modified protein in both early and late atheromatous lesions in the thoracic aorta of the Watanabe heritable hyperlipidaemic (WHHL) rabbit, the animal model for familial hypercholesterolaemia (FH).

The two antibodies appear to bind to the same regions of the intima, although the technique uses adjacent arterial sections. Specifically, they appear in the elastic lamina and the smooth muscle layers. This is evidence that LDL bound to endothelium can become modified. There is no detectable amount of malondialdehyde-modified LDL in the plasma in these animals, suggesting that the release of malondialdehyde in vivo may be restricted to the arterial wall.

Similar experiments on normolipidaemic white rabbits demonstrated only slight aortic staining for malondialdehyde protein or ApoB-100. Since Watanabe rabbits lack the LDL receptor, the suggestion is that the endothelium-bound LDL can become modified by a physiological product of lipid peroxidation and later produces cholesteryl ester deposition in macrophage-derived foam cells of the atherosclerotic lesion.

Haberland's monoclonal antibody preparation against malondialdehyde-modified LDL appears to be specific for the cross-linked lysines, as there is cross-reactivity between modified LDL (or other proteins) and native or otherwise oxidised LDL, HDL, albumin or gamma-globulin.

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MEASUREMENT OF URINARY CAFFEINE METABOLITES REFLECTING
THE IN VIVO XANTHINE OXIDASE ACTIVITY IN PREMATURE INFANTS
WITH RDS AND IN HYPOXIC STATES OF CHILDREN

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Grant et al. have demonstrated that after caffeine intake the ratio of 1-methyluric acid (MU) and 1-methylxanthine (MX) in the urine indicates the total xanthine oxidase (XO) activity of the organism. Application of this method to premature infants with severe respiration distress syndrome (17 cases), to children in need of intensive care (18 cases) in the critical phase and during the reparation of the same patients, to shock-tolerant patients (6 cases) and to healthy children (15 cases) revealed that the total XO activity can increase extremely in severe acute clinical states, whereas a considerable decrease can be detected during reparation, while in shock-tolerant states it can become markedly low.

The investigations explain the favourable XO-inhibiting effect of allopurinol, demonstrated in states of experimental and clinical shock. The low XO activity observed in shock-tolerant conditions may be a result of intrinsic compensatory mechanisms.

INTRODUCTION

The interest of recent investigations has recently focused on two features of the reaction of XO converting hypoxanthine-xanthine into uric acid. One of these features is the great variability in the activity of the enzyme, depending on the circumstances; it is regarded as one of the characteristic adaptive enzymes. The other feature is that, during the reaction of the 'O' form of the enzyme, four O_2^- free radicals are formed during the oxidation of one molecule of substrate. It has been proved by numerous investigations that, during the prurine catabolism accompanying conditions of hypoxia and shock the superoxide generated is the most important factor responsible for the ischemic reperfusion injury damaging various organs (3, 5). Experimental data and investigations on human subjects have demonstrated that specific XO inhibition by allopurinol moderates, the severity of shock, increases the tolerance to hypoxia, prolongs the survival time of transplants and enhances the protection against active O_2 radicals (1, 2, 5).

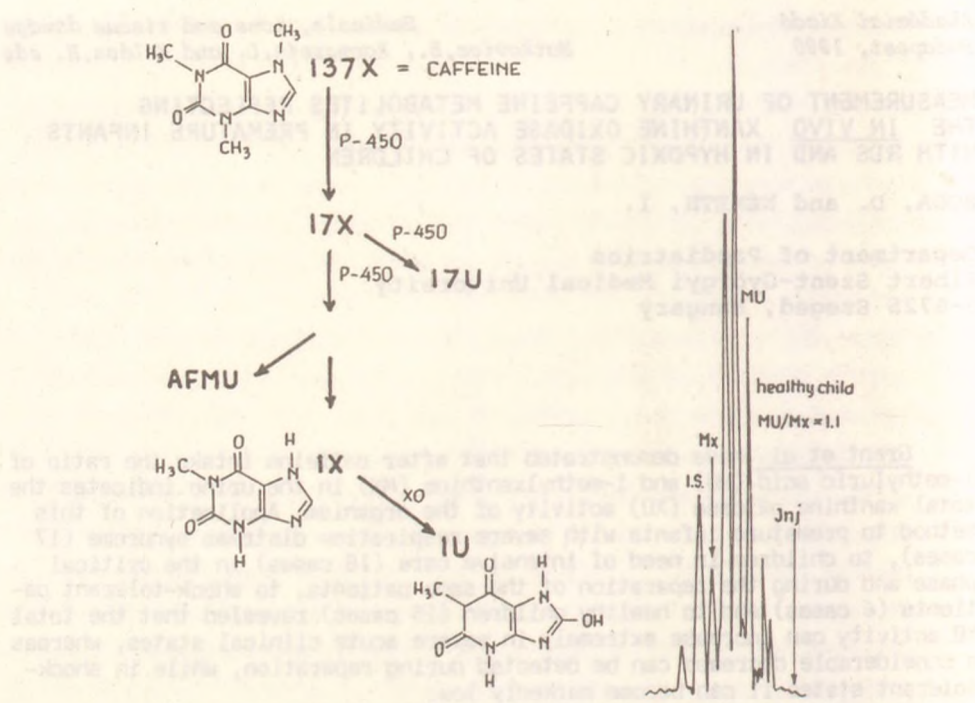


Fig. 1. Caffeine metabolism and XO activity in humans (4)

It was recently proved that, following caffeine intake, the ratio of 1-methyluric acid (MU) and 1-methylxanthine (MX) in the urine indicates the total XO activity (Fig. 1) (4).

This new method has given us the possibility to follow the enzyme activity in clinical practice, and thus the role of XO in pathology and the basis of treatment based on enzyme inhibition can be investigated in an objective way.

MATERIAL AND METHODS

Our investigations have been performed on the following groups of patients: On 17 newborns undergoing intensive care, suffering from respiration distress syndrome (RDS) and in need of high concentration oxygen treatment.

On 19 patients treated in the paediatric intensive care unit because of various severe basal diseases, during the critical phase and during the reparation period of the same patients.

On 6 patients in a shock-tolerant state. This phenomenon, which is well known in experimental pathology, has been encountered during clinical treatment as well. Anergy is characteristic in these cases. In spite of their poor condition, the patients tolerate various repeated surgical interventions, sepsis, etc. surprisingly well.

On 15 children of various ages in need of a hospital check-up but otherwise healthy.

During the caffeine test according to Grant et al. (4), the patients were given 10-20 mg/kg caffeine parenterally or orally according to their condition, and the MU/MX ratio was measured in the urine 4 hours later. Immediately after collection, the urine samples were adjusted to pH 3.5 and were stored at -20°C until analysis. The molar ratio MU/MX was calculated via high pressure liquid-chromatography on 0.2-0.4 ml urine samples. 0.15 ml of internal standard was added (4-amino-antipyrine dissolved in chloroform 120 mg/ml) the solution was extracted with 1 ml of a mixture of chloroform: isopropyl alcohol (9:1), the extract was evaporated in a nitrogen current, the samples were transferred to a mobile phase containing phosphate buffer: methanol (85:15) and, after separation on a reverse phase column (C_{18} , 10 μm charge), the MU and MX concentrations were determined by comparison with the internal standard. The results seen in Fig. 1 were obtained by measuring the extinction values at 270 nm.

RESULTS

The ratio MU/MX, indicating the degree of XO activity in the urine, was 1.11 ± 0.52 ($x \pm \text{S.D.}$) in normal children; this is slightly higher than the values measured by Grant et al. (4) in adults.

In Fig. 2, the XO activity values measured during 3 successive days in newborns with RDS can be seen as a function gestational age. The XO activity is extremely high mainly in patients younger than 32 weeks of gestational age, and does not decrease during the subsequent days. A decrease in XO activity ensues only in premature infants with a longer gestational age and suffering from RDS; a considerable improvement in their condition was experienced during the treatment.

Figure 3 compares the XO activities of the same patients (measured via the ratio MU/MX in the urine) with the simultaneously measured serum uric acid level. A statistically significant correlation can be observed ($p < 0.001$; $r = 0.579$; $n = 44$).

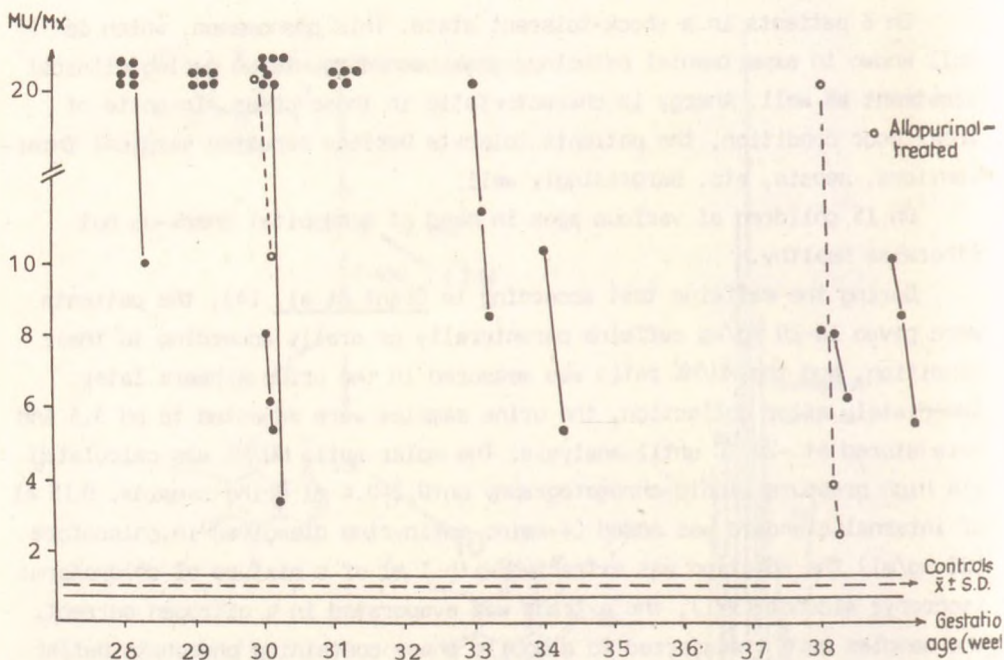


Fig. 2. Ratios MU/MX for newborns in the first 2-3 days of life

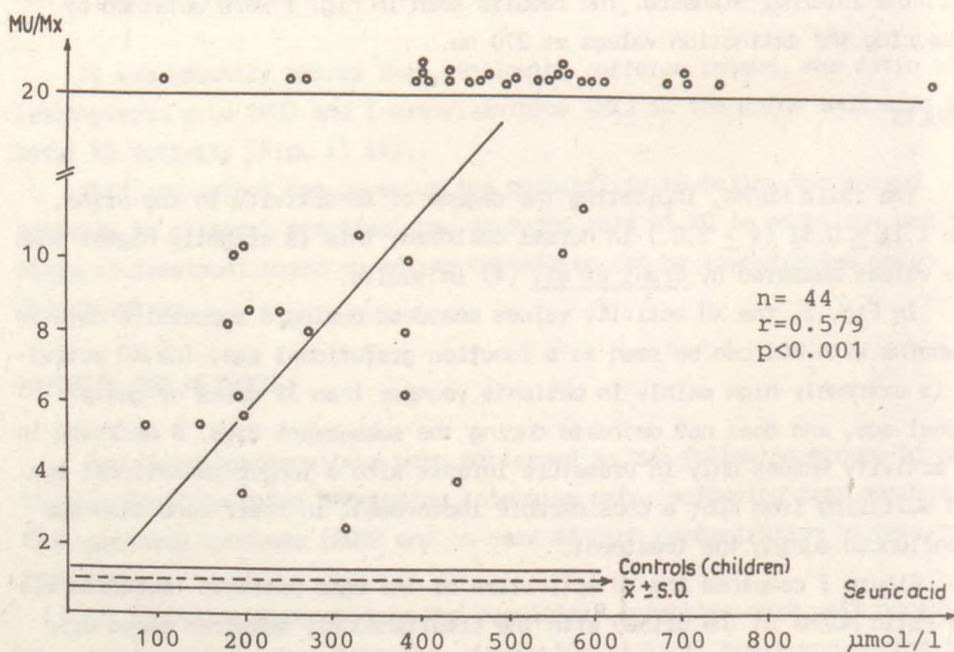


Fig. 3. Correlation between ratio MU/MX and serum uric acid concentration

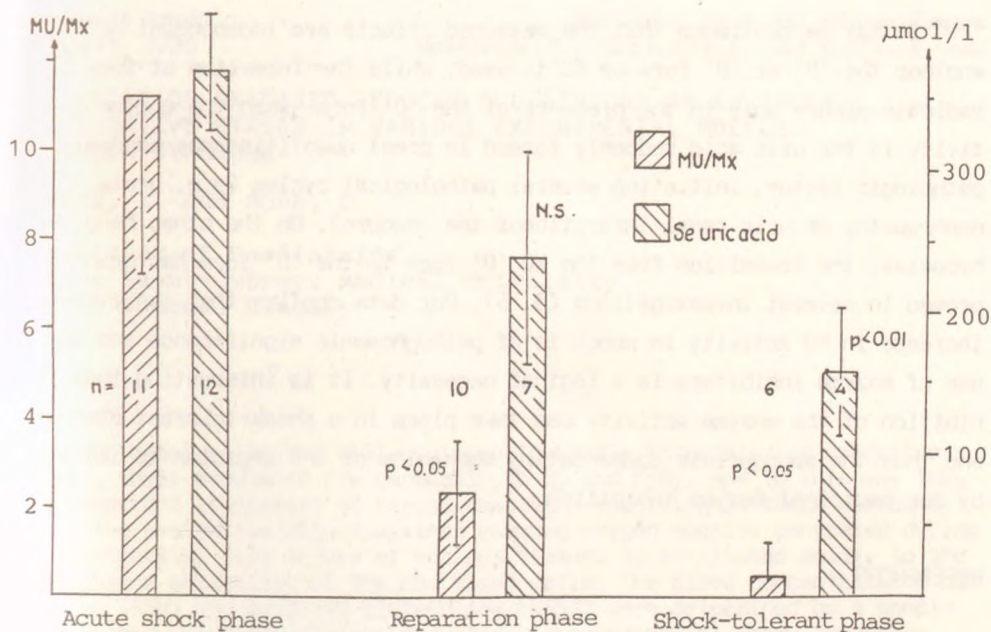


Fig. 4. Ratios MU/MX and serum uric acid concentrations in critically ill patients

In Fig. 4, the XO activities and serum uric acid concentrations of patients in the intensive care unit are grouped according to the stages of the shock condition. In the acute shock phase, considerable increases can be observed in both the XO activity and the serum uric acid level. During the stage of reparation, there is a greater decrease in the XO activity than in the serum uric acid level. In some patients who developed a shock-tolerant state, the XO activity became very low and the serum uric acid level also decreased considerably.

The specific enzyme inhibiting effects of allopurinol on the changes in XO activity and serum uric acid level of the patients during treatment with intravenous allopurinol were also checked.

DISCUSSION

It seems to be proved that the metabolite ratio MU/MX, and its regularity depending on the clinical condition, really is an indicator of XO activity. This is demonstrated even by the fact that the XO inhibiting effect of allopurinol is indicated by this method in a very sensitive way.

It may be mentioned that the measured effects are independent of whether the 'D' or 'O' form of XO is used, while the formation of free O_2 radicals occurs only in the presence of the 'O' form. When the enzyme activity is the uric acid suddenly formed in great quantities may become a pathologic factor, initiating several pathological cycles (e.g. urate nephropathy or uric renal infarction of the newborn). On the other hand, in hypoxias, the transition from the XO 'D' form to the 'O' form has been proved in several investigations (3, 5). Our data confirm that the extreme increase in XO activity in shock is of pathognomonic significance and the use of enzyme inhibitors is a logical necessity. It is interesting that inhibition of the enzyme activity can take place in a shock-tolerant state, and thus the appropriate compensating mechanism of the organism is imitated by our medicinal enzyme inhibition.

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THE RATIO OF OXIDIZED/REDUCED GLUTATHIONE AS AN INDEX OF OXIDATIVE STRESS IN VARIOUS EXPERIMENTAL MODELS OF SHOCK SYNDROME

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Red blood cells are well equipped to handle intracellular oxidative stress, their membranes are permeable to O_2 and H_2O_2 , and in this way they are important regulators of oxygen reactions occurring in their surroundings. The protective effect against reduced oxygen species generated during the endothelial cell injury of various tissues is attributed mainly to the glutathione metabolism of the red blood cells. The blood concentrations of reduced (GSH) and oxidized glutathione (GSSG) were determined by a sensitive method using glutathione reductase in experimental shock syndromes induced by paraquat toxicity, tourniquet ligation and endotoxin in rats, by bleeding and reperfusion in dogs and by transient ligation of the thoracic aorta in newborn piglets. Under these conditions, the concentration of GSSG was elevated and that of GSH was lowered, resulting in an increase in the redox ratio: $GSSG/(GSH + GSSG) \times 100$ (GSSG/GSH), particularly during reperfusion. Determination of GSSG/GSH seems to give a reliable index for the assessment of the degree of oxidative stress "in vivo".

INTRODUCTION

The primary protection against toxic oxygen metabolites is provided by the glutathione redox cycle. Elevation of the ratio GSSG/GSH in cells was proven to be a direct measure of the oxidant stress following renal ischemia and diquat toxicity in rats (3). Previously, plasma GSSG concentrations were reported to be a sensitive index of whole-body oxidative stress in rats as induced by oxidant drugs (1). Unlike rats, which have $\approx 5 \mu\text{mol}$ GSH/GSSG in their plasma, human plasma contains negligible amounts of glutathione. However, significant oxidation of the glutathione in human blood (erythrocytes) as a result of submaximal physical exercises has recently been shown (2).

The purpose of our investigation was twofold:

1. To examine whether there is any change in the GSSG/GSH of whole blood under marked oxidative stress in various experimental shock syndromes.

2. If so, to clarify whether there is a correlation between the change in GSSG/GSH measured simultaneously from the whole blood and the plasma in rats.

MATERIAL AND METHODS

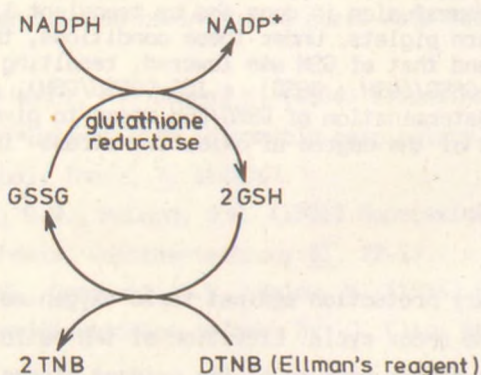
Experimental shock models

1. Paraquat toxicity in rats: after 1 hr (n = 5), 2 hrs (n = 5) and 4 hrs (n = 5) of 0.29 mmol/kg of Gramoxone^R administered i.p. Controls (n = 5).

2. Tourniquet shock in rats: just after the release of a 4-hr ligature (n = 10) and also after a 4-hr reperfusion (n = 10).

3. Endotoxin shock in rats: 4 hrs after 10 mg/kg E. coli 089P₂ administered i.v. (n = 10). Controls (n = 10).

4. Hemorrhagic shock in dogs, performed by bleeding and reinfusion (n = 5). Samples were taken at a blood pressure of 40 mm Hg, at maximum



rate of formation is recorded spectrophotometrically at 412 nm during 6 min

GSH + 2 GSSG = "GSH equivalents"

GSSG = GSH + NEM (Akerboom and Sies: Methods Enzymol. 77: 373, 1981)

separation of NEM by Sephadex G-10

(Güntherberg and Rapaport: Acta Biol. Med. Germ. 20:559, 1968)

Fig. 1. Enzymic assay of total and oxidized glutathione contents (Tietze, F.: Anal. Biochem. 27, 502, 1969)

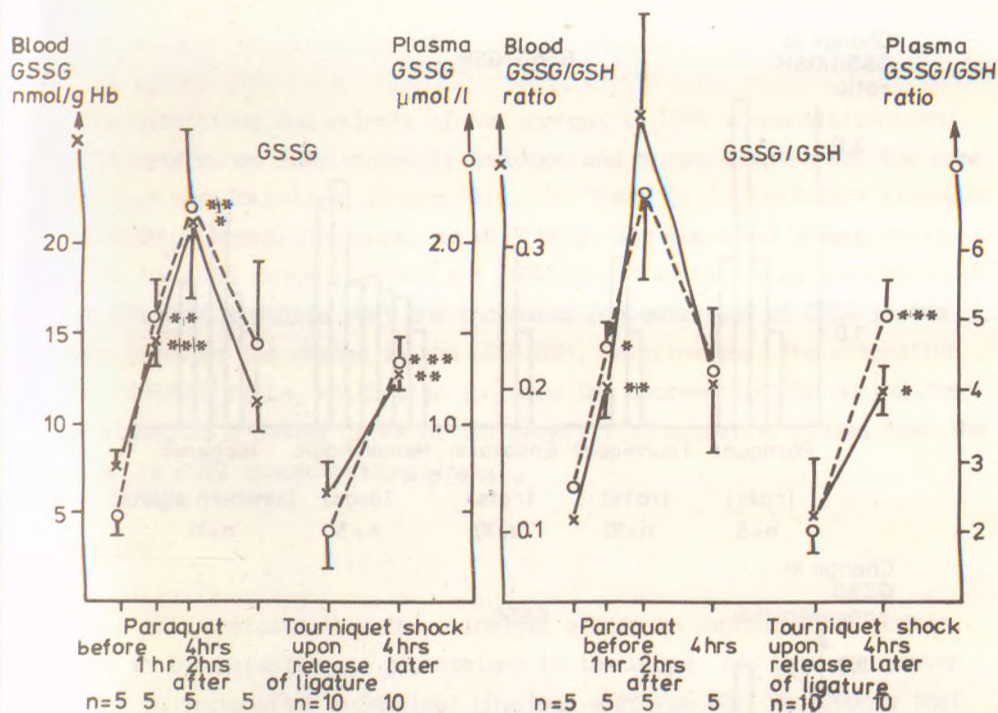


Fig. 2. Concentrations of GSSG and GSSG/GSH in blood and plasma in rats

Blood
GSSG/GSH
redox ratio

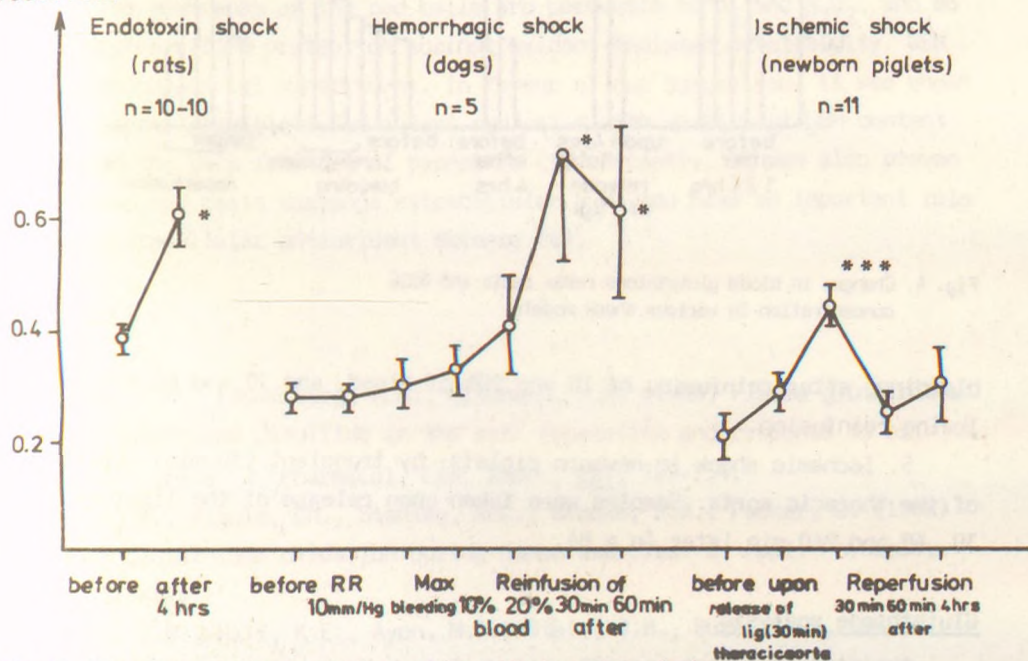


Fig. 3. Alterations in blood glutathione redox ratio in various shock

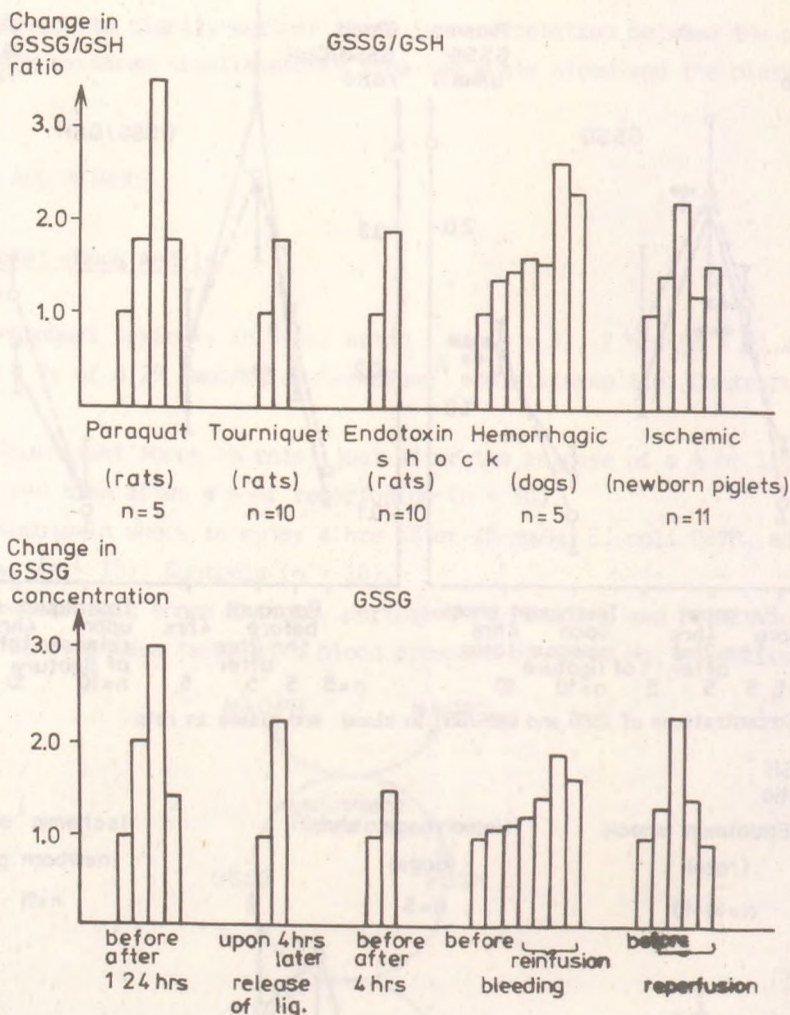


Fig. 4. Changes in blood glutathione redox ratio and GSSG concentration in various shock models

bleeding, after reinfusion of 10 and 20% of blood, and 30 and 60 min following reinfusion.

5. Ischemic shock in newborn piglets: by transient (30-min) ligation of the thoracic aorta. Samples were taken upon release of the ligature and 30, 60 and 240 min later (n = 8).

Glutathione analysis

See Fig. 1

RESULTS

The directions and extents of the changes in GSSG concentrations and GSSG/GSH determined simultaneously in blood and plasma samples are the same in paraquat and tourniquet shocks (Fig. 2). There is a significant elevation of GSSG/GSH in endotoxin shock, as well (Fig. 3). The simultaneous elevations of the GSSG concentrations and GSSG/GSH in various experimental shock models (Fig. 4) indicate that the increased concentration of GSSG is the primary cause of the change in the GSSG/GSH. Nevertheless, the alteration in the GSSG/GSH ratio, which also includes the decrease in GSH during the shock state, is a better index of the severity of oxidative stress than the increase in GSSG concentration alone.

DISCUSSION

Our data indicate that the oxidative stress in various shock models results in the oxidation of glutathione in the blood. The same was proven in human subjects after submaximal physical exercise (2). We suppose that oxygen-derived free radicals are generated from the injured endothelial cells and polymorphonuclear granulocytes in the surroundings of the red cells. The membranes of the red cells are permeable to O_2^- and H_2O_2 , and so erythrocytes afford protection against oxidant-mediated cytotoxicity, GSH being the beneficial constituent. In favour of our suggestion, it was shown that tracheal insufflation of intact red cells with sufficient GSH content protected the rats from lethal hyperoxia (5). Recently, it was also proven that human red cells scavenge extracellular H_2O_2 and have an important role in the extracellular antioxidant defense (6).

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INVESTIGATIONS ON SOME LIPID PEROXIDATIVE PARAMATERS OF GEESE

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No data are available on the lipid peroxidative (LP) parameters in geese. Therefore, in 30 healthy, 8-week-old, Landes-type geese with an average body weight of 4000 g, we measured the glutathione peroxidase (GSHPX) activity and malonyldialdehyde (MDA) concentration in the blood plasma and in the hemolysate of the erythrocytes, and also the superoxide dismutase (SOD) activity of the whole blood.

The geese were divided into 3 equal groups, and fed with granulated food with different (14, 16 or 18%) crude protein contents.

The LP parameters varied greatly in the groups and the differences showed no clear tendencies.

The results will be used as basic data in our future experiments on geese with fatty liver.

The LP results in healthy geese varied as shown in Table 1.

Table 1. LP parameters in geese blood

Range		
GSHPX	Blood plasma	7.22-8.09 U/g protein
	Hemolysate	7.83-8.16 U/g hemoglobin
MDA	Blood plasma	0.05-0.07 $\mu\text{mol/g}$ protein
	Hemolysate	0.22-0.26 $\mu\text{mol/g}$ hemoglobin
SOD	Whole blood	38.7-48.5 U/l
	hemolysate	

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STATE OF ANTIOXIDATIVE PROTECTIVE SYSTEM OF BLOOD DURING AGEING

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Ageing can be defined as a physiological process which governs the enhanced instability and vulnerability to pathological processes.

Numerous data now available indicate with a high degree of probability that more or less random endogenous free radical reactions can play a significant role in the degradation of biological systems during ageing. It is reasonable to expect that inhibitors of free radical reactions (biological antioxidants and the protective enzyme system) may increase the duration of the useful life of humans, by resulting in a decreased rate of biological degradation.

Limitation of free radical reactions in the organism via:

- 1) the O_2 metabolism
- 2) bioantioxidants and the enzyme system,
- 3) the lipid metabolism.

Lipids vary markedly in their tendency to be involved in free radical reactions with molecular oxygen:

- 1) cholesterol and esters are practically indifferent substances, with very low rates of peroxidation,
- 2) triglycerides and free fatty acids are the main energy substrates for free radical production,
- 3) phospholipids are antioxidants.

Hormonal regulation of lipid metabolism

ACTH - the cholesterol level is decreased, and the phospholipid concentration is increased; this plays a protective rather

than a promoting role as concerns the production of free radicals.

Cortisol — the cholesterol, phospholipid and esterified fatty acid levels are increased.

Adrenaline - the triglyceride and free fatty acid contents of the serum are increased; this has a promoting effect on the production of free radicals.

We have investigated the state of the oxidative protective system in blood (catalase and SOD activities and the formation of lipid peroxidation products (LPP) during ageing. Moreover, we have determined the lipid fraction content of the serum, considering that the lipid metabolism can prevent free radical attack on body constituents. We have compared these data and have found the correlations between them. Measurements were made on elderly patients with different diseases who were being treated in the gerontological wards of the hospital.

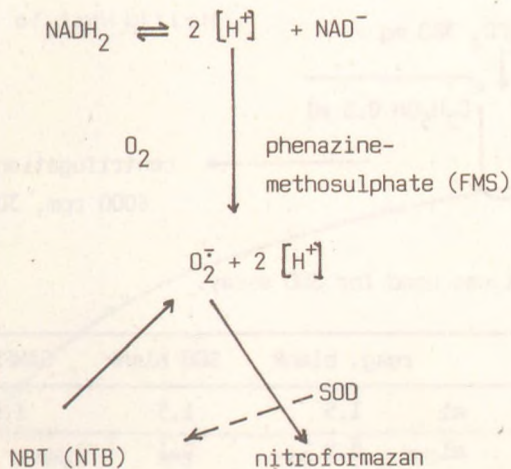
MATERIALS AND METHODS

The data were obtained on 140 persons aged between 66 and 93 years. The differences between the two sexes (88 females and 52 males) and the various age groups were studied: 66-70 years old - 14 persons, 71-80 years - 48 persons, 81-85 years - 38 persons, 86-90 years - 28 persons, 90 years - 12 persons. During the study period, none of the patients took any medication for 24 hours before blood collection. Blood and serum samples from 50 normal healthy individuals were also assayed, to establish reference values.

- 1) Catalase in blood was determined according to (4).
- 2) Lipid peroxidation products were determined according to (3).
- 3) Cholesterol and HDL-cholesterol were determined via the Galenopharm test (5).
- 4) Triglycerides (TG) were determined according to (1).

SOD activity was determined by our own modification (2), on the following basis:

A stable solution of superoxide radicals was generated aerobically by the reaction between phenazinemethosulphate and NADH. An infusion of such a solution into buffered aqueous media permitted the demonstration that $O_2^{\cdot -}$ can reduce nitrotetrazolium blue:



This reduction is abolished by SOD. Thus, when a two mixtures containing an O_2^- -forming system together with NTB are incubated under identical conditions, except that one contains SOD and the other does not, a difference absorbance develops between them due to the prevention of O_2^- -mediated NTB reduction absorbance. From the difference spectrum between the sample and reference at 540 nm, the O_2^- -dependent NTB reduction degree was calculated.

Reagents: 1) 0.15 M phosphate buffer, pH = 7.8;

2) TRIS-EDTA buffer, pH = 8.0 (EDTA - 37 mg, TRIS - 24 mg, in 100 ml dist. water);

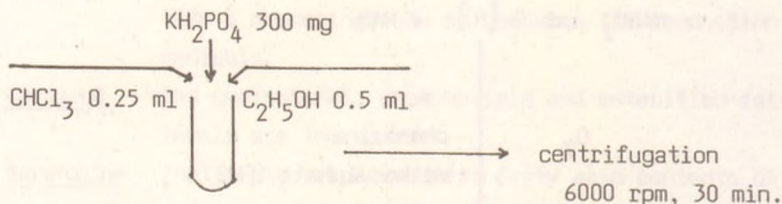
3) reagent I (DTA - 37 mg, NTB - 300 mg, FMS - 55 mg, in 300 ml phosphatebuffer);

4) NADH_2 solution (NADH_2 - 152 mg, in 100 ml TRIS-EDTA buffer);

5) SOD standard, 10000 U/mg (Miles, USA).

Human blood samples

Whole blood was obtained by venipuncture; coagulation was prevented with EDTA. 0.9 ml dist. water was added to 0.1 ml blood, followed by haemolysis.



The clear supernatant was used for SOD assay.

		reag. blank	SOD blank	SAMPLES
Reagent I	ml	1.5	1.5	1.5
H ₂ O	ml	0.2	0.1	-
Samples	ml	-	-	0.1
NADH ₂	ml	-	0.1	0.1

Absorbance was measured after incubation for 3 min at room temperature.

Calculation: $\frac{A_0^{SOD} - A_S}{A_0^{SOD}} \cdot 100 = \text{percentage inhibition of level of nitroformazan colour development.}$

Via the percentage inhibition for a sample its SOD activity can be estimated from the standard calibration curve (Fig. 1).

RESULTS

- 1) Influence of age (Figs 2-7).
- 2) Influence of sex (Figs 8-13).
- 3) Role of free radical reactions in promotion of cataract (Figs 14-16).

Column 1: mean value for 71-80-year-old patients without cataract.

Column 2: mean value for patients of the same age with cataract.

- 4) Correlation between the level of lipid peroxidation and the cholesterol concentration in the serum (Fig. 17).

DISCUSSION

1) The LPP level and the state of the protective enzyme system are more favourable in women than in men of the same age.

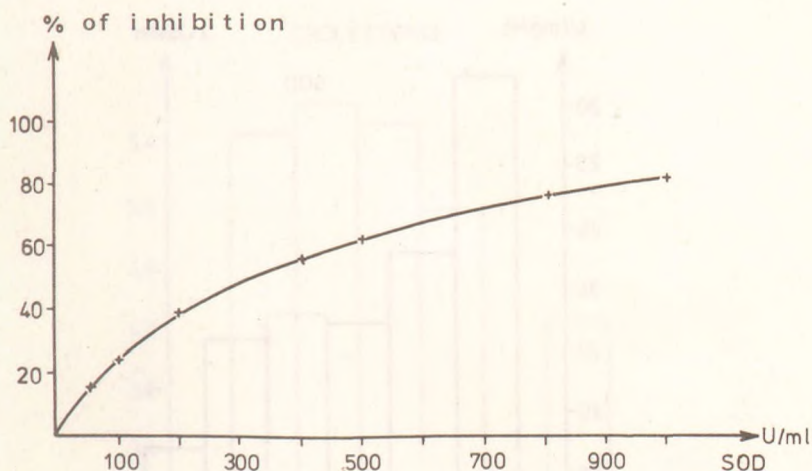


Fig. 1.

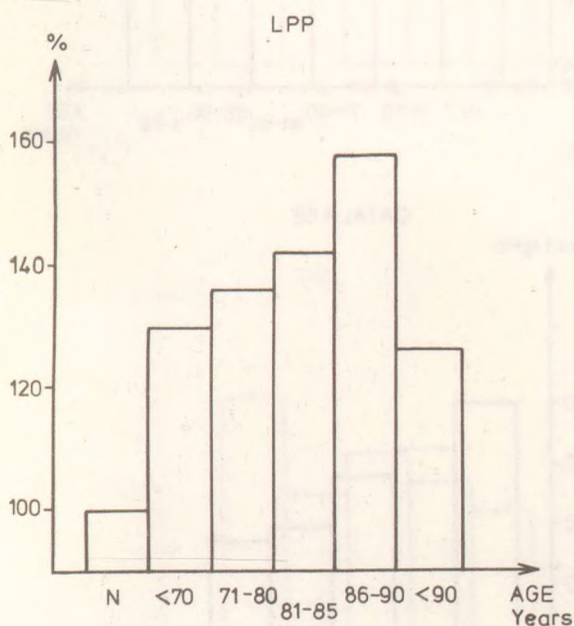


Fig. 2.

2) The results show that the SOD and catalase activities in the blood decrease significantly with age, especially after the age of 90 years. Similarly, we have found that the lipid peroxidation degree is not elevated as much as could be expected.

The rapid and ready induction of SOD and catalase in the cell organelles indicates the action of an adaptation mechanism.

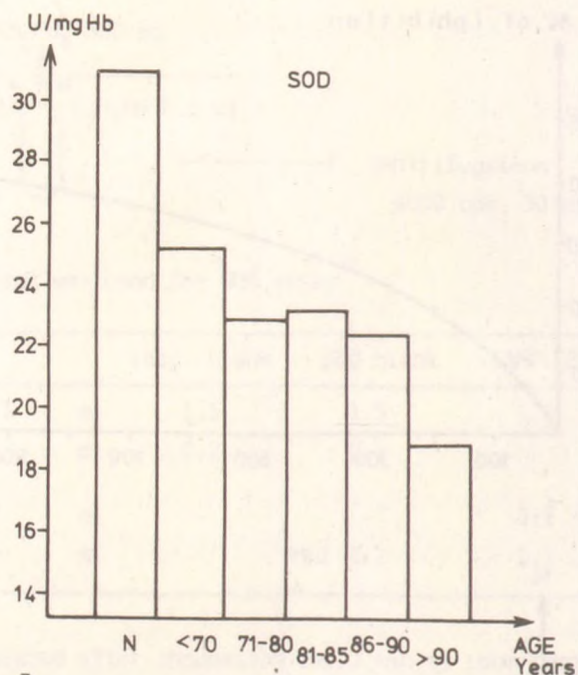


Fig. 3.

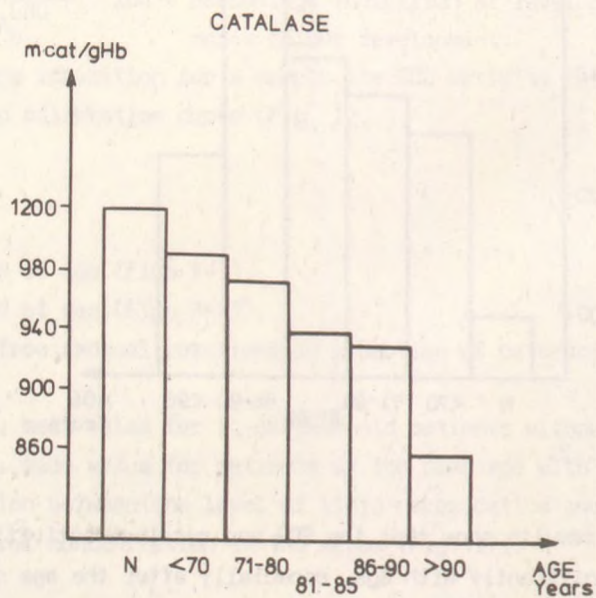


Fig. 4.

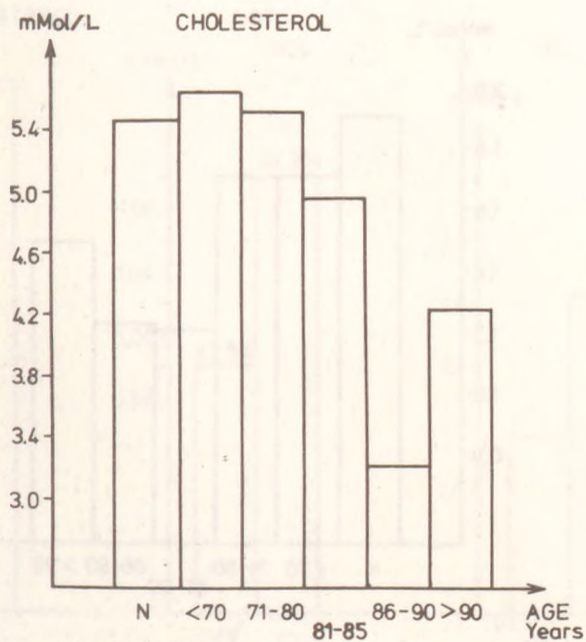


Fig. 5.

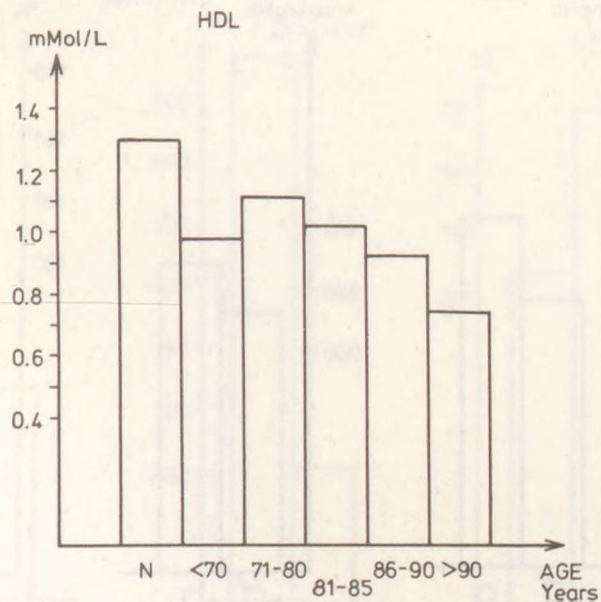


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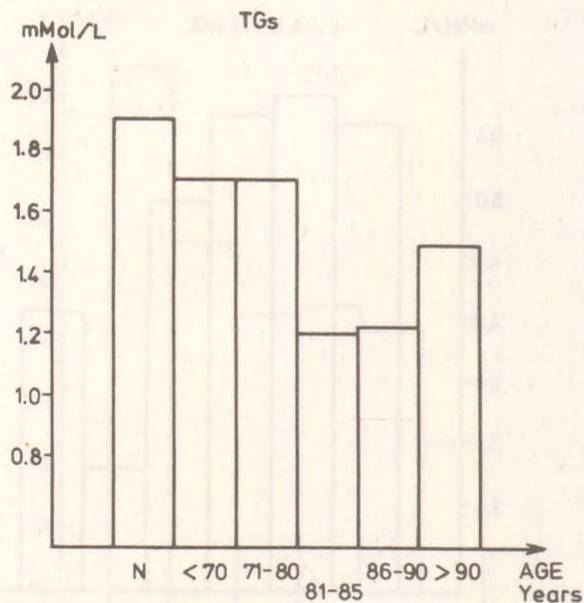


Fig. 7.

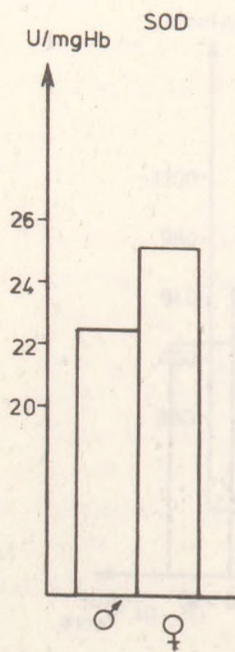


Fig. 8.

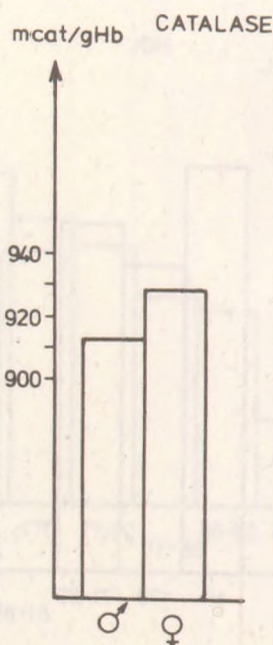


Fig. 9.

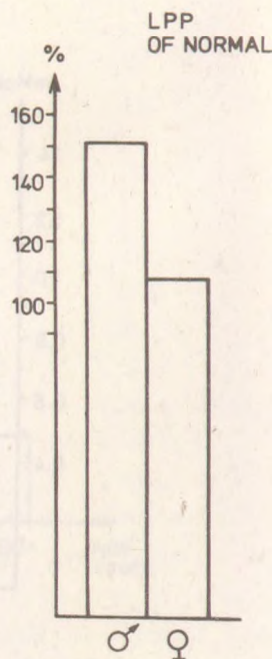


Fig. 10.

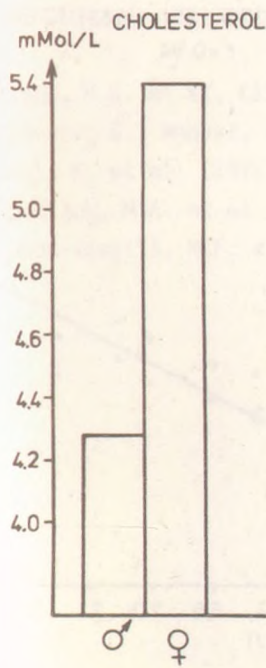


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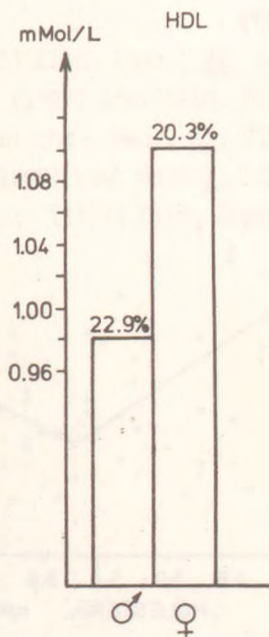


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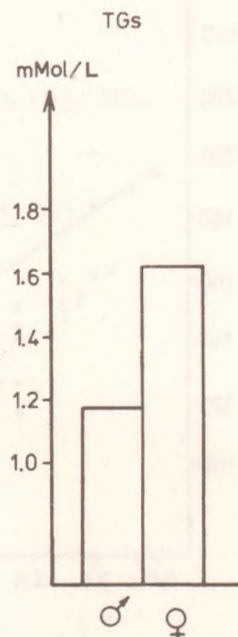


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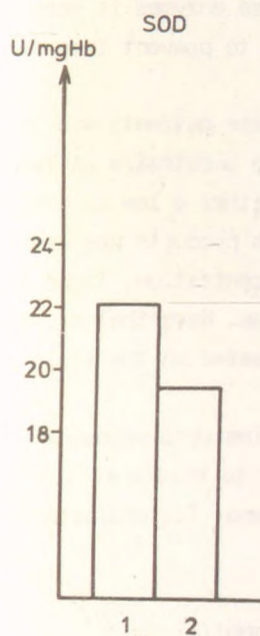


Fig. 14.

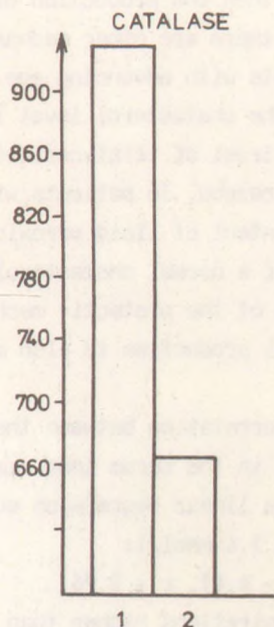


Fig. 15.

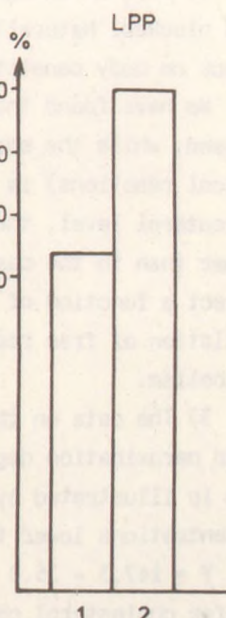


Fig. 16.

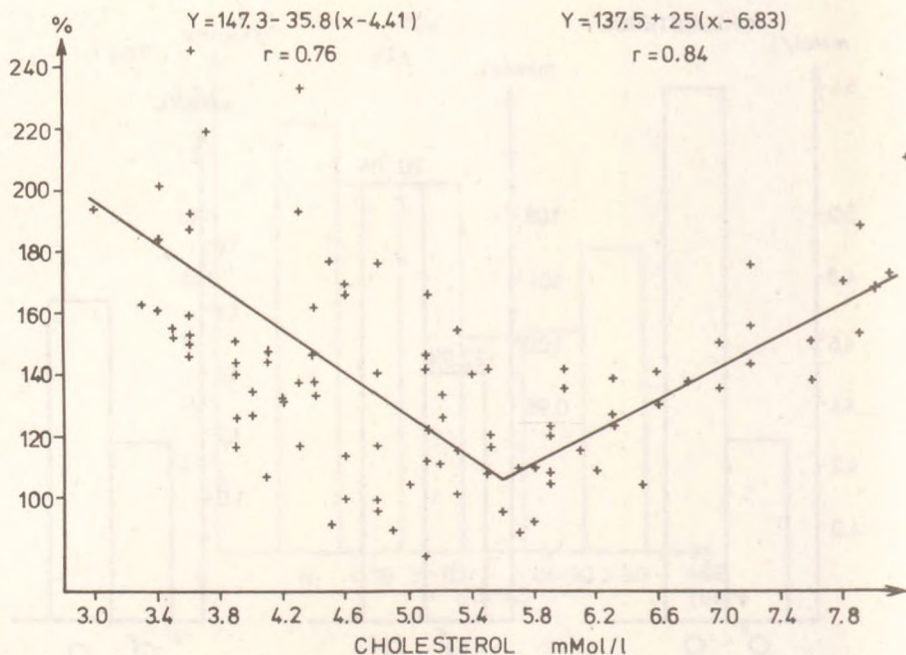


Fig. 17.

If this is damaged, then the production of these enzymes is more or less blocked. Naturally, there are other mechanisms to prevent free radical attack on body constituents with advancing age.

We have found that the cholesterol level in these patients was increased, while the serum level of triglycerides (the substrates of free radical reactions) is decreased. In patients with either a low or a high cholesterol level, the content of lipid peroxidation products was usually higher than in the case of a normal cholesterol concentration. These data reflect a function of one of the protective mechanisms. Nevertheless, the regulation of free radical production is also manifested in the lipid metabolism.

3) The data on the correlation between the cholesterol values and the lipid peroxidation degree in the serum lend support to this possibility. This is illustrated by two linear regression equations; for cholesterol concentrations lower than 5.6 mMol/l:

$$Y = 147.3 - 35.8 (X - 4.4), r = 0.76,$$

and for cholesterol concentrations higher than 5.6 mMol/l:

$$Y = 137 + 25 (X - 6.83), r = 0.84.$$

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CORRELATIONS BETWEEN LIPID PEROXIDATION,
NUTRITIONAL STATE AND REDOX STATE IN DIFFERENT MUSCLES
OF THE FROG

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Changes in the lipid peroxidation and redox state in cellular objects and tissues can be regarded as non-specific modulators of the specific activities (1-4). Certain data indicate that free radical generation and the succeeding lipid peroxidation are connected with changes in the redox state of the cellular redox systems, and opposite changes in the redox state can be evoked by electron acceptors or by electron donors having extremely different chemical structures (5-10).

We previously reported a negative correlation between the malondialdehyde (MDA) content and the redox-state potentials in different muscles of the frog (10). Since we further observed nutritional state-dependent differences in the MDA contents of the samples, and such differences were known in connection with the redox state of cellular redox systems (11-12), we have examined the effects of starvation on the MDA content and the redox state, and how this effect is influenced by the presence of an electron acceptor, methylene blue (MB), or an electron donor, ascorbate (Asc).

Isolated rectus abdominis muscles, stomach smooth muscles and the heart of frog (*Rana esculenta*) were used for the experiments, as detailed elsewhere (10). The muscles from fed animals and from others starved for 100±5 or 130 days were preincubated for 15 minutes either in frog Ringer, or in Ringer containing MB1 (7.7×10^{-5} M), MB2 (2.7×10^{-4} M), Asc1 (5×10^{-4} M) or Asc2 (5×10^{-3} M) solution. After preincubation, the MDA concentrations were determined and the redox-state potentials (E'_0) were measured in the same homogenates with the use of platinum plate and Ag/AgCl electrodes (10). The correlation coefficient (R) between E'_0 and the MDA concentration was computed as a function of E'_0 , and the significance of R was expressed by P to test whether the correlation is zero or not.

In the fed animals, the highest MDA content was found in the heart (h), and the lowest in the stomach (s) (Table 1). After 100 days of chronic starvation (CS), the MDA content was decreased in each of the muscle homogenates. The decrease depended linearly on the amount of MDA found in the muscle of the fed animals, but the decrement was not significant in the s.

With the exception of MB1 in the s, starvation augmented the MDA-decreasing effect of MB significantly (see the differences between 3 and 2 in Table 1). However, the decreasing effect of MB1 was significant only in the s. With the exception of MB1 in the h, MB augmented the MDA-decreasing effect of CS significantly (differences between CS and 2). It seems evident that MB is the least effective on the h.

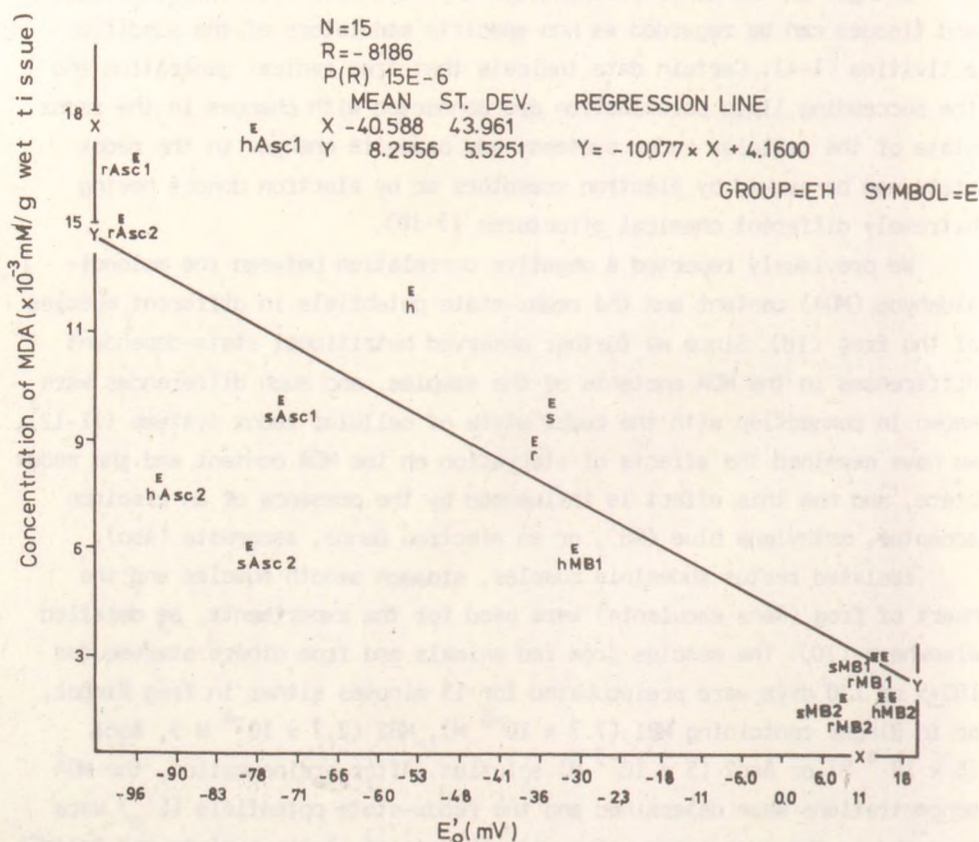


Fig. 1 Correlation between the changes in E'_0 and MDA concentration in starved frogs. The symbols E represent the experimental values for homogenates of the rectus abdominis muscle (r), stomach muscle (s) and heart (h), and the corresponding treatment with the redox compounds as indicated in the text. R = correlation coefficient, P(R) = significance of R.

Table 1. Effects of chronic starvation for 100 days (CS), methylene blue (MB1 = 7.7×10^{-3} M; MB2 = 2.7×10^{-4} M), ascorbate (Asc1 = 5×10^{-4} M; Asc2 = 5×10^{-5} M) on the MDA contents of different frog muscles. MDA concentrations determined in muscle homogenates of fed frogs are indicated in the first column and are taken as 100%. n values ranged from 4 to 15 p is not indicated if it is less than 0.001 n.s. = non-significant.

1. Muscle homogenizate and MDA content taken as 100% in 10^{-5} mW/g wet tissue $\pm$ S.E.M.		Differences in MDA contents related to the control			Differences between		
		2. in the case of 1		3. in the presence of redox agent alone	3 and 2		sum of effect of CS or redox agent alone and 2
		p >	as percentage of the control		p >	p >	
rectus abdominis (r) 20.46 $\pm$ 2.53	CS	-55.1	0.01	none	-	-	-
	CS+MB1	-85.6		- 34.0	- 51.6	- 30.5	- 3.5
	CS+MB2	-88.7		- 69.8	- 18.9	- 33.6	- 36.2
	CS+Asc1	-22.5	n.s.	+ 39.4	- 61.9	+ 32.6	+ 6.8
	CS+Asc2	-31.1	n.s.	+285.2	-316.4	+24.0	+261.3
stomach (s) 13.00 $\pm$ 1.52	CS	-23.3	n.s.	none	-	-	-
	CS+MB1	-69.8	0.002	- 41.7	- 28.1	- 46.5	+ 4.8
	CS+MB2	-84.9		- 62.5	- 32.4	- 61.7	- 0.9
	CS+Asc1	-22.1	n.s.	+ 4.4	- 26.5	+ 1.2	+ 3.2
	CS+Asc2	-58.3	0.01	+322.1	-380.4	-35.0	+372.3
heart (h) 31.73 $\pm$ 8.26	CS	-58.6	0.002	none	-	-	-
	CS+MB1	-79.5		- 1.7	- 77.8	- 20.9	+ 20.8
	CS+MB2	-93.0		- 61.4	- 31.6	- 34.4	- 27.0
	CS+Asc1	-43.5	0.05	+124.5	-168.0	+15.1	+109.4
	CS+Asc2	-76.2		+201.6	-277.8	-17.6	+219.2

When the duration of CS was extended to 130 days, the decrease in MDA after MB1 administration markedly exceeded the 100-day starvation value; after MB2 administration, MDA was not detectable in either homogenate (data not given).

CS is generally able to counteract with an increasing effect of Asc on the MDA level (see differences between 3 and 2 in Table 1). In the case of rectus abdominis muscles (r), there is a significant excess of MDA in relation to the CS group (+32.6 and 24%, respectively; see differences between CS and 2), but the increasing effect of Asc is not manifested here either in relation to the fed controls (in column 2). As the MDA increasing effect of Asc1 depends linearly on the MDA content found in the muscles from fed frogs (column 3), the decreasing effect of CS on the increasing effect of Asc1 follows the same sequence (differences between 3 and 2). While the increasing effect of Asc1 is significant only on the h, where CS is also the most effective (columns 3 and 2), the decreasing effect of CS on the increasing effect of Asc1 did not prove significant in the s (differences between 3 and 2), where both Asc1 and CS were ineffective per se. The effectiveness of Asc1 and Asc2 shows a dual character, since Asc2 is the least effective in the h and the most effective in the s in increasing MDA (column 3).

After starvation for 150 days, MDA was not detectable in either homogenate even in the presence of Asc. Therefore, the possibility of MDA formation can be eliminated completely by starvation in a normal oxygen atmosphere (not indicated).

After Asc administration, E'_0 changed with a negative sign (Fig. 1), but, owing to the CS, the MDA concentrations were not increased so much as in the case of the fed animals. In the starved frogs, a -0.82 negative correlation coefficient was obtained between the changes in E'_0 and MDA concentrations.

To summarize the results, in muscles with higher potential, MB is more effective, but CS is less effective in lowering the MDA level; Asc1 is not able to exert its increasing effect, but such an action of Asc2 is more pronounced here. In turn, in muscles which have a lower potential, MB is less effective, but CS is more effective in lowering the MDA level; the effect of Asc1 is more pronounced, but the action of Asc2 is less.

From the results, the following conclusions were drawn:

1. The MDA-lowering action of an electron acceptor increases after starvation, and starvation can cause a decreased MDA content, even in the

presence of an electron donor. Accordingly, the redox state-modifying effect of the electron sources formed upon the feeding can be a condition in the formation of MDA.

2. As the negative correlation between the redox state potentials and the MDA concentrations exists even after opposite change of the redox state by redox-active compounds or by nutritional conditions, the specific reactions leading to the generation and/or elimination of free radicals fundamentally depend on the redox state of the muscles, independently of the factor.

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FORMATION OF FREE RADICALS INDUCING MEMBRANE DAMAGE IN FOODS

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INTRODUCTION

The formation of free radicals in the living organism is essential for maintenance of the essential functions of normal physiological conditions. The functional balance is preserved by defending mechanisms of chemical and enzymatic origin, and their deterioration or deficiency results in an overbalance of the free radicals. A surplus of reactive free radicals and their intermediates will damage the membrane structure. The changes in the permeability of the tissues will cause degenerative processes and later various diseases in the living organism; in a similar way, detrimental changes can occur in the quality of foods. The metabolites of free radicals in food can play a role in the aetiology of various diseases in vivo (e.g. tumourigenic and atherogenic processes).

Figure 1 introduces the free radical and lipid peroxidation reactions in animal foods, including the regulating and defending mechanisms. The free radicals activate the polyunsaturated fatty acids of lipids and produce conjugated-diene-type compounds. The next step is the formation of lipid peroxides and later the aldehydes, one characteristic representative of which is malondialdehyde (MDA).

One group of defending mechanisms involves the antioxidant scavenger compounds, among them ascorbic acid and tocopherol. As part of the enzymic defence, superoxide dismutase (SOD) breaks down the primary form of free radicals, and glutathione peroxidase (GSH_{px}) decomposes the lipid peroxides and hydrogen peroxide.

Our aim was to find suitable methods of characterizing the changes caused by free radicals and lipid peroxidation in foods.

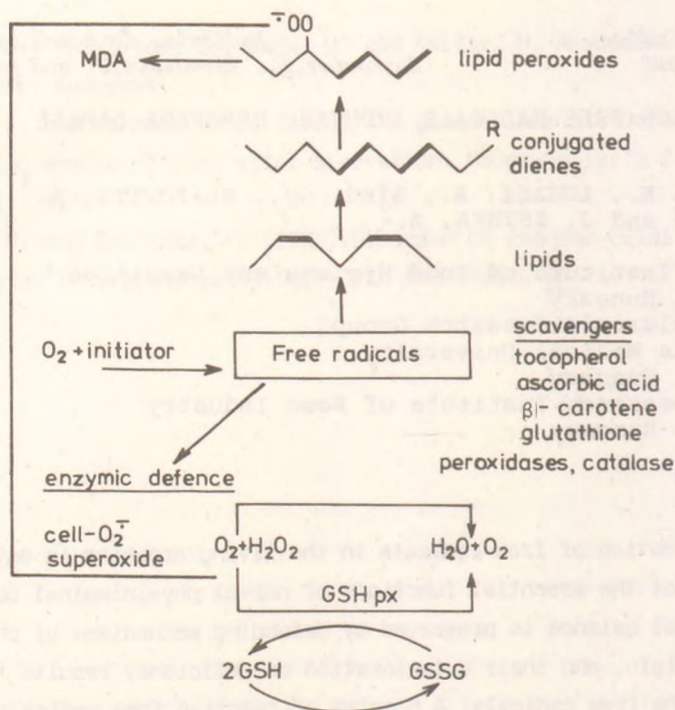


Fig. 1. Pathway of free radical reactions in tissues

METHODS

For the investigation of free radical reactions, the chemiluminescence intensity was measured. The principle of the method is that the photosensitizer luminol induces free radical reactions. The free radicals are promoted to an excited atomic state, from which they return to the ground level with the emission of light, which is measured with an appropriate instrument.

Two principles of the chemiluminescence technique were used: first the direct evaluation of free radicals; second: the scavenging capacity of the samples. In the second case, we had the possibility to measure the scavenging capacity of the superoxide radical by evaluating the decrease in chemiluminescence intensity due to the adrenaline-luminol reaction. Similarly, the scavenging capacity of the peroxide radical was determined via the reaction between luminol and hydrogen peroxide.

For the determinations, an MMT microprocessed CLD-1 luminometer (MEDICOR-MEDILAB) was used.

MDA was determined photometrically with the thiobarbituric acid reagent (Ohkawa, 1979).

Conjugated dienes were assayed via the absorbance at 233 nm.

The activity of SOD was measured by the method fo Winterbourn et al. (1975), using the inhibition of adrenaline oxidation.

The activity of GSH_{px} was evaluated by the photometric measurement of reduced glutathione with 5,5'-dithio-bis-(2-nitrobenzoic acid) (Naguchi et al. 1973).

RESULTS AND DISCUSSION

Figure 2 shows the amount of free radicals characterized by the chemiluminescence intensity in liver samples. The formation of free radicals increases proportionally with the fat content of the liver. In accordance with these results, the increased fat content in the liver and the membrane damage caused by defrosting reduced the free radical scavenging activity in the tissues; this is valid for both the adrenaline and hydroge peroxide systems (Fig. 3).

It can be seen in Fig. 4 that sunflower-seed oil produced free radicals in measurable amounts, and these were elevated when the oil had been heated several times. Margarine did not produce free radicals, probably due to its enriched tocopherol content.

In the flour of cereal grains, we were not able to detect free radicals, but only the scavenging capacity of these foods in the adrenaline system.

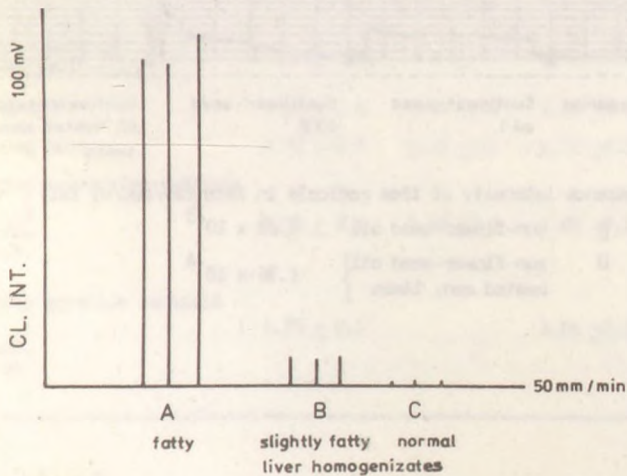


Fig. 2. Chemiluminescence (CL) intensity of free radicals in chicken liver (mV/sec/g)

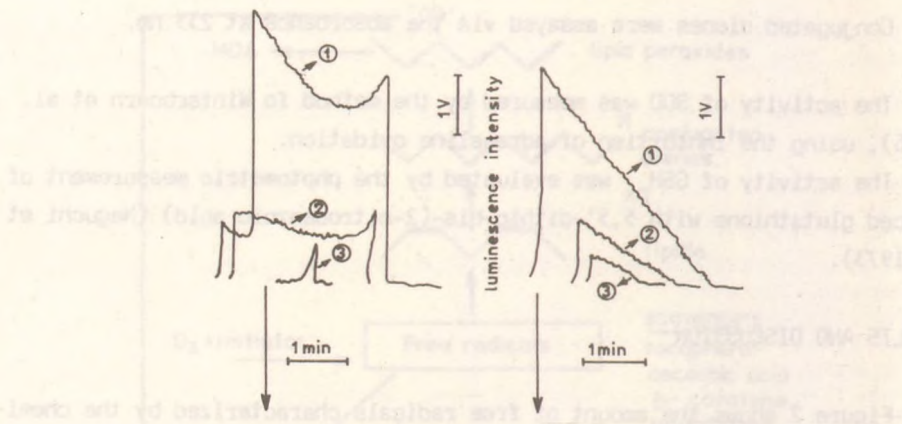


Fig. 3. Chemiluminescence intensity of the scavenger capacity in liver

- | | |
|-------------------------------|---------------------------------|
| 1. 30 ul 10^{-4} M H_2O_2 | 1. 50 ul 10^{-3} M adrenaline |
| 2. +20 ul 3% goose-liver hom. | 2. +20 ul 3% stored beef hom. |
| 3. +20 ul 3% ox-liver hom. | 3. +20 ul 3% fresh beef hom. |

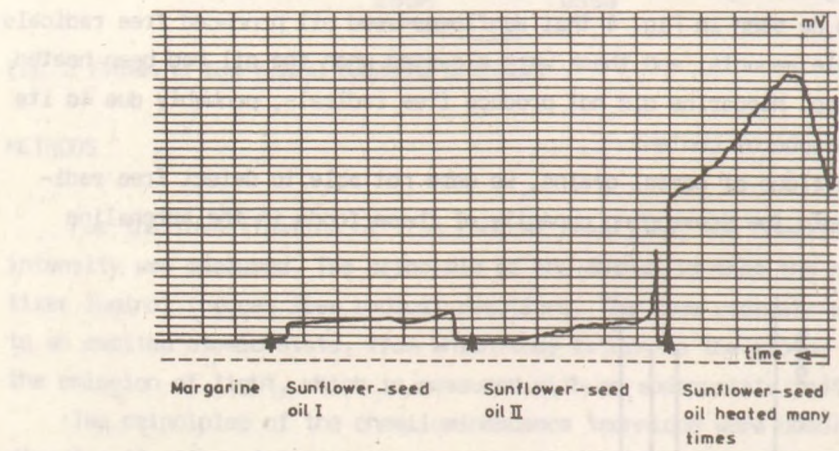


Fig. 4. Chemiluminescence intensity of free radicals in fats (mV/sec/ml fat)

lard	0	sun-flower-seed oil	1.68×10^{-3}
butter	0	sun-flower-seed oil	1.76×10^{-4}
margarine		heated many times	

Figure 5 shows that wheat germ has a much higher free radical scavenging capacity than that of wheat flour.

In fresh meat samples MDA and the conjugated dienes gave a fairly good correlation with the fat content (Fig. 6). The two reactive intermediates also correlated well with each other. This means that the lipid peroxidation starts already in fresh meat and its extent is proportional to the amount of fats.

A feasible way to damage the biomembranes of meats is to freeze and defrost them time after time. In order to initiate free radical and peroxidation reactions, we carried out 5 consecutive deep-freezing and defrosting cycles with beef an pork.

The levels of MDA and conjugated dienes were increased after the membrane damage only in beef; in the fatty pork samples they had high values originally (Table 1). The enzyme protection was severely damaged after the intervention, and the same held for the activity of scavenging superoxide radical.

Table 1. Effects of biomembrane damage on the enzymic defence and on the reactive intermediates of free radicals

	Beef (n = 5)		Fatty pork (n =4)	
	Membrane damage		Membrane damage	
	before	after	before	after
MDA nmol/100 g	8.75 ± 3.1	11.4 ±1.0	22.9 ±5.6	25.4 ±9.7
Conjugated dienes A ₂₃₃ /g/100 ml dioxane	0.041± 0.02	0.107±0.04	0.452±0.23	0.437±0.37
SOD U/mg prot.	2.12 ± 1.0	1.95 ±0.2	2.38 ±0.8	1.99 ±0.4
GSH _{px} U/mg prot.	0.95 ± 0.5	0.70 ±0.3	0.76 ±0.3	0.62 ±0.2
Scavenging superoxide radicals <u>mV.sec.10⁶</u> 1mg prot.	0.96 ± 0.4	1.59 ±0.2	1.85 ±0.2	1.58 ±0.1
Scavenging peroxide radicals <u>mV.sec.10⁶</u> 1mg prot.	1.95 ± 0.3		1.86 ±0.2	

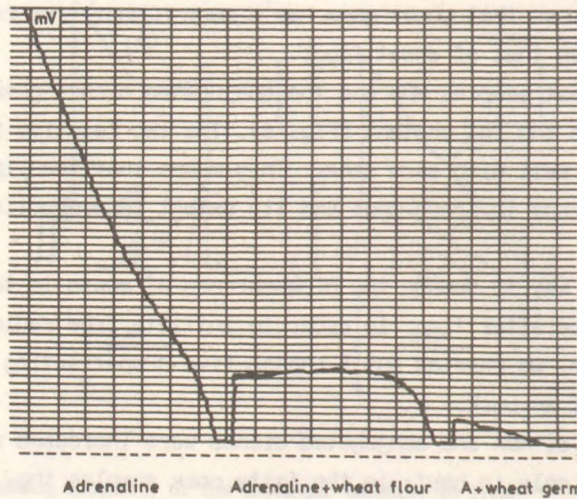


Fig. 5. Chemiluminescence intensity of the scavenger capacity in cereals (mV/sec/gr)

wheat bran	450×10^4	maize flour	526×10^4
wheat flour	570×10^4	wheat germ	820×10^4

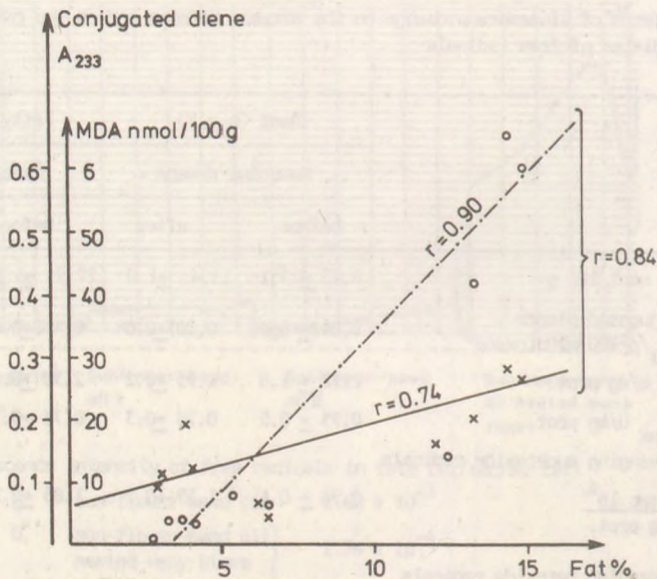


Fig. 6. Changes in MDA and conjugated diene levels in fresh meats as a function of fat content
 x—x MDA o-.-.-o Conjugated diene

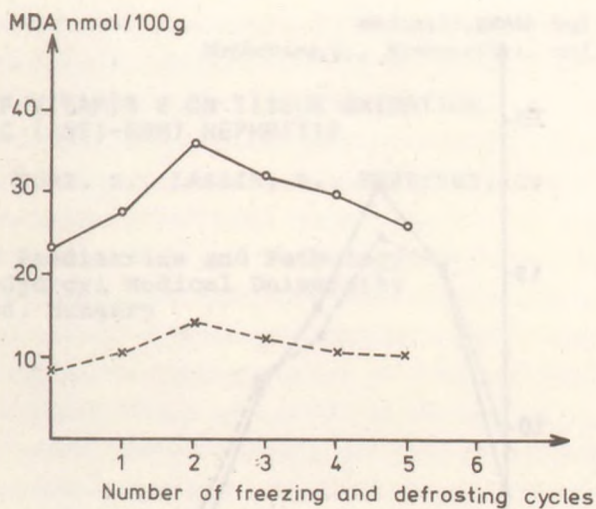


Fig. 7. Effect of biomembrane damage on the MDA level

o—o fatty pork x—x beef

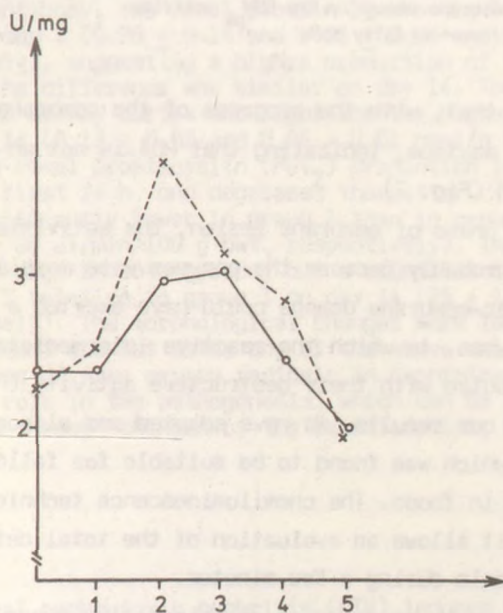


Fig. 8. Effect of biomembrane damage on the SOD activity

x—x beef o—o fatty pork

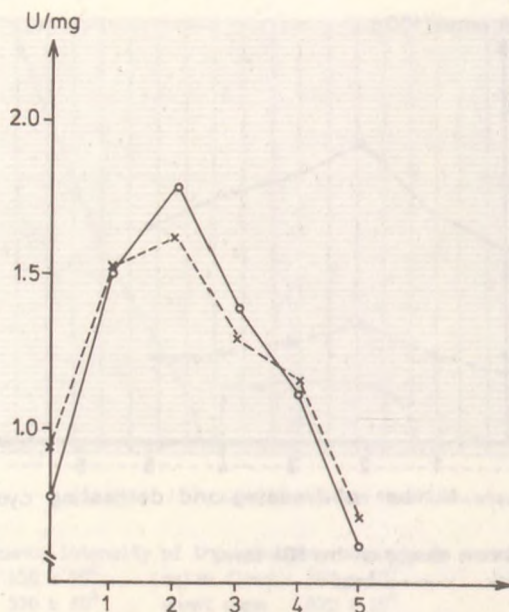


Fig. 9. Effect of biomembrane damage on the GSH_{px} activity
 x-----x beef o-----o fatty pork

It was found that, with the progress of the membrane damage the MDA level displayed a maximum, indicating that MDA is not an end-product of lipid peroxidation (Fig. 7).

In the first phase of membrane lesion, the activities of both SOD and GSH_{px} increased, probably because the enzymes were more available for extraction. Further membrane damage could have exerted a denaturing effect on the enzymes, to which the reactive intermediates of free radicals might have contributed with their destructive activity (Figs 8 and 9).

To summarize our results, we have adapted and elaborated various methods, each of which was found to be suitable for following the free radical reactions in foods. The chemiluminescence technique can be highlighted, because it allows an evaluation of the total defence activity against free radicals during a few minutes.

This work is to be regarded as an orientating experiment. For food industrial practice, the elaboration of new standardized methods and further experiments are needed.

THE EFFECTS OF VITAMIN E ON TISSUE OXIDATION IN NEPHROTOXIC (ANTI-GBM) NEPHRITIS

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ABSTRACT

The effects of vitamin E on tissue oxidation in nephrotoxic (anti-GBM) nephritis. The effects of vitamin E on the tissue oxidation, prostaglandin metabolism, kidney function and morphology were studied in rats with nephrotoxic nephritis. 36 nephritic animals received no treatment (group 1), while 36 were treated with vitamin E (group 2). 24 h after the administration of anti-GBM antibody, the renal protein SH was significantly lower in group 1 than in group 2 (0.70 ± 0.16 and 1.08 ± 0.06 mmol/100 g kidney tissue, respectively), suggesting a higher production of free oxygen radicals in group 1. The difference was similar on day 14. The thromboxane B_2 (TxB_2) level of the kidney cortex was significantly higher in group 1 than in group 2 on day 14 (0.18 ± 0.08 and 0.05 ± 0.01 nmol/g kidney tissue, respectively). The renal prostacyclin (PGI_2) production increased in both groups during the first 24 h, and decreased thereafter. The creatinine clearance was significantly lower in group 1 than in group 2 on day 1 (40 ± 30 and 204 ± 60 μ l/min/100 g bwt, respectively). The protein excretion was initially high in both groups, but a significant decrease was detected in group 2 relative to group 1 on day 14 (25 ± 18 and 92 ± 38 mg/24 h, respectively). The morphological changes were less severe in group 2. Vitamin E treatment did not alter any of the above values in healthy animals. The release of free oxygen radicals in nephrotoxic nephritis might play an important role in the pathogenesis, which can be influenced by free radical scavengers through changes of the Tx metabolism, kidney function and morphology.

INTRODUCTION

In experimental nephrotoxic nephritis (NTN) induced by the binding of anti-glomerular basement membrane antibody to the glomerular basement membrane (GMB), a proliferative glomerulonephritis develops with deposition of fibrin in the glomeruli, resulting in capillary lumen obliteration and progressive deterioration of the renal function. In the first (heterologous) phase, the heterologous antibody binds to the kidney GMB and induces acute

damage, mediated by the accumulated polymorphonuclear cells and complement (1). This event is associated with a release of inflammatory mediators and proteases, and with a marked increase in oxygen uptake and the activation of membrane-associated NADPH oxidase, usually resulting in the production of reactive oxygen products, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH\cdot$) and hypochlorous acid ($HOCl$) (2).

These reactive oxygen products are directly cytotoxic to normal cells (3, 4), and in vivo studies have demonstrated a critical role of these products in immune complex-induced vasculitis (5-7). The protection afforded against free oxygen radicals by the antioxidant enzymes superoxide dismutase (SOD) (5) or catalase (8, 9), or the use of chelates of iron or scavengers (10), can decrease the inflammatory reaction or prevent tissue injury. The delayed phase of the disease (autologous phase) is due to the host's immune response to heterologous antibody bound to the GBM. In this phase, proteinuria and renal insufficiency are associated with macrophage infiltration, fibrin formation and extracapillary proliferation (11).

The aim of our studies was to investigate the effect of vitamin E, a natural lipid phase antioxidant, on the kidney function, prostaglandin metabolism, tissue oxidation and histological injury in the heterologous phase of nephrotoxic serum nephritis.

MATERIAL AND METHODS

Glomeruli were isolated from healthy rat kidneys by the sieving technique and sucrose solution gradient centrifugation described by Portis et al. (12). GBM was obtained from the isolated glomeruli by sonication. Sonicated GBM was lyophilised. Anti-rat GBM antibody was produced in rabbits by intradermal injection of 10 mg GBM in an emulsion of Freund (in complete adjuvant once a week. After 5-7 weeks, the nephrotoxicity of the rabbit-anti-rat-GBM serum was determined via the dose required to induce proteinuria after intravenous injection into rats. The rabbits were bled, and the serum was collected. Complement was inactivated by heating at $56^\circ C$ for 30 minutes, and absorbed against rat red blood cells. The globulin fraction was obtained by precipitation with saturated ammonium sulphate. The precipitate was resuspended in phosphate-buffered saline solution and passed through a 0.22 μ filter. Anti-GBM antibody was administered to rats as a globulin fraction (anti-GBM-IgG). 5 mg/100 g bwt anti-GBM-IgG was administered i.v. into CFY female rats weighing 200-250 g. The animals were

treated or non-treated with 5 mg vitamin E/100 g bwt/day. The therapy was started 3 days prior to the administration of anti-GBM-IgG and the treated and non-treated animals were killed 3 h, 24 h or 14 days subsequently. There were 12 animals in each group. The 24 h urine was collected for creatinine clearance (C_{creat}) and urinary protein excretion (U_{prot}) measurements. Serum and urine creatinine were measured by the standard Popper-Mandel-Mayer method, and urinary protein was determined quantitatively by the biuret method following precipitation with 10% trichloroacetic acid. Protein SH and non-protein SH (reduced glutathione: GSH) in the renal tissue were analysed by the Sedlak technique with DTNB, Ellman's thiol reagent (13). The effects of the 2-week vitamin E treatment on C_{creat} , U_{prot} , GSH and protein SH were studied in healthy rats too. The prostacyclin (PGI_2) of the kidney cortex was measured as its stable hydrolysis metabolite (6-keto- $\text{PGF}_{1\alpha}$), and thromboxane A_2 ($\text{Tx}A_2$) was measured as $\text{Tx}B_2$ by specific radioimmunoassay (14, 15). Renal histological studies were carried out by light, immunofluorescence and electron microscopy. For light microscopy, formalin-fixed sections were processed by routine methods and stained with haematoxylin and eosin, trichrome staining according to Crossmon, silver pating by Jones, and PAS staining. For immunofluorescence studies, fluorescein-labelled goat anti-rabbit IgG was used in a dilution of 1:10 for direct immunofluorescence. For electron microscopy, renal tissue was fixed in glutaraldehyde and embedded in Durcupan.

The Student t-test (two-tailed analysis) was used to compare the differences in data derived from the various groups of animals.

RESULTS

In all rats, acute glomerulonephritis developed 24 h following anti-GBM-IgG administration. This was characterised by heavy proteinuria (102 ± 67 mg/24 h, $x \pm \text{SD}$) relative to the level prior to anti-GBM-IgG administration (2 ± 0.3 mg/24 h, $p < 0.001$). Those animals which were treated with vitamin E had a slightly lower level of protein excretion (75 ± 38 mg/24 h) than that of the non-treated group. On day 14, the difference between the treated and the non-treated group (25 ± 18 and 92 ± 38 mg/24 h, respectively) became significant ($p < 0.001$). In those non-nephritic animals which participated in vitamin E treatment for 14 days, the protein excretion was not changed (2 ± 1.6 mg/24 h) (Fig. 1).

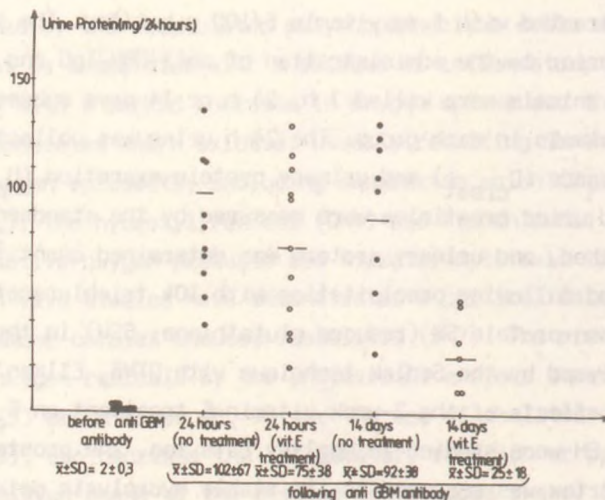


Fig. 1. Changes of urinary protein excretion in treated (vitamin E) and non-treated anti-GBM nephritis

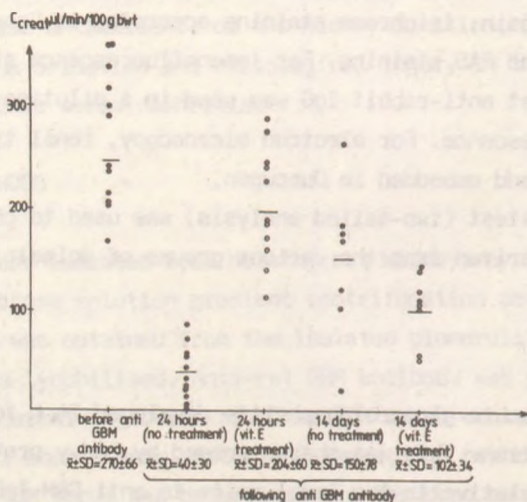


Fig. 2. Changes of creatinine clearance before and after anti-GBM-IgG administration (with and without vitamin E)

The level of C_{creat} became significantly lower in the non-treated nephritic rats (270 ± 66 and 40 ± 30 $\mu\text{l}/\text{min}/100$ g bwt before and 24 h after anti-GBM-IgG administration, respectively). C_{creat} remained significantly higher in the vitamin E-treated nephritic rats (204 ± 60 $\mu\text{l}/\text{min}/100$ g bwt,

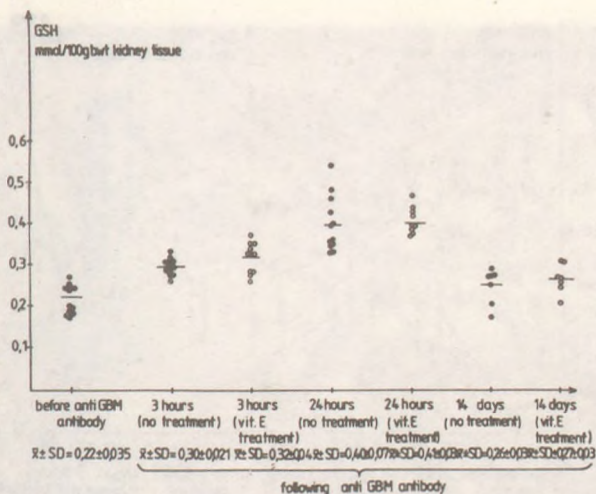


Fig. 3. Kidney non-protein-SH (GSH) values in treated (vitamin E) and non-treated anti-GBM nephritis

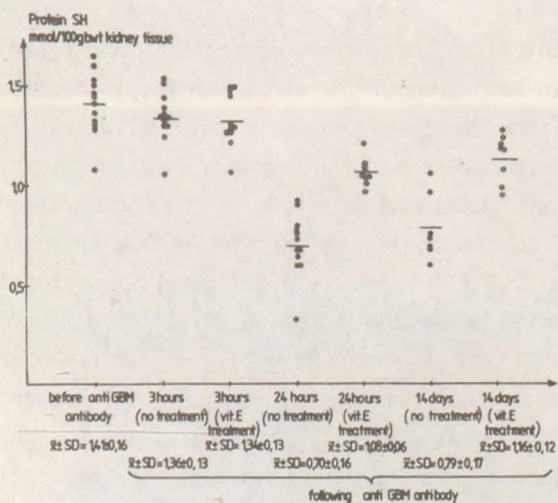


Fig. 4. The effect of vitamin E treatment on kidney protein-SH concentration in anti-GBM-nephritis

$p > 0.001$). 14 days later, there was no significant difference in C_{creat} between these groups (150 ± 78 and $102 \pm 32 \mu\text{l/min/100 g bwt}$ for the non-treated and treated nephritic animals, respectively). The 14-day vitamin E treatment itself did not alter C_{creat} in the healthy rats ($271 \pm 53 \mu\text{l/min/100 g bwt}$) (Fig. 2). Figures 3 and 4 show the changes in the GSH and

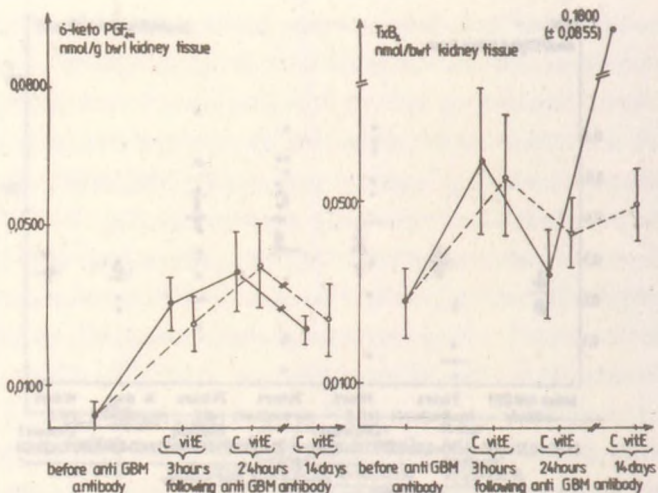


Fig. 5. The values of 6-keto-PGF₁ α and TxB₂ in the renal cortex before and after anti-GBM-IgG administration in treated and non-treated (vitamin E) rats.

C = control

protein-SH concentrations of the kidney prior to and following anti-GBM-IgG administration in both groups. The renal GSH level increased gradually in the first 24 h, then decreased nearly to the initial value on day 14. No significant difference was observed between the two groups (Fig. 3). In the first 24 h the protein-SH concentration of the kidney tissue decreased more markedly in the non-treated nephritic animals than in the treated rats (0.70 ± 0.16 and 1.08 ± 0.06 mmol/100 g kidney tissue for the non-treated and treated animals, respectively, $p < 0.001$). The difference was also significant ($p < 0.001$) on day 14 (0.79 ± 0.17 and 1.16 ± 0.12 mmol/100 g kidney tissue for the non-treated and treated rats, respectively). The GSH and protein-SH concentrations in the kidneys of the healthy rats treated with vitamin E for 14 days were the same as in the nephritic parallels treated with vitamin E.

The concentrations of renal cortical 6-keto-PGF₁ α and TxB₂ changed in parallel in the treated and non-treated nephritic rats. The level of the PGI₂ metabolite increased gradually in the first 24 h, then decreased by day 14. There was no significant difference between the two groups. Nevertheless, after the first 24 h the renal cortical TxB₂ production increased markedly in the non-treated animals, and a highly significant difference was seen by day 14 as compared with the vitamin E-treated group

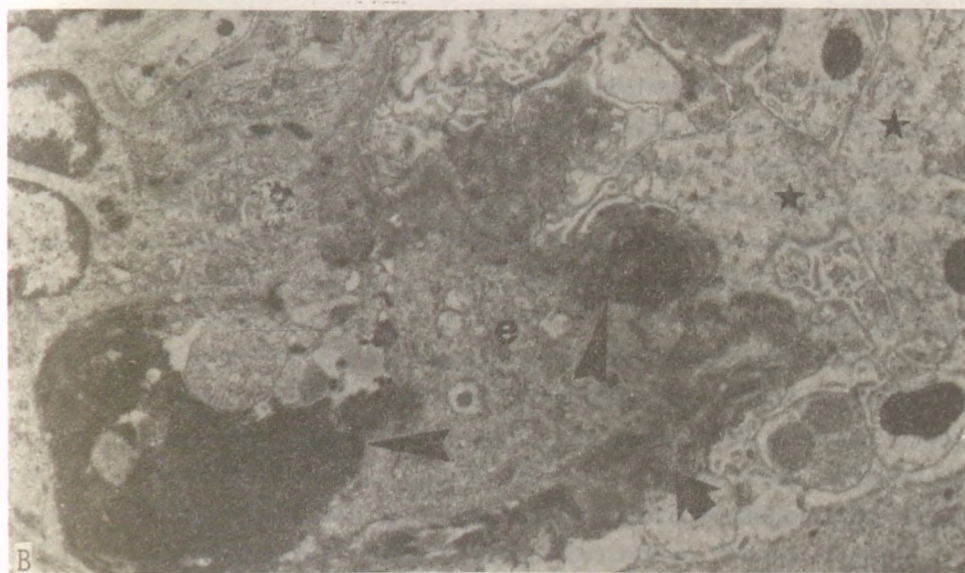
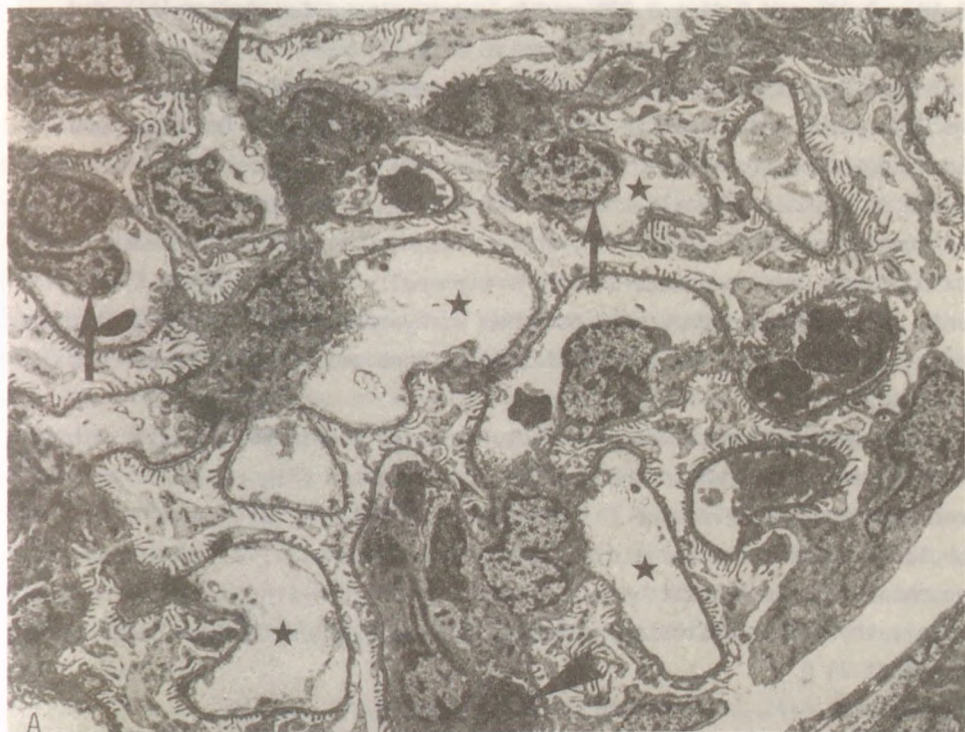


Fig. 6. a) Anti-GBM nephritis treated with vitamin E (24 h after anti-GBM-IgG administration). Well-preserved glomerular structure with opened capillary loops asterisks, with polymorphonuclear leukocytes (arrow-head) and mononuclear cells (arrows). (x 4000)
 b) Anti-GBM nephritis without treatment (24 h after anti-GBM-IgG administration). Electronmicroscopic picture showing wrinkling of glomerular basement membrane (arrow), fibrin thrombi in capillary loop (arrowheads), marked cellular swelling in the cytoplasm of podocytes (asterisks) and presumably endothelial cells (e). (x 10 000)

(0.18 ± 0.085 and 0.05 ± 0.008 nmol/g kidney tissue for the non-treated and treated nephritic groups, respectively) (Fig. 5).

The immunofluorescence investigation revealed a linear staining along the GBM, which had a similar intensity in the two groups. The light and electronmicroscopic studies indicated that the histological damage was less severe in animals with NTN treated with vitamin E, as compared with the non-treated animals. After 24 h, white blood cells, and particularly granulocytes, were accumulated in the glomeruli. Without vitamin E treatment, the capillary loops were occluded by hypertrophised endothelial cells and other materials. The endothelial cells were swollen and the GBM was wrinkled.

The mesangial matrix was markedly expanded and intraglomerular fibrin thrombi were also seen in the kidneys of this group (Fig. 6a, 6b). Hyalin-drop degeneration developed in the tubular epithelium, with some focal tubulo-interstitial sclerosis in both groups. On day 14, a more increased mesangial cell proliferation was observed in the non-treated group. In both groups, the tubulo-epithelial alterations were similar to those seen after the first 24 h.

DISCUSSION

The results of this study demonstrate the deterioration of the kidney function 24 h following anti-GBM-IgG administration. The decrease in C_{creat} was significantly smaller (about 50%) in the glomerulonephritis group treated with vitamin E than in the non-treated animals. Nevertheless, 2 weeks later, there was no significant difference between these two groups; at this time the value of C_{creat} was 50% smaller in both groups than that observed before treatment.

The severity of the proliferative glomerulonephritis was characterised by heavy proteinuria appearing in the first 24 h. This was not reduced by vitamin E during the first day following anti-GBM-IgG injection, but a significantly lower protein excretion was observed in the same group 14 days later. Similar results were observed by Bertani et al. (16).

The histological damage was less severe in NTN animals treated with vitamin E as compared with the non-treated nephritic animals. After 24 h, white blood cells, and particularly granulocytes, were accumulated in the glomeruli. Without vitamin E treatment, the capillary loops were occluded with intraglomerular fibrin deposits, the endothelial cells were swollen and the mesangial matrix expanded.

Anti-GBM-nephritis is a neutrophil-dependent model of immune complex-induced tissue injury. These cells produce an array of potent biological mediators, including arachidonate metabolites, platelet activating factors, proteases cationic proteins and reactive oxygen products. Reactive oxygen products appear to be central to the development of the kidney damage. The most likely mechanism of this activation is through immune complex attachment to the cell membrane via the Fc receptor (17, 18). Inflammatory cells produced by platelets enhance the oxidant burst activity of IgG immune complex-stimulated neutrophils. This platelet stimulatory factor is a platelet-derived adenine nucleotide (ATP, ADP) (19).

Neutrophils appear to be the source of the oxidants in IgG-induced injury in anti-GBM-nephritis. Hydrogen peroxide, the OH radical and myeloperoxidase products such as hypochlorous acid (HOCl) may be important in the pathogenesis (17). Treatment of animals with catalase is markedly protective, with 100% suppression of proteinuria (8). In our experiments, the renal protein-SH level as an indicator of the antioxidant effect was significantly higher on days 2 and 14 in the group of NTN animals treated with vitamin E.

The GSH in the kidney was higher in both groups 3 and 24 h after anti-GBM-IgG administration, but no difference was observed between the results for the treated and non-treated groups.

Several sources of PG and TxA_2 synthesis can be implicated in this model of glomerular immune injury. Blood-borne cells (leukocytes, monocytes and platelets) recruited to sites of antigen-antibody interaction and complement activation along the GBM could account for the enhanced rates of PG and TxA_2 synthesis. An endogenous glomerular source of prostanoids is also possible, since both epithelial and mesangial cells are capable of synthesising PG and thromboxanes (20, 21).

Mesangial cell proliferation is a common feature of nephrotoxic glomerulonephritis, and was seen during the heterologous phase in our experiment. The syntheses of TxB_2 and 6-keto-PGF₁ α were increased within 3 h. Without vitamin E treatment, the TxB_2 level continued to increase after the first 24 h. Vitamin E therapy inhibited the further enhancement in TxB_2 production, but no difference was seen between the values of 6-keto-PGF₁ α in the two groups of animals. By day 14, the level of 6-keto-PGF₁ α was lower than after the first 24 h, but it was still higher than before anti-GBM-IgG administration. Lianos et al. (22) observed increased syntheses of TxB_2 and PGF₂ α in the kidney within 2 h, while

while PGE_2 and 6-ket-PGF $_{1\alpha}$ were enhanced 3 h following nephrotoxic serum (NTS) infusion. On day 2, TxB_2 showed the greatest increment, and throughout the rest of the 2-week course only the TxB_2 synthesis remained enhanced.

A key feature of anti-GBM-IgG-induced haemodynamic changes is the reduction of the whole kidney and single nephron glomerular filtration rate (GFR). Biochemical inhibition of TxA_2 syntheses in the glomeruli prevented the expected decrements of GFR, renal plasma flow (RPF) and the filtration fraction 2 and 3 h after NTS infusion. In the study of Lianos et al. (22), a partial recovery of the GFR occurred at 3 h, despite further increments in glomerular TxA_2 synthesis. This recovery coincided with significant increments in the syntheses of glomerular PGE_2 and 6-keto-PGF $_{1\alpha}$.

These observations support a role for these vasodilatory PG-s in mediating the partial recovery in GFR and RPF 3 h after NTS infusions. In our study, a significantly lower C_{creat} was observed during the first 24 h in the non-treated nephritic animals than in the vitamin E-treated rats, but at the same time this was associated with significantly higher renal 6-ket6-PGF $_{1\alpha}$ and TxB_2 levels in both groups. On the other hand, a higher renal TxB_2 concentration on day 14 was not accompanied by a significantly lower C_{creat} in the non-treated group as compared with the vitamin E-administered animals. These results suggest the role of other vasocactive substances influencing the effects of PGI_2 and TxA_2 . Nevertheless, the kidney protein-SH was significantly lower, and the morphological changes were more severe in the non-treated nephritic kidneys on either day 2 or 14. In our study, the effect of free oxygen radicals was well characterised by the increased GSH and the decreased protein-SH levels in the kidney tissue. These were associated with early increases in the PGI_2 and TxA_2 syntheses.

The effects of the free oxygen radicals, the early damage to the kidney function and morphology, and the consecutive proteinuria, were influenced advantageously by vitamin E. These findings suggest that the release of free oxygen radicals in anti-GBM-nephritis might play an important role in the pathogenesis, which can be influenced by free radical scavengers through changes of the Tx metabolism, kidney function and morphology.

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EFFECTS OF IONISING RADIATION,
H₂O₂ AND S-2-AMINOETHYL ISOTHIOURONIUM BROMIDE,
HBr ON THE SURFACE OF RED BLOOD CELLS

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INTRODUCTION

The outer surface of the plasma membrane has both cationic and anionic groups, which under physiological conditions produce a net negative surface potential of the cells (1-3).

The negative surface potential is changed after chemical or physical alteration of the cell surface structure (4, 5) or change of the functional status of the cells (6-8).

Because of the important role of the plasma membrane in the functional integrity of cells, the cell membrane is the main target of ionising radiation (9-11) and/or active oxygen radicals (12-14). The main aim of recent work was to investigate whether measurement of the cell surface net negative potential could be a useful method of obtaining information about the altering effects of ionizing radiation or active oxygen radicals.

For this purpose, we have measured the electrophoretic mobility (EPM or EFM) of cells. This parameter is proportional to the surface potential of the cells.

METHODS

With sodium citrate (3.3%)(Richter Pharmaceuticals) as anticoagulant, blood (blood:sodium citrate ratio 9:1) was collected from the male CFLPY mouse. After washing (in PBS, 10 min., 4 °C, 1000 rpm, three times) the blood cells (RBCs) were resuspended in MEM without glutamine (Phylaxia) or in PBS. The cell concentration in the first group was 5x10⁶/ml and in the second group 10⁶/ml.

The RBCs in the MEM were irradiated with different ^{60}Co gamma doses (0.5, 1.0, 1.5, 2.0, 2.5, 5.0 and 10.0 Gy). After washing and resuspending of the cells, the EPM of the RBCs was determined in all cases, 1, 2 and 3 hrs after irradiation.

The RBCs in PBS were treated with different concentrations (M-M) of S-2-ethylaminoisothiuronium bromide. HBr (AET, Serva) or with different concentrations (0.05, 0.025, 0.005 or 0.0025%) of H_2O_2 . One group received H_2O_2 after 5 minutes of preincubation with mol AET.

The electrophoresis was carried out by the microscopic method described earlier (15). The transition time of 20-30 were as follows: 10 mA, 50 μ , 22 $^\circ\text{C}$. Statistical analysis was carried out with Student's two-sample test.

RESULTS

^{60}Co gamma-irradiation, even in a relatively small dose, produces a decrease of the EPM of RBCs. Higher doses induce greater decreases of the EPM. The effect can already be seen 1 hour after irradiation, but after 2-3 hours, after irradiation the effect is more pronounced ($p < 0.05$) (Fig. 1).

H_2O_2 significantly decreases the EPM of RBCs only at higher concentrations (0.025-0.05%). (The decrease in EPM at shorter incubation times is greater ($p < 0.05$) (Fig. 2).

RBCs were incubated with different concentrations of AET for different periods of time. As can be seen in Fig. 3, AET within a wide concentration range has no significant effect on the EPM of RBCs ($p < 0.05$).

When H_2O_2 was added to the RBCs after preincubation with mol AET, the EPM of the RBCs decreased significantly ($p < 0.01$), even at low H_2O_2 concentration. During the incubation time, the EPM changed significantly. The EPM at the 5th minute was lower at all H_2O_2 concentrations used than after 60 minutes ($p < 0.05$) (Fig. 4).

AET at higher concentration (M conc.) exerts H_2O_2 -scavenging activity, as shown by the H_2O_2 -induced luminol-dependent chemiluminescence. Lower concentration (M conc.) of AET exerted co-oxidant features, increasing the emitted light generated by the reaction of H_2O_2 with luminol (Fig. 5).

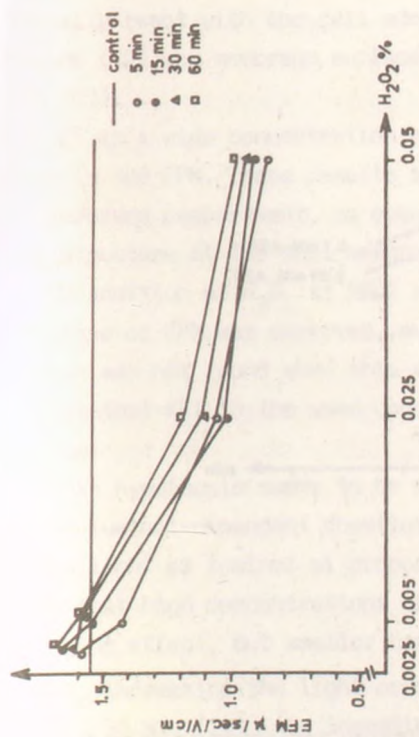


Fig. 2.

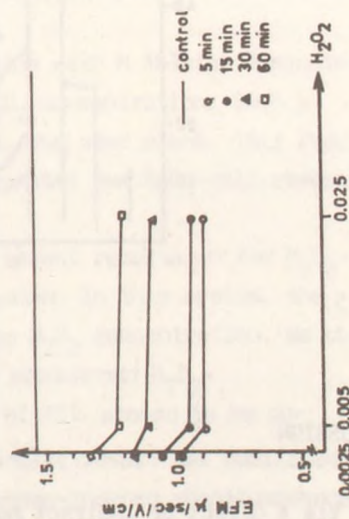


Fig. 4.

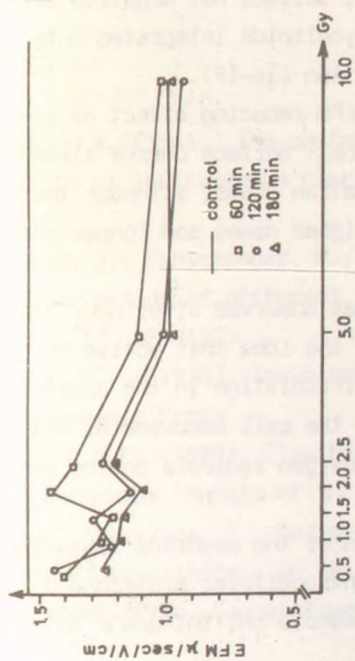


Fig. 1.

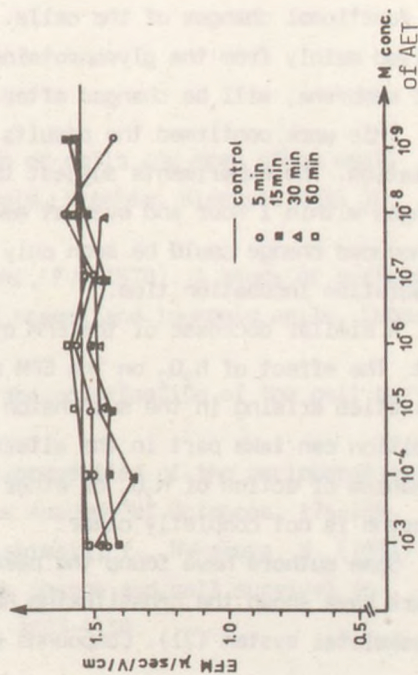


Fig. 3.

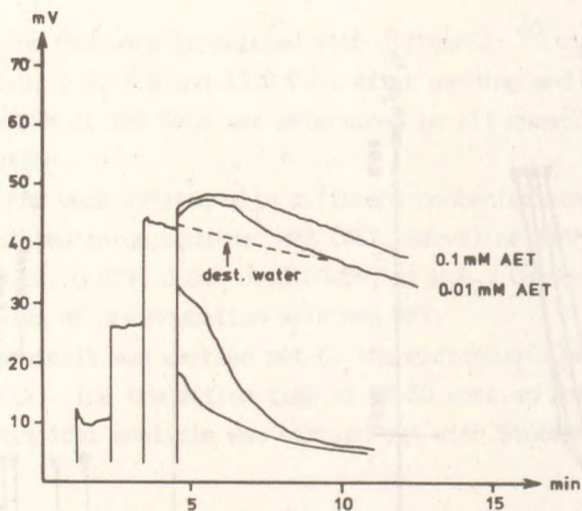


Fig. 5.

DISCUSSION

Via a direct or indirect mechanism, ionizing radiation is able to alter the structure of the cell membrane, causing a number of morphological and functional changes of the cells. The cell surface net negative charge, derived mainly from the glycoproteins and glycolipids integrated into the cell membrane, will be changed after irradiation (16-19).

This work confirmed the results on the EPM-reducing effect of ionizing radiation. The experiments suggest that the cell surface charge already changes within 1 hour and even at small radiation doses, although the most pronounced change could be seen only after higher doses and longer post-irradiation incubation time.

A similar decrease of the EPM of RBCs was observed after H_2O_2 treatment. The effect of H_2O_2 on the EPM supports the idea that active oxygen derivatives arising in the suspension during irradiation in the aerated condition can take part in the alteration of the cell membrane of RBCs. The mechanism of action of H_2O_2 or other active oxygen radicals on the cell membrane is not completely clear.

Some authors have found the peroxidation of the membrane lipid (20); others have shown the crosslinking between intracellular protein and the cytoskeletal system (21). Compounds able to adsorb on, integrate into, or

chemically react with the cell membrane compartment are able to change the membrane (22) and membrane surface structure, causing changes of the EPM of cells (23).

AET in a wide concentration range did not lead to a decrease or increase in the EPM. These results suggest that AET does not react with the cell membrane compartment, or even if it does then it does not alter the basic structure of the cell membrane.

On addition of H_2O_2 to RBCs after incubation with M AET for 5 minutes, a decrease of EPM was observed, even at low H_2O_2 concentration. Such a decrease was not found when this amount of H_2O_2 was used alone. This result suggests that AET in the used concentration promotes the H_2O_2 -cell membrane reaction.

This hypothesis seems to be supported by recent results on the H_2O_2 -induced luminol-dependent chemiluminescence system. In this system, the emitted light of luminol is proportional to the H_2O_2 concentration. We observed that high concentrations of AET have a pronounced H_2O_2 -scavenging effect, but smaller concentrations of AET proved to be co-oxidant, increasing the light production. A similar result was published by Budavári et al. (24), who investigated the zymosan-induced chemiluminescence of polymorphonuclear granulocytes in the presence of exogenous and endogenous scavengers.

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BLOOD LIPID PEROXIDATIVE (LP) PARAMETERS IN DAIRY COWS WITH VARIOUS LIVER LIPID CONTENTS

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INTRODUCTION

We have relatively little knowledge on the LP processes, physiological or pathological, of ruminants, even though it has long been known that an increased LP plays a great role in Se and vitamin E deficiency syndromes in all species (7).

In dairy cows around calving, a physiological fatty liver develops, i.e. the total lipid content of the fresh liver is increased up to 100-120 g/kg (10-12%). This is a temporary phenomenon, however, which disappears 1-2 months after calving, and from then on the lipid content of the liver is about 4-6% again, which is a typical value for healthy mammals (3). It is mainly in high-producing dairy cows that we can see a liver with a much higher fat content and various clinical symptoms as a consequence. This is called a fatty liver syndrome or a lipid mobilization syndrome, which is not regarded as an independent liver disease, but as a general functional disorder in the whole lipid metabolism of an animal (6).

LP processes have an important role in the pathogenesis of fatty liver of various origins, for example the fatty liver in alcoholic humans or the fatty liver developing in all species after CCl_4 toxicosis. Accordingly, we have studied the blood LP parameters in dairy cows with various liver lipid contents after parturition.

MATERIALS AND METHODS

The lipid contents of liver biopsy samples and the LP parameters of the blood were determined in dairy cows from two large herds one week after

calving. Twenty-six healthy cows from Herd A and 11 healthy cows from Herd B were investigated. The lipid contents of liver biopsy samples were extracted and determined gravimetrically in the chloroform-methanol-water system described by Atkinson et al. (1). Of the LP parameters, the activity of glutathione peroxidase (GSHPX) was determined according to Lawrence and Burk (4), and that of catalase according to Beers and Sizer (2). Thiobarbituric acid reactive metabolites, expressed as malonyl-dialdehyde (MDA), were measured photometrically (5).

On the basis of the fat contents of the liver biopsy samples, two groups were formed retrospectively for each herd, a fatty liver group and a control one.

RESULTS

For Herd A, 11 cows belonged to the fatty liver group and 15 cows to the control group. The LP parameters are shown in Table I.

The results indicate increased LP processes in the fatty liver group.

Table I. Blood LP parameters of Herd A

	Control group (n=15)	Fatty liver group (n=11)	t-test
Liver lipid, %	9.88	16.13	p < 0.001
Blood plasma MDA, $\mu\text{mol/l}$	2.73	5.77	p < 0.001
Hemolysate MDA, $\mu\text{mol/l}$	1.56	4.06	p < 0.001
Blood plasma GSHPX, U/g protein	0.33	0.20	p < 0.01
Hemolysate GSHPX, U/g protein	3.04	2.64	NS
Hemolysate catalase, B.U./g protein	105	80	p < 0.01

For Herd B, where both the type of cow and the nutritional methods differed from those in Herd A, the liver fat contents were significantly higher than those found for Herd A. For Herd B, all 6 animals in the fatty liver group and all 6 in the control group exhibited higher liver lipid contents than the respective groups of Herd A. The lipid parameters of Herd B are shown in Table II.

Table II. Blood LP parameters of Herd B

	Control group (n=5)	Fatty liver group (n=6)	t-test
Liver lipid, %	13.4	20.93	p < 0.001
Blood plasma MDA, $\mu\text{mol/l}$	2.62	1.95	NS
Hemolysate MDA, $\mu\text{mol/g Hb}$	0.34	0.32	NS
Blood plasma GSHPX, U/g protein	0.64	0.60	NS
Hemolysate GSHPX, U/g Hb	11.31	9.85	NS
Hemolysate catalase B.U.* /g Hb	250	249	NS

*B.U. = Bergmeyer unit

For Herd B, we could not find such significant differences between the fatty liver group and the control group as those found between the respective groups of Herd A.

DISCUSSION

The LP results obtained for the two herds indicate that the LP processes can differ even with cows in identical physiological state.

These differences can be explained by the difference in the type of cow, the nutritional system, the milk production, etc.

For Herd A, the differences in liver lipid contents and in LP parameters run parallel.

We assume that for Herd B, that we did not find significant differences between the LP parameters in the fatty liver and the control groups was due to the already elevated liver fat contents, even in the control group the fact.

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LIPID PEROXIDATION IN SERONEGATIVE SPONDYLARTHROSIS

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The importance of toxic oxygen reduction products in the pathogenesis of inflammatory joint disease is increasingly recognised. Exposure to superoxide radical and hydrogen peroxide in vitro leads to the degeneration of hyaluronic acid, collagen and cartilage constituents. However, little is known about the production of these free radicals in seronegative spondylarthritides and the assessment of the basic defense mechanism against them. The aim of our study was to collect more information about the biochemical changes due to lipid peroxidation in the serum and synovial fluid of ankylosing spondylitis (AS) and psoriatic patients (PS).

Thirteen male patient with definite AS according to the New York criteria, and fourteen patients with PS, were included in the study. The following laboratory parameters were measured: malondialdehyde, SH concentration, glutathione peroxidase, catalase and peroxidase.

The results suggest that there are no major differences in the lipid peroxidation activity of the serum between the AS and PS groups. In the synovial fluid, the MDA content was higher in the AS than in the PS group, which might reflect a locally increased inflammatory process. The SOD activity in the synovial fluid was higher than that in the serum. This extracellular activity was relatively low, but it may be potentially important as an antiinflammatory agent. A significant increase in lipid peroxidation (MDA) and a decrease in scavenger enzyme activity were found in both groups of patients compared to the controls.

AUTOXIDATION TEST FOR MEASURING THE LIPID PEROXIDATION CAPACITY OF LIVER TISSUE

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The superoxide dismutase activity and the kinetics of lipid peroxide formation during incubation at 37 °C for 3 h under aerobic conditions were measured in liver tissue homogenates of young and old female BALB/c mice. The autoxidation test was used to measure the lipid peroxidation capacity of liver tissue.

The lipid peroxidation capacity was found to be remarkably high in young mice and low in old ones. It was reduced in young mice pretreated with allopurinol.

Superoxide dismutase levels were equal in young and old mice. A hypothesis was set up in order to explain the biochemical mechanism of the lipid peroxidation capacity of liver tissue in mice of different ages.

INTRODUCTION

Lipid peroxidation has long been known to be responsible for numerous effects observed in biological systems and provides a very attractive hypothesis for explaining many diseases and the ageing phenomenon (8).

Oxygen radicals could be responsible for lipid peroxidation, the degradative oxidation of polyunsaturated fatty acids leading to destruction of the membrane structure, to the deterioration of membrane-bound enzymes and eventually to cell death too.

Although there is circumstantial evidence to support the role of lipid peroxidation in tissue injury associated with ageing and many diseases, it has been technically difficult to demonstrate directly the lipid peroxidation capacity or peroxide-producing potential of different tissues.

Much evidence indicates that the sensitivity of tissue homogenates to autoxidation in vitro reflects their normal in vivo sensitivity to similar oxidative processes (3).

Aerobically incubated tissue homogenates spontaneously undergo autoxidation. Thus, the autoxidation of aerobically incubated liver tissue

homogenate in our experiments appears to represent the net sensitivity of a tissue against oxidative damage.

METHODS

Examinations were carried out on young (1.5-month-old) and old (24-month-old) inbred female BALB/c mice. First, the liver tissue homogenates prepared from untreated young and old mice were compared.

One group of young mice, however, was treated with allopurinol (Milurit, Chemical Works of Gedeon Richter Ltd., Budapest, Hungary). These animals received 100 mg/kg allopurinol by esophageal cannula 12 and 1 hours before slaughtering. The sham-treated group of young mice received physiological saline by cannula.

The mice were killed and determinations were carried out on the homogenates of the liver. The crude liver homogenates were prepared in 20 vols. of 0.05 M K_2HPO_4 solution (pH 7.8). The homogenates were centrifuged at 8000 g for 15 min and aliquots of the supernatant or its appropriate dilutions with the phosphate buffer were used for superoxide dismutase (SOD) and protein measurements.

The SOD activity was measured by the Misra and Fridovich (14) method, using the epinephrine-adrenochrome reaction. The protein contents of the homogenates were determined by the method of Lowry et al. (11).

The lipid peroxidation capacity of the mouse liver tissue homogenates was measured via the thiobarbituric acid colour reaction (10). Tissues were thawed, minced and washed in ice-cold 0.15 M NaCl solution to remove all traces of blood. Minimal amounts of blood were found to have no significant effect on the rate of peroxidation. One gram of tissue (wet weight) was added to 19 ml of 0.15 M NaCl solution and homogenized; the supernatant was used after centrifuging for 5 min at 400 g and at 4 °C. The supernatant of the tissue homogenate was suspended in a suitable amount of 0.15 M Tris-HCl buffer at pH 7.4 so as to give a final protein concentration of 4.5 mg/ml. This incubation mixture in an Erlenmeyer flask with a glass stopper was placed in ice.

No difference was found in TBA values with or without the use of antibiotic for up to 3 hours of incubation.

After the zero-time sample had been taken, the flasks were incubated at 37 °C for 3 hours in a rotary water bath.

Two-ml samples were withdrawn from the flasks at timed intervals and

the TBA assay method of Hunter et al. (10) was employed with only minor modification to follow lipid peroxide formation.

The uncorrected absorbance measurements at 532 nm indicate the total TBA-reacting material, expressed in μM malondialdehyde per mg protein. Although the TBA assay does not measure total peroxides, it does provide a reasonable approximation of the relative peroxide concentration produced during incubation (17). The TBA-reactive material correlates well with the production of chemiluminescence, oxygen consumption, the loss of unsaturated fatty acids (17) and the concentration of peroxides determined by other methods (18).

RESULTS

Typical data on the kinetics of autoxidation of liver tissue homogenates for young and old BALB/c mice are shown in Fig. 1. There is a significant difference in the rate of reaction after the first hour, as the plateau value has already been reached in the liver homogenates of old mice, but the rate of reaction increases further as a function of time in the homogenates of young ones. The amount of peroxides detected at zero time was nearly the same in both age groups.

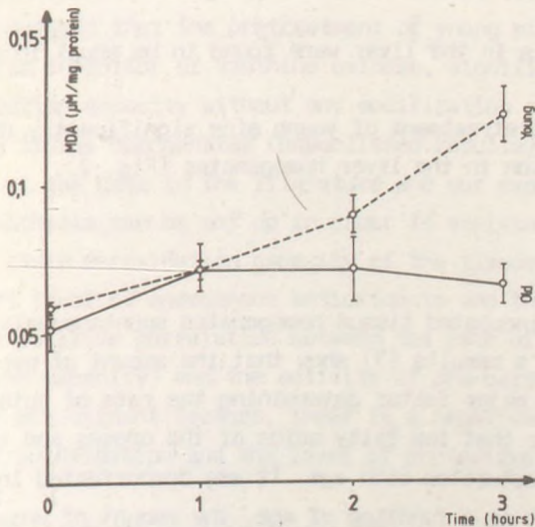


Fig. 1. Liver lipid peroxidation capacity in young and old female BALB/c mice

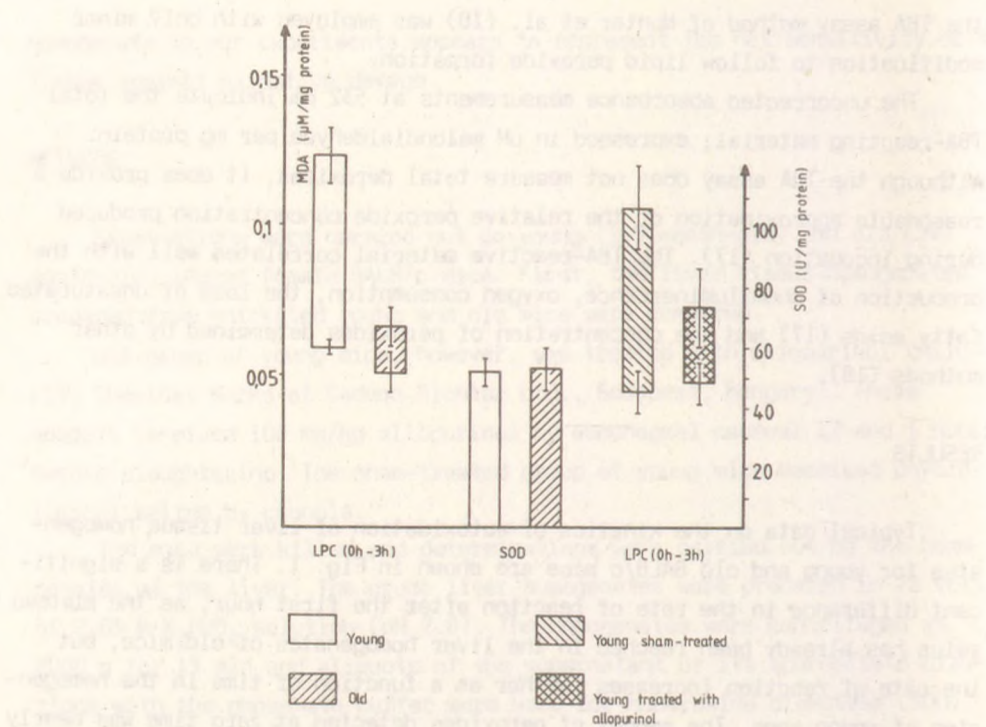


Fig. 2. Liver lipid peroxidation capacity (LPC) and SOD activity in young, old, sham-treated and allopurinol-treated young female BALB/c mice.

The SOD levels in the liver were found to be equal in young and old mice.

Allopurinol pretreatment of young mice significantly decreased the rate of autoxidation in the liver homogenates (Fig. 2).

DISCUSSION

Aerobically incubated tissue homogenates spontaneously undergo autoxidation. Cutler's results (3) show that the amount of peroxidizable substrate may be the major factor determining the rate of autoxidation. It is generally accepted that the fatty acids of the organs and tissues of the body become more saturated with age. It was demonstrated in Hall's (7) investigations, that as a function of age, the amount of unsaturated fatty acids decreases due to the diminished activity of the desaturases. Fatty acid compositional changes have also been reported to occur with age in the microsomal and mitochondrial membranes of the liver of both rats and mice

(6, 9). Thus, whereas there may be a general trend for fatty acid compositional changes in tissues with age, the reduction in the lipid peroxidation capacity in liver homogenates of old mice can be regarded as a consequence of the reduced amount of polyunsaturated fatty acids. This hypothesis has been supported by demonstrating a positive correlation between the lipid peroxidation capacity and the level of arachidonic acid in liver homogenates of old CBA/Ca mice treated with 2-mercaptoethanol (15).

A study of the literature, together with the data discussed above, raised a hypothetical question about the source of superoxide radicals in the autoxidation of liver homogenates. Xanthine oxidase was the first documented biological source of $O_2^{\cdot-}$ (13). The liver was found to be particularly rich in this enzyme, although much evidence suggested that the *in vivo* form of the enzyme is not the superoxide-producing oxidase, but rather an NAD^+ -reducing dehydrogenase.

When liver tissue is homogenized, the dehydrogenase converts rapidly to the oxidase as a result of sulfhydryl oxidation or limited proteolysis (1, 4). McCord and Roy (12) have presented evidence that the conversion of xanthine dehydrogenase to xanthine oxidase does in fact occur *in vivo* in ischemic tissues. The allopurinol-induced specific inhibition of xanthine oxidase provides a limited generation of $O_2^{\cdot-}$ and an effective protection against shock and oxidative damage in ischemic tissues (2, 5, 16).

Our results suggest that the pretreatment of young mice with allopurinol, a specific inhibitor of xanthine oxidase, significantly decreases the lipid peroxidation capacity without any modification of the SOD level measured in liver tissue homogenates (unpublished results).

As a result of the data in the literature and our experimental results, the following hypothesis can be set up in order to explain the biochemical mechanism of the lipid peroxidation capacity of the tissues (Fig. 3).

At a constant level of endogenous antioxidants and the repair mechanisms, there is a positive correlation between the rate of autoxidation (lipid peroxidation capacity) and the activity of pro-oxidant factors. At a constant level of pro-oxidant factors, there is a negative correlation between the rate of autoxidation and the level of protective, scavenging and repair mechanisms.

The lipid peroxidation capacity of the tissues reflects the state of a finely regulated counterbalance of the pro- and antioxidant factors in the organisms.

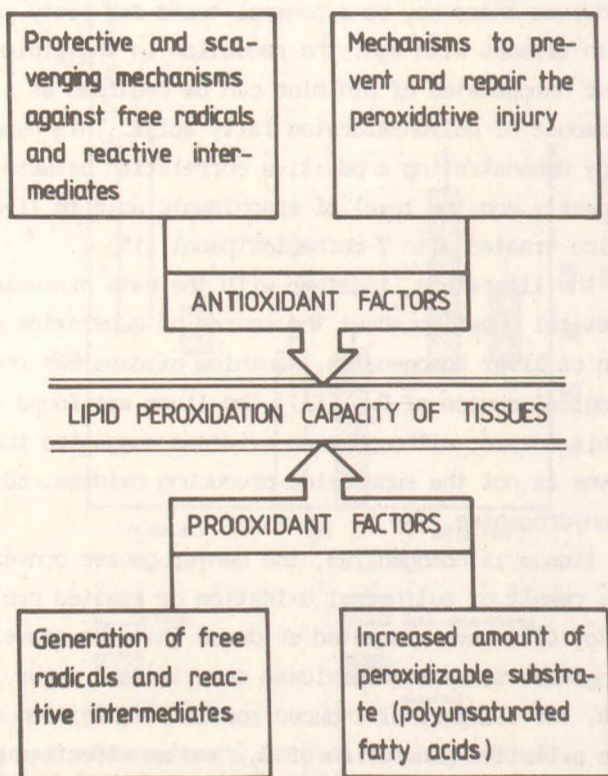


Fig. 3. Antioxidant and pro-oxidant factors controlling lipid peroxidation capacity of tissues

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INFLUENCE OF SUPEROXIDE DISMUTASE AND ILOPROST
ON ISCHEMIA-INDUCED ARRHYTHMIAS AND
POSTISCHEMIC MYOCARDIAL FUNCTION

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SUMMARY

The regional systolic shortening (RSS) in "open chest" rats was measured directly in the temporarily ischemic region. Superoxide dismutase (SOD) was injected into the carotid artery during the 5th min both of coronary occlusion (CO) and of the reperfusion (R) period. Iloprost infusion into the carotid artery began 5 min after CO and lasted for 2 hours. The postischemic myocardial function (PIMF) was improved by 2 x 12,000, 2 x 20,000 and 2 x 40,000 U/kg SOD. Iloprost in a dose of 200 ng/kg.min was also effective. Iloprost in a dose of 100 ng/kg.min combined with 2 x 20,000 U/kg SOD produced the same effects as SOD alone. The arrhythmia score (AS) in the early phase of ischemia was lowered significantly by SOD in doses of 20,000 and 40,000 U/kg. Iloprost had no antiarrhythmic effects in this model.

INTRODUCTION

The myocardial function is reduced after short ischemia (15 to 20 min; "stunned myocardium") (Ito et al. (1); Greenfield et al. (2); Farber et al. (3). The application of SOD or Iloprost enhances the PIMF (Przyklenk et al. (4); Gross et al. (5); Farber et al. (3)). Moreover, both drugs may reduce the infarct size in cases of longer (1-2 hours) ischemia: SOD: Fincke et al. (6); Näslund et al. (7); Ambrosio et al. (8); Werns et al. (9); Chambers et al. (10); Jolly et al. (11); Iloprost: Chiariello et al. (12); Simpson et al. (13); De Langen et al. (14).

The reduction in infarct size need not to be accompanied by an improvement of the PIMF in the central parts of the ischemic area. However, this seems possible, because SOD and Iloprost may influence the free radical-dependent reperfusion syndrome.

The arrhythmic events in the early phase of ischemia are reduced by the application of SOD (Riva et al. (15); Bernier et al. (16)). For Iloprost,

this effect also seems to be possible (Cocker et al. (17); De Langen et al. (14)). Therefore, in this study we measured the RSS directly in the inner zone of the temporarily ischemic area after one hour of CO. Furthermore, we investigated the influence on the early ischemic arrhythmias. It likewise seemed interesting to evaluate the effects of human and bovine copper-zinc SOD in comparison with Iloprost. In addition, the effect of the combined application of these drugs was studied.

MATERIALS AND METHODS

Our studies were performed on urethane-anesthetized (1.25 g/kg) Wistar rats of both sexes; body mass 250-300 g. A carotid catheter was connected with a combined system for measuring the pressure, drug injection and continuous infusion (Fig. 1). After thoractomy, the heart was placed in a plastic cradle. The trunk of the left coronary artery was underpassed, using atraumatic suture materials. The temporary CO for one hour was performed by stretching the thread. Thus, the coronary trunk was compressed by a support set against. The RSS was measured directly in the supply area of this coronary artery (see A and B in Fig. 1). A special gage using light-conducting fibers (diameter 0.15 mm) was employed. The principle of this gage is illustrated in Fig. 2.

The light in the quartz fiber Fl_a leaves the fiber in the bend dependent on its radius. The ends of fibers F₂ and F₃ are placed in the myocardium (see A and B in Fig. 2). F₂ and F₃ transmit the myocardial dynamics to fiber Fl and alter the radius of the bend. The light from fiber Fl_b is nearly proportional to the distance A-B.

In order to assess the PIMF, the RSS was determined as a fraction of the preischemic values in the 60th min of the reperfusion period (see Fig. 4). The arrhythmic score (AS) was determined as the number of arrhythmic events per 1,000 heart beats between the 6th and 30th min of ischemia, as an average for this period (See Fig. 5).

The influence of the following drugs was investigated:

- | | |
|----------------------------|---|
| a) Bovine erythrocyte SOD: | "Oxodisprop" preparation |
| (b SOD groups) | (University of Rostock, Institute of Pharmacology and Toxicology; U. ZELCK and U. KARN-STEDT) |
| b) Human erythrocyte SOD: | Preparation: National Institute |

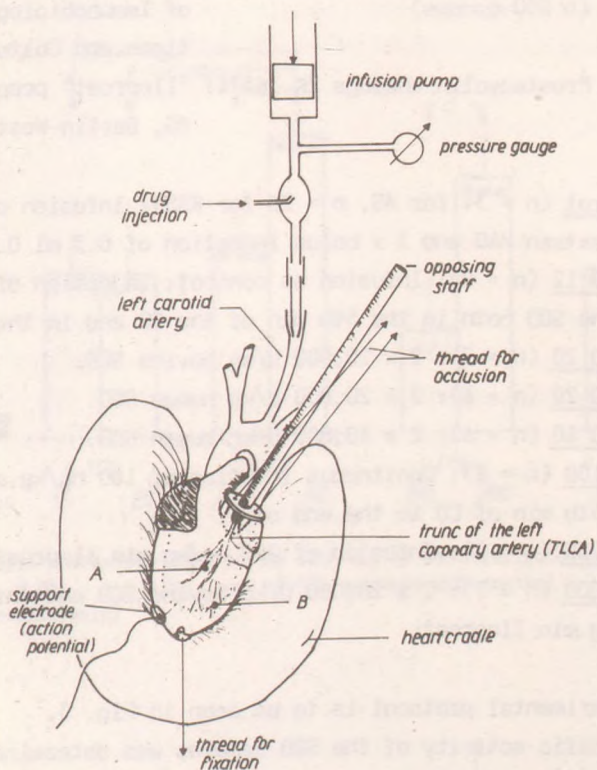


Fig. 1. Scheme of preparation and instrumentation

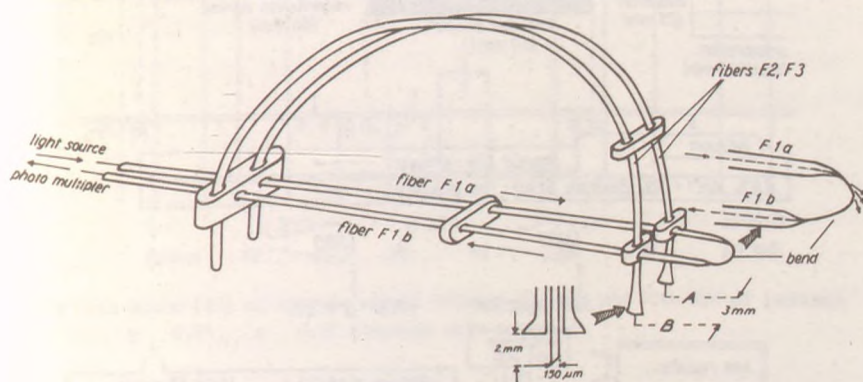


Fig. 2. Principle of the gage for measuring the myocardial dynamics

(h SOD groups) of Immunobiological Preparations and Culture Media, Berlin

c) Prostacyclin analogs ZK 36374: "Iloprost" preparation, Schering AG, Berlin-West

Groups: Control (n = 34 for AS, n = 26 for RSS): Infusion of 0.9% NaCl with 5% Dextran M40 and 3 x bolus injection of 0.5 ml 0.9% NaCl.

B SOD 12 (n = 8): Infusion as control; injection of 12,000 U/kg bovine SOD both in the 5th min of the CO and in the 5th of R.

B SOD 20 (n = 7): 2 x 20,000 U/kg bovine SOD.

H SOD 20 (n = 6): 2 x 20,000 U/kg human SOD.

H SOD 40 (n = 6): 2 x 40,000 U/kg human SOD.

Ilo 100 (n = 6): Continuous infusion of 100 ng/kg.min Iloprost from the 5th min of CO to the end of R.

Ilo 200 (n = 8): Infusion of 200 ng/kg.min Iloprost.

Ilo/SOD (n = 7): 2 x 20,000 U/kg bovine SOD and infusion of 100 ng/kg.min Iloprost.

The experimental protocol is to be seen in Fig. 3.

The specific activity of the SOD samples was determined according to McCord and Fridovich (26) at the beginning of the experiments.

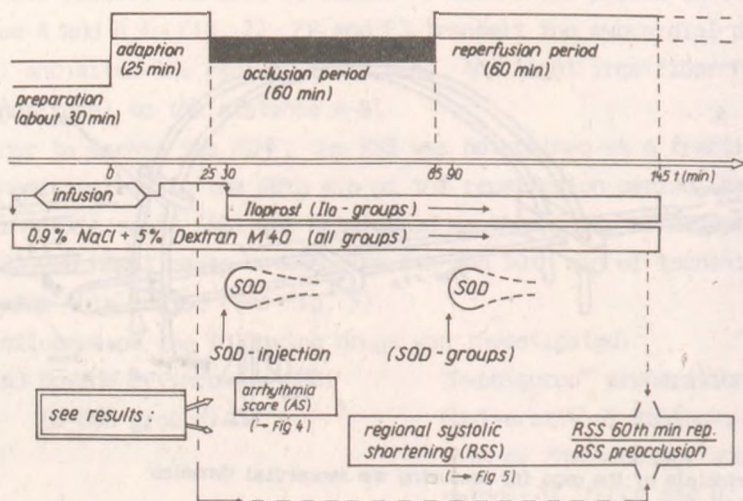


Fig. 3. Experimental protocol

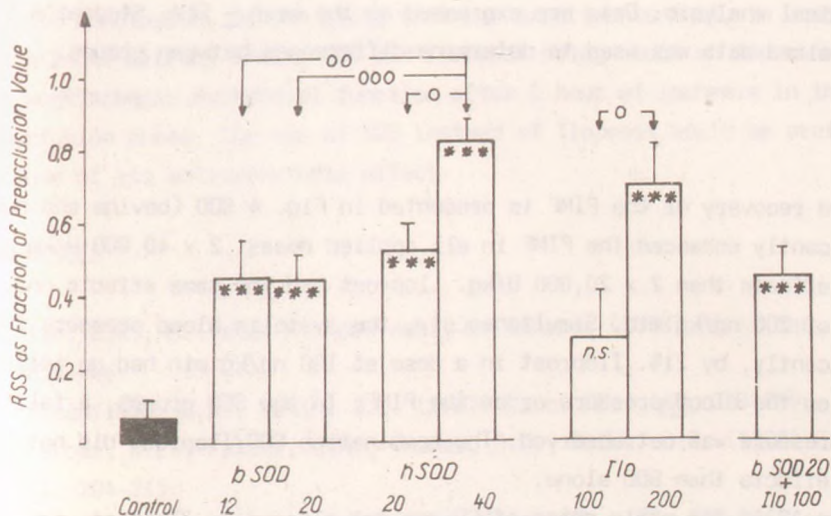


Fig. 4. Regional systolic shortening (RSS) in the 60th min of the reperfusion period ($\bar{x} \pm \text{SEM}$; p 0.05; p 0.01; p 0.001 compared with control () and other groups (o), respectively)

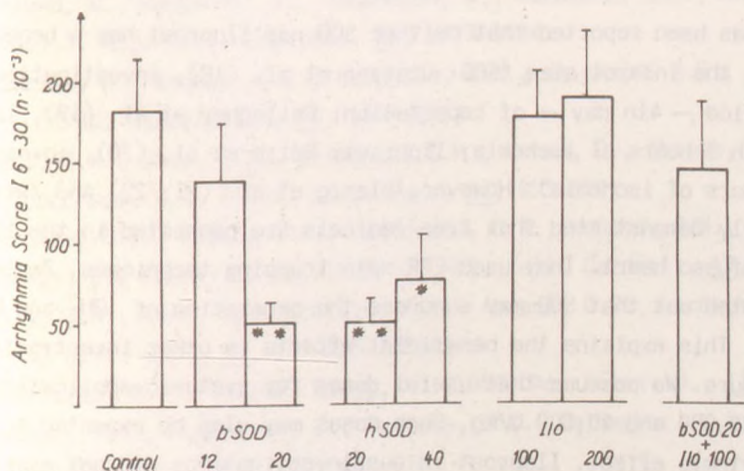


Fig. 5. Arrhythmic score (AS) as average values between the 6th and 30th min of ischemia ($\bar{x} \pm \text{SEM}$; p 0.05, p 0.01 compared with control)

Statistical analysis: Data are expressed as the mean $\pm$ SEM; Student's t-test for unpaired data was used to determine differences between groups.

RESULTS

The recovery of the PIMF is presented in Fig. 4. SOD (bovine and human) significantly enhanced the PIMF in all applied doses. $2 \times 40,000$ U/kg gave better effects than $2 \times 20,000$ U/kg. Iloprost had the same effects only in a dose of 200 ng/kg.min. Simultaneously, the systolic blood pressure fell significantly, by 21%. Iloprost in a dose of 100 ng/kg.min had no influence either on the blood pressure or on the PIMF. In the SOD groups, a fall in blood pressure was not observed. The combination SOD/Iloprost did not yield better effects than SOD alone.

The AS in the early phase of CO was not altered by Iloprost. SOD was effectively antiarrhythmic in doses of 20,000 U/kg and 40,000 U/kg. In combination with Iloprost, the antiarrhythmic effect of SOD disappeared (Fig. 5).

DISCUSSION

It has been reported that neither SOD nor Iloprost has a beneficial effect on the infarct size (SOD: Uraizee et al. (18), investigations in the later period — 4th day — of reperfusion; Gallagher et al. (19), investigations with 3 hours of ischemia; Iloprost: Smith et al. (20), investigations with 5 hours of ischemia). However, Blasig et al. (21, 22) and Zweier (23, 24) clearly demonstrated that free radicals are generated in the ischemic and reperfused heart. They used EPR spin trapping techniques. Zweier (23) also pointed out that SOD may suppress the generation of $\cdot\text{OH}^-$ and $\text{R}^\cdot$ free radicals. This explains the beneficial effects in other investigations as well as ours. We presume that useful doses for systemic application may be between 20,000 and 40,000 U/kg. Such doses may also be expected to have an antiarrhythmic effect. Iloprost in our investigations did not exert an antiarrhythmic effect. In a dose of 100 ng/kg.min, its influence on the PIMF was not evident either. However, at 200 ng/kg.min the PIMF was improved and accompanied by a significant fall in systolic blood pressure. It has been reported to be accompanied by an advantageously reduced left ventricular end diastolic pressure (Cocker et al. (17), Pissarek et al. (25)). In dogs, 100 ng/kg.min preserved high-energy phosphates (Pissarek et al. (25)) or reduced the infarct size (Simpson et al. (13)). It is possible that higher doses are necessary for rats.

In conclusion, we can state from our experiments that, in addition to the effects already known, SOD and Iloprost bring about an improvement of the postischemic myocardial function after 1 hour of ischemia in the early reperfusion phase. The use of SOD instead of Iloprost would be preferable because of its antiarrhythmic effect.

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SERUM ANTIOXIDANT ACTIVITY IN PREMATURE BABIES

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ABSTRACT

The autooxidation of bovine brain homogenate proved to be a good model for determination of the antioxidant capacity of sera. This was investigated in parallel with the levels of ceruloplasmin and apotransferrin in the sera of 35 healthy children and 20 premature babies (gestation age 28-33 weeks). The antioxidant capacity is very low in premature babies and has its minimum value on day 4 of life. The lipid peroxidation of the bovine brain homogenate can be inhibited in vitro by the addition of exogenous antioxidant. In this assay, MTDQ-DS, Cavinton^R, DPA-HCl, and vitamins E, A and C showed different antioxidant activities.

During the investigation of lipid peroxidation in tissue homogenates, it was observed that serum can inhibit this process (1, 6). Certain serum protein, primarily ceruloplasmin and apotransferrin (having ferroxidase activity and the ability to bind ferric ion), have high antioxidant activities, similarly to other biological fluids (cerebrospinal and synovial fluid, colostrum). It is well known that variations in serum antioxidant activity have considerable clinical relevance (3, 5). This was the main reason for the standard biological antioxidant assay developed by Stocks et al. (3).

Spontaneous autooxidation of bovine brain homogenate proved to be a good model of lipid peroxidation in tissues and also for investigation of the inhibitory effects of several biological fluids and different drugs. Using this method, Sullivan and Newton demonstrated that the antioxidant capacity of the cord serum of new-born infants increased with the birth weight, and was much lower than in adults (4).

We set out to establish whether or not the antioxidant capacity is a characteristic value for premature babies. First, we measured the anti-

oxidant capacity of the sera in parallel with the levels of ceruloplasmin and apotransferrin in 35 healthy infants and children ranging in age from 1 day to 13 years (normal controls). We then determined these parameters in 20 premature babies (gestation age 28-33 weeks) on days 1, 4, 7 and 14 day of life, together with the blood-gas parameters (pO_2 , pCO_2 and pH). The premature babies were patients with several different problems in our perinatal intensive centre. Although they received similar medical treatment, they constituted a rather inhomogeneous non-healthy population. In addition, we investigated in vitro the inhibitory effects of different drugs (D-penicillamine.HCl, Cavinton, vitamins E, C and A, MTDQ-DS, salicylic acid and D-mannitol) on the lipid peroxidation of bovine brain homogenate.

METHODS

The antioxidant activity was determined by the modified method of Stocks et al. (3). After the centrifugation of native blood at 0-5 °C, serum samples were stored for 1-7 days at -20 °C. Bovine brain homogenate (1:10 w/v) in phosphate-buffered saline (pH 7.4) was stored at -20 °C for 6 weeks. Different volumes of serum (80, 40, 20, 10 or 5 μ l) or nothing (control tubes) were added to 2 ml of bovine brain homogenate at room temperature, the contents were vortex mixed for 10 seconds, and duplicate samples were incubated for 1 hour at 37 °C. To determine the starting autooxidation value 1 ml of cold TCA (30%) was added to zero-time samples to stop autooxidation and thereafter these tubes were kept at 0-5 °C instead of being incubated.

The malonyldialdehyde concentrations in zero-time and incubated samples were estimated via the thiobarbituric acid reaction (2). TCA (1 ml) was added to 2 ml of bovine brain homogenate, and the precipitated proteins were removed by centrifugation at 0-5 °C. 2 ml of the supernatant and 0.5 ml of 10 g/l thiobarbituric acid solution (in 0.05 M sodium hydroxide) were then mixed and heated for 15 min at 100 °C. The absorbance (A) at 532 nm was measured with a Specord M-40 spectrophotometer. The extent of residual autooxidation can be characterised by the value of

$$\frac{A(\text{sample}) - A(\text{zero-time sample})}{A(\text{control}) - A(\text{zero-time sample})}.$$

This was plotted against the amount of serum present in the sample. The x intercept of the straight line (fitted by linear regression) is the

serum volume (v_0) required for maximal inhibition of lipid peroxidation in the bovine brain homogenate. v_i is inversely related to the antioxidant capacity.

D-penicillamine.HCl (Knoll AG), Cavinton (Richter), D-mannitol (Merck), salicylic acid, ascorbic acid (Reanal) and MTDQ-DS were dissolved in 1/15 M phosphate-buffered saline (pH 7.4), vitamin A (Egis) was dissolved in oleum helianthi, and vitamin E (Fluka) was dissolved water-ethanol (1:1) containing 10% Tween-20.

The concentrations of ceruloplasmin and apotransferrin were determined by using a Beckman Immunochemistry Analyzer type II nephelometer.

RESULTS

In accordance with the results of Sullivan and Newton (4), we found that the antioxidant capacity in 35 healthy children (aged from 1 day to 13 years) increased with age. In normal control children (aged from 1 day to 4 weeks) the volume of serum needed for maximal inhibition of bovine brain autooxidation (v_0) is high (62 ± 6 ul), i.e. the antioxidant capacity is low. The antioxidant capacity increases during the first 4 months of age, and then remains unchanged until 13 years of age (average value of v_0 : 15 ± 2 ul) (Fig. 1).

Premature babies with a gestation age of 28-33 weeks and a birth weight of 720-2700 g have a very low antioxidant activity, which increases with the gestation age and birth weight (Figs 2 and 3). We found a characteristic change in the antioxidant capacity in one group of 9 premature babies (gestation age 28-33 weeks, birth weight 1200-2000 g). Their antioxidant capacity displayed a local minimum on the 4th day (Fig. 4). As they often suffered from hypoxia, acidosis and asphyxia during the first 4 days, this low antioxidant capacity is probably caused by several factors. Among other parameters, the antioxidant capacity too shows that the 4th day is a critical time for prematures.

Our results indicate that the antioxidant capacity of the serum seems to have considerable clinical relevance in prematures.

The apotransferrin concentration of Premature babies exhibits a maximum value on the 4th day. The ceruloplasmin level generally increases during the first 14 days of life, but declines on the 4th day (Fig. 5).

Stocks et al. (3) and Sullivan (4) reported that the lipid peroxidation of bovine brain homogenate can be inhibited in vitro by the addition

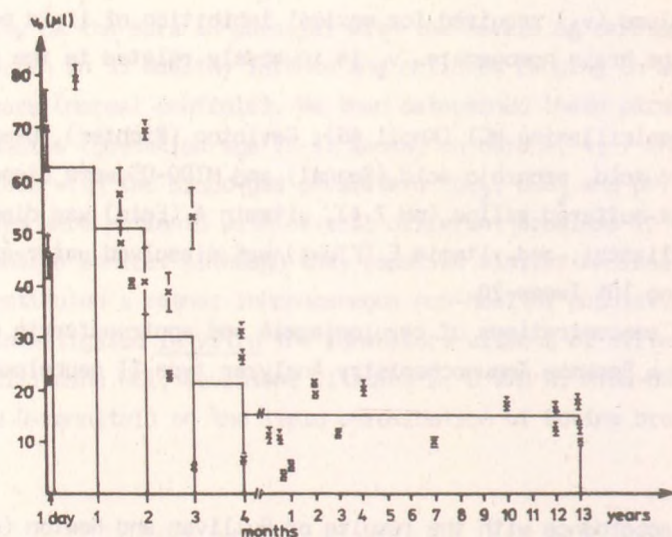


Fig. 1. Antioxidant capacity of normal control children

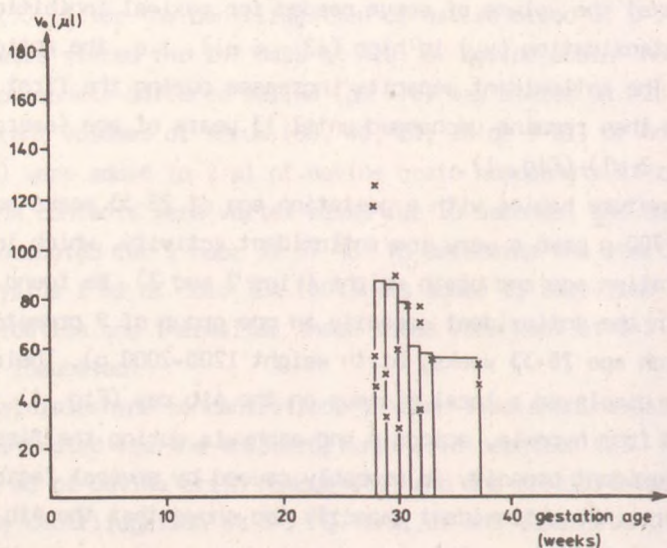


Fig. 2. Antioxidant capacity of premature babies

of exogenous antioxidant (Vitamin E, DPA, desferoxamine mesylate or iron chelating reagents).

We measured the residual autooxidation in the presence of vitamins E, A and C, Cavinton, D-penicillamine.HCl, MTDQ-DS, salicylic acid and D-mannitol. The inhibitor concentration relating to 50% inhibition ($C_{1/2}$) proved that they were good antioxidants (Figs 6-8).

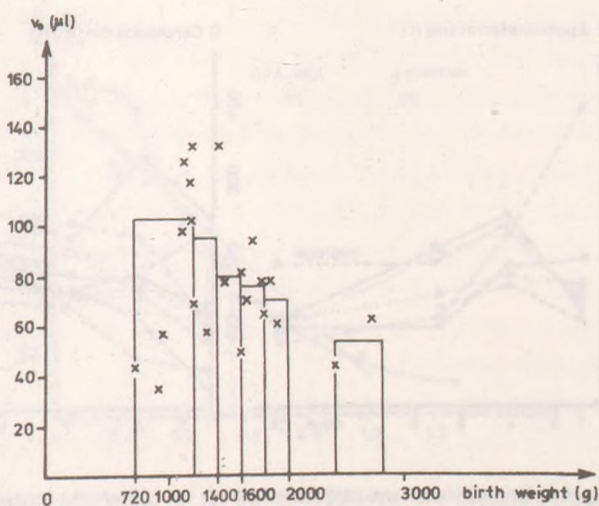


Fig. 3. Antioxidant capacity of premature babies with different birth weights

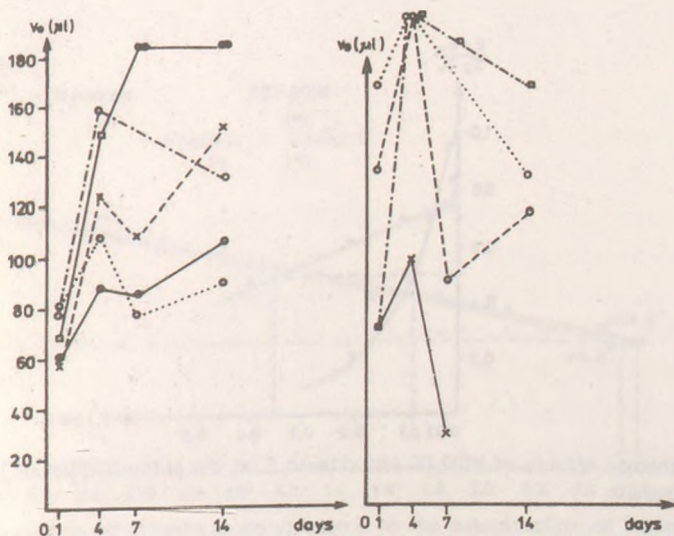


Fig. 4. Antioxidant capacity of 9 premature babies during the first 14 days of life

	$C_{1/2}$ (mM)
MTDQ-DS	0.08
vitamin E	0.35
Cavinton	0.50
DPA-HCl	0.56
vitamin A	2.55
vitamin C	2.60

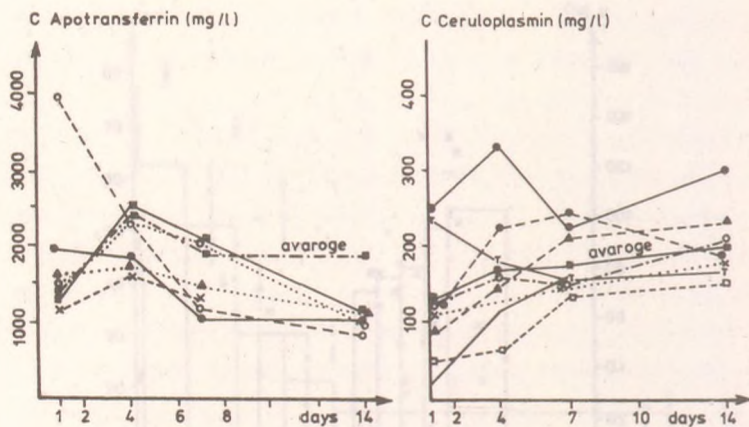


Fig. 5. Serum apotransferrin and ceruloplasmin levels in premature babies during the first 14 days of life

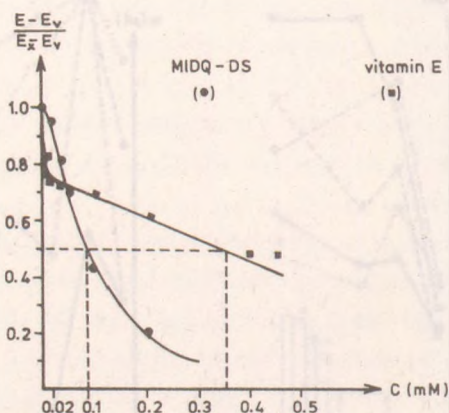


Fig. 6. Inhibitory effects of MTDQ-DS and vitamin E on the autooxidation of bovine brain homogenate

As vitamin E could be dissolved only in water-ethanol + Tween-20 solution, and vitamin A in oleum helianthi, we measured the inhibitory effects of the solvents too, but they did not cause a marked inhibition in this low concentration range.

Salicylic acid (0.1-4.4 mM) and D-mannitol (1-50 mM) did not show any antioxidant effect in this in vitro system.

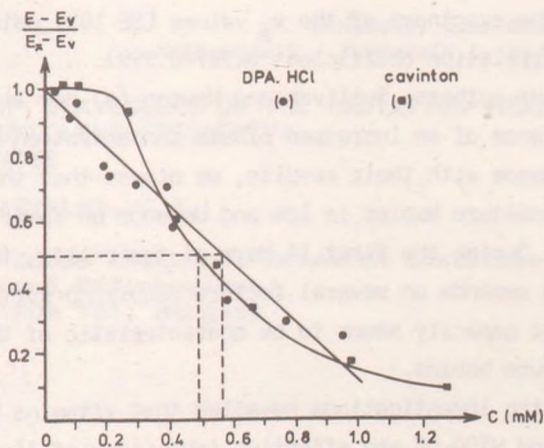


Fig. 7. Inhibitory effects of DPA-HCl and Cavinton on the autooxidation of bovine brain homogenate

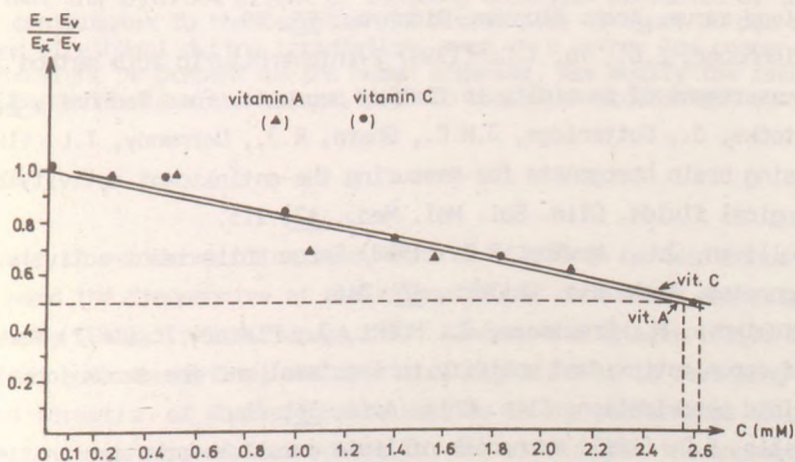


Fig. 8. Inhibitory effects of vitamin A and vitamin C on the autooxidation of bovine brain homogenate

DISCUSSION

As the rate of lipid peroxidation depends on the iron ion concentration, we could determine the autooxidant capacity only in those sera that did not undergo haemolysis. Iron ions liberated from haemoglobin probably cause an unrealistically high lipid peroxidation in bovine brain homogenate.

This limited the exactness of the v_o values (SE 10%) obtained by linear regression (correlation coefficient 0.967-0.999).

Among other authors, Sullivan and Newton (4) pay attention to the central importance of an increased plasma concentration of iron.

In accordance with their results, we proved that the antioxidant capacity in premature babies is low and depends on their birth weight and gestation age. During the first 14 days of their life, this parameter is changeable and depends on several factors requiring further investigation. The antioxidant capacity seems to be characteristic of the general condition of premature babies.

Our in vitro investigations revealed that vitamins E, A and C, Cavinton, DPA.HCl and MTDQ-DS are effective inhibitors of lipid autooxidation.

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THE EFFECTS OF OH[•] SCAVENGERS ON THE RADIATION-INDUCED INCREASE OF MICRONUCLEUS FREQUENCY IN HUMAN LYMPHOCYTES

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SUMMARY

The frequency of micronuclei in cytokinesis-blocked blood lymphocytes was studied when X-irradiation was performed in the presence of ethanol and dimethylsulfoxide. Protective effects were observed in both cases. It is suggested that the indirect effect of ionizing radiation prevented by the scavengers contributes to the formation of micronuclei by appr. 60 per cent. The presence of ethanol during irradiation, even in a rather low concentration which might be present in the human organism, may modify the results of dose assessments via micronucleus tests in radiation accidents.

INTRODUCTION

According to earlier publications, ethanol (EtOH) in certain concentrations lowers the frequencies of radiation-induced chromosome aberrations, e.g. dicentrics (Sasaki and Matsubara 1977, Almássy and Köteles 1981). The phenomenon has both theoretical and practical significance in revealing the mechanism of formation of aberrations as well as in the performance of dose assessments in cases of radiation accidents. The effect upon the appearance of micronuclei in lymphocytes has been studied, as this assay is becoming a rather simple and promising test in dose assessment (Almássy et al. 1987, Kormos and Köteles 1988). In addition, the effect of another OH[•] scavenger, dimethylsulfoxide (DMSO), has also been studied.

MATERIALS AND METHODS

Blood samples were taken from healthy donors 18 to 23 years of age, under sterile conditions, with heparin as anticoagulant. X-irradiations were performed in Falcon plastic dishes with a THX-250 equipment (200 kV,

20 mA, 60 cm distance, 1 mm Cu filter, dose rate $0.287 \text{ Gy} \cdot \text{min}^{-1}$). The volumes of samples were 10 ml each. EtOH and DMSO were applied before irradiation. The chemicals were of analytical grade.

Lymphocytes were cultured in RPMI medium complemented with 15% bovine serum. Stimulation was initiated by 1 μg per ml phytohemagglutinin-P. Incubation at 37°C lasted for 72 hours. At the 44th hour of incubation, 3 μg per ml cytochalasin-B was added. The cells were treated with 0.075 M KCl for 5 minutes after incubation, then fixed twice with a 1:3 mixture of 96% acetic acid and methanol. After spreading on glass, the cells were stained with Giemsa. Five hundred cytochalasin-B-blocked (CB) were scored for micronuclei. The frequencies were expressed per mille.

RESULTS AND DISUCCION

The effects of various doses of X-irradiation upon lymphocytes irradiated and cultured thereafter in vitro are demonstrated in Fig. 1. The dose-effect relationship of micronucleus formation proved to be of a linear-quadratic character between 0.5 and 4.0 Gy. The parameters of the yield equation, i.e.

$$Y = c + \alpha D + \beta D^2$$

are as follows: c , the population background, is 11.3 ± 4.3 , $\alpha = 139.0 \pm 44.0$ and $\beta = 36.8 \pm 18.6$. When the blood samples were irradiated in the presence

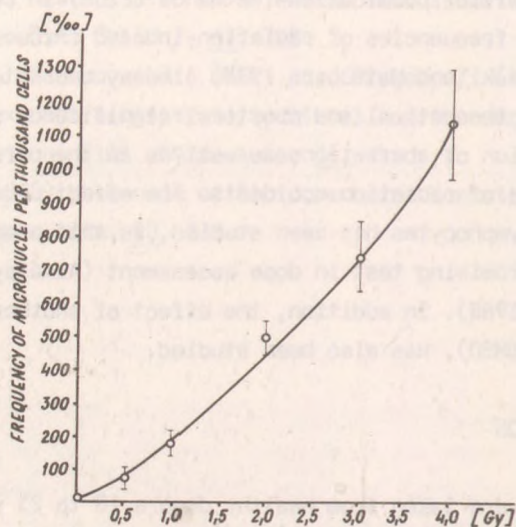


Fig. 1. The dose-response curve for the formation of micronuclei in blood lymphocytes X-irradiated and cultured in vitro

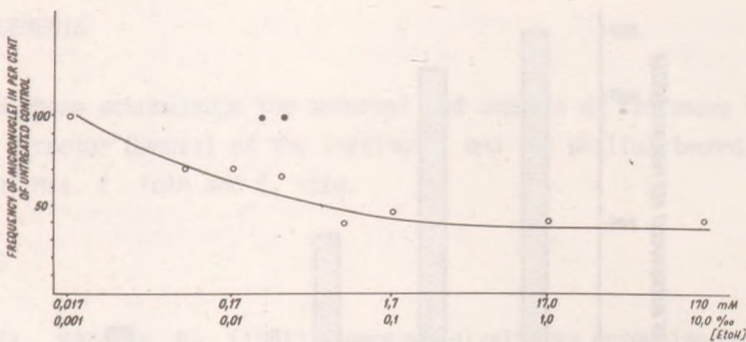


Fig. 2. The effects of various concentrations of ethanol (o) and acetaldehyde (●) upon the frequencies of micronuclei in human lymphocytes when present during irradiation

of various concentrations of EtOH, the frequency of micronuclei gradually decreased, as seen in Fig. 2. No further decrease could be observed when the frequency sank to 40 per cent. This suggests that the indirect effect of radiation in micronucleus contributes to appr. 60 per cent. Figure 2 also demonstrates that acetaldehyde, the metabolic breakdown product of EtOH, in a concentration which might be present in the blood, does not exert a protective effect. The question was next raised as to whether EtOH protects the formation of micronuclei equally at low and high doses. Blood samples were therefore irradiated with various doses in the presence of

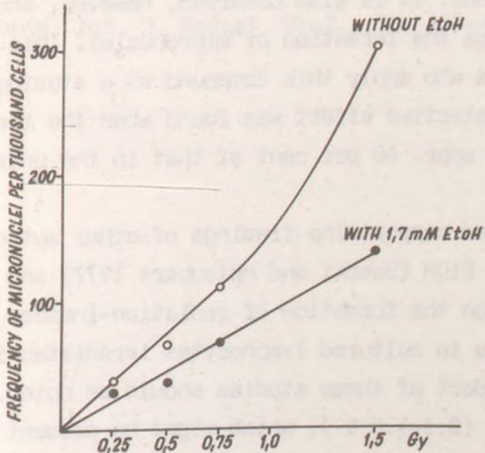


Fig. 3. Dose-response relationship of micronucleus formation in the presence of 1.7 mM ethanol

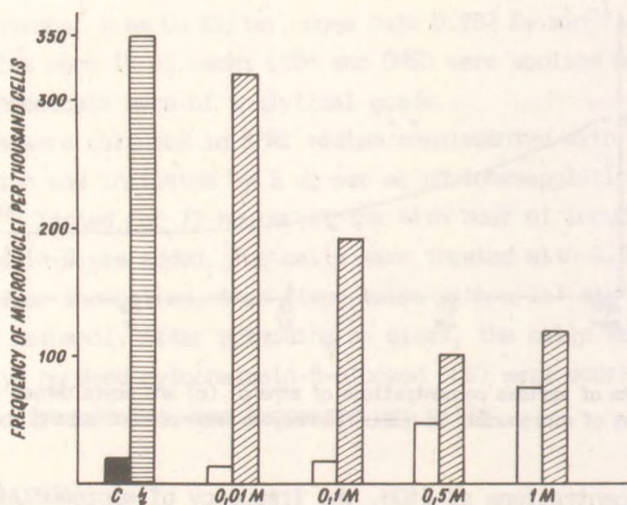


Fig. 4. The effects of various concentrations of dimethylsulfoxide upon the frequencies of micronuclei in lymphocytes when present during irradiation.

■: unirradiated control, ▨: irradiated with 1.5 Gy,
□: treated only with DMSO, ▩: irradiated with 1.5 Gy in the presence of DMSO

1.7 mM EtOH. The data are presented in Fig. 3. It can be seen, that the linear-quadratic dose-response curve became linear, suggesting a higher efficiency of protection at higher doses than at lower ones.

The effect of another hydroxyl scavenger, DMSO was also investigated. Figure 4 demonstrates that increasing concentrations of DMSO decrease the frequency of aberrations. It is also observed, however, that DMSO itself, from 0.5 M on, provokes the formation of micronuclei. This observation is a warning sign for those who apply this compound as a storing medium for cells. The maximum protective effect was found when the frequency of micronuclei was reduced to appr. 40 per cent of that in the untreated and irradiated sample.

The data presented support the findings of other authors on the protective effects of EtOH (Sasaki and Matsubara 1977) and DMSO (Littlefield et al. 1988) upon the formation of radiation-induced dicentric chromosome aberrations in cultured lymphocytes irradiated in vitro.

One practical aspect of these studies should be noted. A rather low concentration of EtOH (0.1-1.0 %), which might be present in blood during irradiation, could modify the results of dose assessment through cytogenetic tests, including the micronuclear one, in radiation accidents.

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THE ANTIOXIDANT EFFECT OF BF-100 IN MONGREL DOGS

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INTRODUCTION

In an earlier study, Somogyi et al. /9/ found that BF-100, a synthetic lipopolyamine, dose-dependently inhibited the enzymatically induced (NADPH + Fe^{3+}) lipid peroxidation in rat liver microsomes and that BF-100 inhibited both spontaneous and induced lipid peroxidation in rat brain homogenizate.

It seemed promising to perform further studies on the presumed inhibition of free radical formation by BF-100 in vivo. We applied BF-100 in the form of an i.v. injection and followed the final product of the lipid peroxidation process.

MATERIAL AND METHODS

Studies were made on healthy mongrel dogs of both sexes (weighing 15-20 kg), kept on standard mixed food and water ad libitum.

We first collected control serum samples from the dogs for 3 days. The animals then received an i.v. BF-100 injection, at the 25 $\mu\text{g/kg}$ or the 100 $\mu\text{g/kg}$ dose level.

The arrangement of the experiment allowed a self-controlling comparison, i.e. the pretreatment (control) and post-treatment values of the same animals could be followed.

Serum malondialdehyde (MDA) determination

A generally accepted method is that lipid peroxidation is measured via MDA final product determination in the native serum, the intensity of free radical processes thereby being followed in the organism.

The thiobarbituric acid determination was performed according to the method of Satoh /8/, with the modifications of Varga et al. /10/.

2.5 ml 20% trichloroacetic acid was added to the native serum (0.5 ml), and the mixture was thoroughly stirred at room temperature. Ten minutes later, the solution was centrifuged at 3500 rpm for 10 minutes. The supernatant was poured off, and 2.5 ml 0.05 M sulfuric acid was added to the precipitate. The centrifugation was repeated as above, and the supernatant was poured off again. 2.5 ml 0.05 M sulfuric acid and 3 ml 1% thiobarbituric acid were added to induce the colour reaction (thiobarbituric acid was first dissolved in 2 M sodium sulfate to avoid the disturbing effect of sialic acid), and the tubes were then placed in a hot water-bath for 60 minutes. After the treatment, the tubes were cooled down in cold water and centrifuged at 3500 rpm for 10 minutes. After centrifugation, the clear, colourless supernatant was poured off, and dimethylsulfoxide was added to the small-volume chromogenous precipitate, the final volume being made up to 3.5 ml. This solution was then measured photometrically at 530 nm.

The nmol/ml concentration of MDA in the serum was calculated by means of the following formula:

$$\text{nmol MDA} = \frac{V \times \text{OD}_{530}}{0.152} = \frac{3.5 \times 2 \times \text{OD}}{0.152} = 46.15 \times \text{OD}_{530}$$

where

V = the volume of the chromogenous substance as calculated for 0.5 ml serum.

RESULTS

The following notations are used in the Table and in the Figure:

- I = BF-100 treatment i.v. + blood sampling 6 hours later (means of 3-day values)
- II = Post-treatment values obtained after a 3-day interval (means of 3-day values)
- III = Values obtained during subsequent 7 days immediately following 3-day (repeated) i.v. BF-100 treatment
- IV = Values obtained after a further 3-day treatment (means of 3-day values)
- V = Values obtained after a further 3-week interval (means of 3-day values)

Table 1. Mean Serum Malondialdehyde (MDA) Levels (nmol/ml) in Different Test Periods

BF-100 Dose	C	I.	II.	III.	IV.	V.	
25 µg/kg	7.07	6.18	5.01	3.98	3.10	4.28	$\bar{X}$
	1.60	1.41	0.88	1.45	1.30	2.38	SD
	18	18	18	42	18	18	n
		ns	***	***	***	***	
100 µg/kg	5.69	5.48	4.35	2.71	2.44	3.22	$\bar{X}$
	1.28	1.28	1.39	0.74	0.94	2.16	SD
	18	18	18	42	18	18	n
		ns	**	***	***	***	
	ΔΔ			ΔΔΔ			

* = $p < 0.05$ vs. control
 ** = $p < 0.01$
 *** = $p < 0.001$

Δ = $p < 0.05$ vs. 25 µg/kg

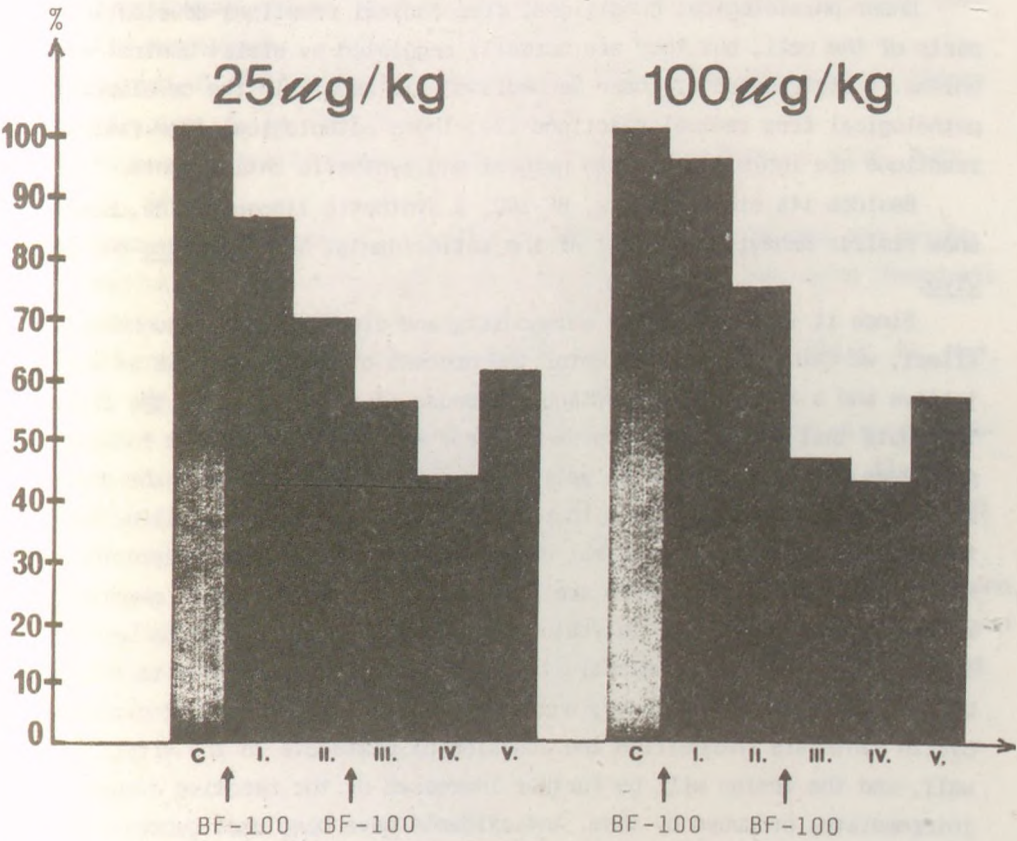


Fig. 1. Trends compared to the control in different periods

The Table 1 shows the mean serum MDA levels. These levels display a decreasing trend even around the first BF-100 treatment (I), both the 25 and the 100 $\mu\text{g/kg}$ groups, and the fall is sharper after the 3-day interval, following the treatment (II). The decrease in the serum MDA level is the most marked after repeated BF-100 treatment (III-IV), and it remains below the control value even as late as 3 weeks after the treatment (V).

The Figure 1 shows the changes in the MDA level, taking the mean control value as 100%. At both dose levels, the percentage decrease becomes more marked after the second BF-100 treatment (III), and also 7 days later (IV), and it is still around 60% even as late as 3 weeks after the last treatment (V).

In conclusion BF-100 seems to be a strong lipid peroxidation inhibitor.

DISCUSSION

Under physiological conditions, free radical reactions develop in all parts of the cell, but they are normally regulated by strict control mechanisms. A disturbance of these mechanisms will result in the development of pathological free radical reactions (2). These pathological free radical reactions are inhibited by both natural and synthetic antioxidants.

Besides its other effects, BF-100, a synthetic lipopolyamide, seems to show similar behaviour to that of the antioxidants, both in vitro and in vivo.

Since it is a calmodulin antagonist, and also shows an immunomodulant effect, we think that it can enter the process of carcinogenesis as a protective and a therapeutic substance, because it is verified by the literature data that the carcinogens exert their effects through free radical processes (1, 2, 6, 7). It is well known that the antioxidants and diets minimizing lipid peroxidation increase the mean expectancy of life, and this has been verified by animal experiments (3). However, in arteriosclerosis, the arterial walls are permanently irritated by the reaction of molecular oxygen with the polysaturated fatty acids in the serum and the arterial wall (4), and the activity of prostacyclin synthetase is inhibited by the free radical reactions, and thus the decreasing or ceasing prostacyclin synthesis intensifies the adhesion of platelets to the arterial wall, and the lesion will be further increased by the reactive oxygen intermediates produced by them. Antioxidants have been used successfully in the treatment of animal experimental and human arteriosclerosis (2). In our

further studies we intend to investigate the effects of BF-100 in experimental arteriosclerosis.

Naturally, our results and observations require further studies, but we hope that the circle of substances offering protection against the toxic effects of reactive oxygen intermediates may be expanded by a new synthetic antioxidant.

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ANTIOXIDANT ENZYMES AND LIPID PEROXIDATION IN THE HEMOLYSATES OF TUMOROUS PATIENTS

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ABSTRACT

The lipid peroxidation and the antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) were compared in the erythrocyte hemolysates of normal controls and 20 patients suffering from gastrointestinal, hepatic, pancreatic and bronchial carcinomas. The lipid peroxidation was significantly elevated, while the superoxide dismutase and catalase activities were significantly lower in the tumorous red blood cell hemolysates.

INTRODUCTION

During the "metabolism" of molecular oxygen, various radicals are formed, these being responsible for the toxicity of oxygen. Besides the radicals, a great amount of hydrogen peroxide (H_2O_2) is formed. These oxygen products are eliminated by a special enzyme system, the "antioxidant" enzymes. Superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (i.e. glutathione peroxidase; GP-ase; EC 1.11.1.9) and catalase (C-ase; EC 1.11.1.6) are the most important members of the enzymatic antioxidant defense (1). Reduced glutathione (GSH) is also important as a determinant of the therapeutic effectiveness of different anticancer drugs and as a water-soluble part of the cell (2-4).

The aim of our present investigation was to compare the antioxidant enzyme activities and lipid peroxidations (LP) in the red blood cell (RBC) hemolysates of different tumor-bearing patients and normal persons. The different tumors were: tu. recti, pulmoni, hepatis, cerebri, etc. All of the tumors were in the second stage and were operated on.

MATERIALS AND METHODS

Blood was taken for patient care from the veins of the subjects in both groups, and was inhibited from coagulation by the addition of heparin (G. Richter Pharmaceutical Works, Budapest, Hungary).

The blood was centrifuged at $1000 \times g$ for 10 min. and the plasma and the buffy coat were removed by aspiration. The RBC were subsequently washed twice with 2 vol of 154 mmol/l sodium chloride solution at about pH 7. Following this, the RBC were hemolysed with a twofold volume of distilled water. With the exception of SOD activity determinations, aliquots of whole or centrifuged hemolysate were used for the estimations.

Before SOD activity determination, the hemolysate was treated with ethanol: chloroform (2:1) (1 ml hemolysate + 0.75 ml ethanol: chloroform, and after centrifugation aliquots of the final supernatant were used for SOD assay (5). SOD (in this case Cu, Zn-SOD) activity was determined via its inhibition of the epinephrine-adrenochrome transformation (6, 7).

C-ase activity was measured spectrophotometrically at 240 nm in aliquots of the supernatants of the hemolysates. The C-ase activity is given in Bergmeyer units (BU) (7, 8).

GP-ase activity was determined by the combined "chemical method" in aliquots of the hemolysates. Cumene hydroperoxide and reduced glutathione (GSH) were used as substrates and after a given time of incubation, the GSH residue was measured with the Ellman reagent (9-11).

The LP values of the hemolysates were estimated in aliquots of the whole hemolysates via the thiobarbituric acid reaction at 532 nm. Malonyl-dialdehyde diethylacetal was used for the preparation of the calibration curve (12).

The protein content was determined by using the Folin reagent (13).

The purest reagents were used for enzyme activity and other measurements without further purification; they were products of Merck (Darmstadt, FRG), Sigma (St. Louis, USA) and Reanal (Budapest, Hungary).

The results were subjected to statistical evaluation with Student's *t* test. All data are given as means $\pm$ SD. In the enzyme activity measurements and estimations of LP, the differences between duplicate measurements were never in excess of 5%.

RESULTS

The Table 1 shows the results. The LP is enhanced in the RBC hemolysates of tumorous patients. The GP-ase activity is higher, while the SOD and C-ase activities are significantly lower in the RBC of the tumorous group than in the controls.

Table 1 Comparison of the levels of antioxidant enzymes and lipid peroxidation normal subjects and in tumor-bearing patients before operation (means $\pm$ SD).

Groups	LD nmol MDA/hem.	GP-ase U/ml hem.	SOD U/ml hem.	C-ase BU/ml hem.
Tumorous	112.0	2.14	368.3	1.03
patients	31.82	0.58	271.0	0.38
(n = 20)				
Controls	67.4	2.57	716.5	2.05
(n = 11)	15.9	0.26	92.5	0.68
t-test	p > 0.001	p < n.s.	p < 0.001	p < 0.001

DISCUSSION

Di Ilio et al. (14) analysed the GP-ase, glutathione S-transferase and glutathione reductase activities in normal and neoplastic human breast cancer tissues.

Oberley (15) surveyed oxygen toxicity and the susceptibility to lipid peroxidation, the role of the superoxide radical in carcinogenesis and the use of SOD in the treatment of cancer, stating that the tumor cells are low in the antioxidant enzymes, SOD, C-ase and GP-ase.

In our tumorous group, an enhanced erythrocyte LP (indicative of tissue destruction) and decreased antioxidant enzyme (SOD and C-ase) activities were observed, while the GP-ase activity was not changed significantly.

Further examinations must be made to evaluate the prognostic value of the levels of the LP and the antioxidant enzymes in the hemolysate of the erythrocytes.

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RED BLOOD CELL AND PLASMA LIPID PEROXIDATION AS AN INDICATOR IN DISEASES

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ABSTRACT

The lipid peroxidation (LP) of red blood cell (RBC) haemolysates is easily determined. Variation of the LP is characteristic in certain disease forms. A study has been made of the LP of RBC haemolysates from children with hereditary Duchenne's muscular dystrophy or cystic fibrosis, their parents, premature and full-term neonates, and children with hyperbilirubinemia. In some cases the LP of the plasma was also measured. The LP was similarly determined in the RBC haemolysate from chronic uraemic patients before and after dialysis. The variation in the LP of the blood haemolysates appears to be a convenient tool for the confirmation of the diagnoses, and for the demonstration of an oxidative metabolic predominance.

INTRODUCTION

We earlier modified the method of Placer et al. (1966) (1) so as to develop a procedure for the simple determination of the lipid peroxidation (LP) for certain diagnostic purposes, and used the method to determine the LP in red blood cell (RBC) haemolysates and plasma. Appropriate control measurements were made in all cases. The calibration curve was prepared with the use of malonyl dialdehyde (MDA)-diethyl acetal by the method of Placer et al. (1), and the results were always calculated in terms of MDA.

The method has long been used in human comparative studies (2-4), and it appears that it can also be applied for diagnostic purposes to confirm the role of the oxidative stress state.

METHODS

Lipid peroxide assay. The essence of the method: MDA (and most of the other oxo compounds formed during the LP) reacts with 2-thiobarbituric acid (TBA) in acidic medium. The yellowish-red product has an absorption maximum at 532 nm. We normally speak of the total TBA-active compounds, which are reported as nmol MDA per L plasma or haemolysate or per mg protein.

Reagent: 10% perchloric acid solution is saturated with TBA, and to 10 parts of this solution 30 parts of 20% trichloroacetic acid is added. This reagent is always freshly prepared by mixing the ready components.

Procedure: 4.5 mL of reagent is added to 0.5 mL of plasma or haemolysate and the mixture is kept in a 100 °C water-bath for 20 minutes. It is next cooled to 4 °C and the precipitate is centrifuged at 2000 g. The solution to be measured is subjected to photometry at 532 nm against a blank containing all of the reagents. Analytically purest reagents were used without additional purification; the reagents were products of Merck (Darmstadt, FRG), BDH (Poole, U.K.) and Reanal (Budapest, Hungary). The protein content was determined by the method of Lowry et al. (5).

Statistical evaluation of the results was performed with the Student t-test. All data are means $\pm$ SD. In duplicate measurements, the differences never exceeded 5%.

RESULTS

The RBC and plasma LP levels for children with Duchenne's muscular dystrophy (DMD) were first compared with those for healthy children. (It should be noted that the expression LP is used in parallel with the term total TBA-reactive substances.) The LP of the haemolysates was significantly higher in children with DMD than in the healthy controls (Table 1).

Table 2 presents the results relating to the LP in the blood of cystic fibrosis (CF) patients, their parents and healthy controls.

The data in Table 3 reveal a significant difference between the LP values for the RBC haemolysates of premature and full-term neonates. As the age increases, the difference tends to decrease.

Table 4 illustrates the differences between the LP values of the RBC haemolysates from normal full-term neonates and hyperbilirubinaemic ones

Table 1. LP of plasma and RBC haemolysates of DMD children and healthy controls (means $\pm$ SD)

Assay	Controls (4-10 years) (n=10)	DMD children (from 6-12 years) (n=20-22)
TBA-reactive plasma products (nmol MDA/L plasma)	15.40×10^3 $\pm 1.50 \times 10^3$	15.45×10^3 $\pm 1.17 \times 10^3$
TBA-reactive products in haemol. (nmol MDA/L haemol.)	201.90×10^3 $\pm 18.90 \times 10^3$	$310.70 \times 10^{3*}$ $\pm 15.10 \times 10^{3*}$

* significant $p < 0.005$

Table 2. LP of plasma and RBC haemolysates of CF children, their parents and healthy controls (means $\pm$ SD)

Assay	Controls			Obligate heterozygote parents (25-30 yrs) (n=13)
	Age group I (4-10 years) (n=10)	Age group II (20-25 years) (n=30)	CF homozyg. children (1-12 years) (n=8)	
TBA-reactive plasma products (nmol MDA/L plasma)	10.43×10^3 $\pm 1.50 \times 10^3$	12.85×10^3 $\pm 3.90 \times 10^3$	$7.90 \times 10^{3*}$ $\pm 1.50 \times 10^3$	$7.5 \times 10^{3*}$ $\pm 2.0 \times 10^3$
TBA-reactive products in haemol. (nmol MDA/L haemol.)	201.9×10^3 $\pm 18.9 \times 10^3$	67.4×10^3 $\pm 1.6 \times 10^3$	$68.4 \times 10^{3*}$ $\pm 1.5 \times 10^3$	68.3×10^3 $\pm 1.9 \times 10^3$

* significant $p < 0.001$

Table 3. LP of RBC haemolysates of premature and full-term neonates (means $\pm$ SD)

Assay	Full-terms neonates (n=14)	Premature infants (born after 30-39 weeks) (n=10)
TBA-reactive products in haemol. (nmol MDA/l haemol.)	191.25×10^3	106.40×10^3
1 day after birth	$\pm 47.07 \times 10^3$	$\pm 33.02 \times 10^3$
3 days after birth	157.10×10^3 $\pm 41.20 \times 10^3$	110.83×10^3 $\pm 27.22 \times 10^3$
5 days after birth	142.52×10^3 $\pm 45.20 \times 10^3$	116.23×10^3 $\pm 36.34 \times 10^3$

* significant $p < 0.01$

** significant $p < 0.001$

Table 4. LP values of hyperbilirubinaemic children 3 days after birth after treatment in different ways, compared with the data for healthy full-term infants (means $\pm$ SD)

Assay	
TBA-reactive products in haemol.	157.10×10^3
(nmol MDA/g protein)	$\pm 41.17 \times 10^3$
3 days	235.00×10^3
after blue light therapy (n=10)	$\pm 67.00 \times 10^3$
Before exchange transfusion (n=10)	118.57×10^3
	$\pm 47.02 \times 10^3$
Before Beflavin treatment (n=8)	141.47×10^3
	$\pm 81.80 \times 10^3$

Table 5. LP values of plasma and RBC haemolysates of uraemic patients before and after haemodialysis (means $\pm$ SD)

Assay	Control group (n=20)	Patients before haemodialysis (n=35)	Patients after haemodialysis (n=35)
TBA-reactive plasma products (nmol MDA/L haemol.)	10.3×10^3 $\pm 3.9 \times 10^3$	12.15×10^3 $\pm 3.90 \times 10^3$	14.0×10^3 $\pm 1.2 \times 10^3$
TBA-reactive products in haemolys. (nmol MDA/L haemol.)	67.4×10^3 $\pm 1.6 \times 10^3$	$128.9 \times 10^{3*}$ $\pm 33.9 \times 10^3$	$157.8 \times 10^{3*}$ $\pm 46.6 \times 10^3$

*significant $p < 0.01$

treated in various ways. The data in all cases relate to the 2nd day after birth. Beflavin (Hoffman-La Roche, Basel, Switzerland) was administered i.v. in a dose of 10 mg/kg. The LP value for the RBC haemolysates was compared with that for the hyperbilirubinaemic infants who received Beflavin (who had also undergone blue light therapy before Beflavin treatments).

Table 5 lists the LP results on the RBC haemolysates of uraemic patients before and after the haemodialysis.

DISCUSSION

The findings from the comparative LP measurements were as follows:

1. Investigations in recent years indicate that an oxidative stress state develops mainly in the muscles. This phenomenon may be a general one, and it may lead overall to rapid degeneration and degenerative destruction of the muscle cells. The presence of significantly higher TBA-active products has been proved in the RBC haemolysates (2, 6).

2. We explain the lower plasma LP and the significantly lower RBC LP in CF patients and in their parents by the slower nature of the metabolism or by the effect of some active scavenger system, due to the enhancement of the activities of the antioxidant enzymes through the significant increases in superoxide dismutase and catalase in the RBC haemolysates (3).

3. A number of important publications appeared in 1986 on the RBC LP of both animal, including rat (8) and rabbit (9), and human (7) neonates. We earlier compared the LP values for plasma and RBC haemolysates from full-term and premature neonates. It may definitely be stated that the RBC LP in full-term human neonates is higher than in adults. In contrast, premature infants display low LP values, though these rise progressively after birth (4).

4. We have attempted to define an important parameter through comparison of the LP in the RBC haemolysates at a given time in hyperbilirubinaemic neonates, where the hyperbilirubinaemia was treated in different ways. Blue light treatment resulted in a significantly higher RBC LP, whereas hyperbilirubinaemic neonates who undergo replacement transfusion and Bephrin treatment have lower RBC LP values than the controls.

5. We experienced that the LP of both the plasma and the RBC haemolysates increases significantly in uraemic patients before haemodialysis (Table 5).

To summarize, it may be said that measurement of the LP of the plasma and the RBC haemolysates is a simple diagnostic procedure, and is suitable, together with the other symptoms, for confirming primarily the diagnosis of an oxidative stress state. These investigations are continuing.

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EFFECTS OF PLASMODIUM INFECTION IN MICE
ON HOST-PARASITE ANTIOXIDANT ENZYMES
AND OTHER PARAMETERS

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SUMMARY

A study was made of how Plasmodium (Pl.) berghei and Pl. vinckei infections influence the metabolism of the host red blood cell and liver antioxidant enzymes and the redox systems in mice. In response to infection with both of the strains, substantial changes resulted in all of the examined parameters.

INTRODUCTION

The Plasmodia are parasitic micro-aerophilic organisms sensitive to hydrogen peroxide and to the radicals formed from molecular oxygen. Oxidative damage to the red blood cells (RBC) promotes resistance to the infection (1-3).

Particularly in the initial stages of their development, the parasites make use of the antioxidant systems of the host organism in order to ensure their multiplication (4).

The aims of our investigations were to follow the changes in the RBC and liver superoxide dismutase (SOD or Cu,Zn-SOD and Mn-SOD; EC 1.15.1.1), catalase (C-ase; EC 1.11.1.6) and glutathione peroxidase (GP-ase; EC 1.15.1.9) activities following infection with the Plasmodia, and to study the development of the reduced glutathione (GSH), lipid peroxidation (LP) and total protein levels with the advance of the parasitism.

MATERIALS AND METHODS

Mice of both sexes of the CFLP strain were used. They received normal mouse chow and water ad libitum. The controls were mice of similar age of

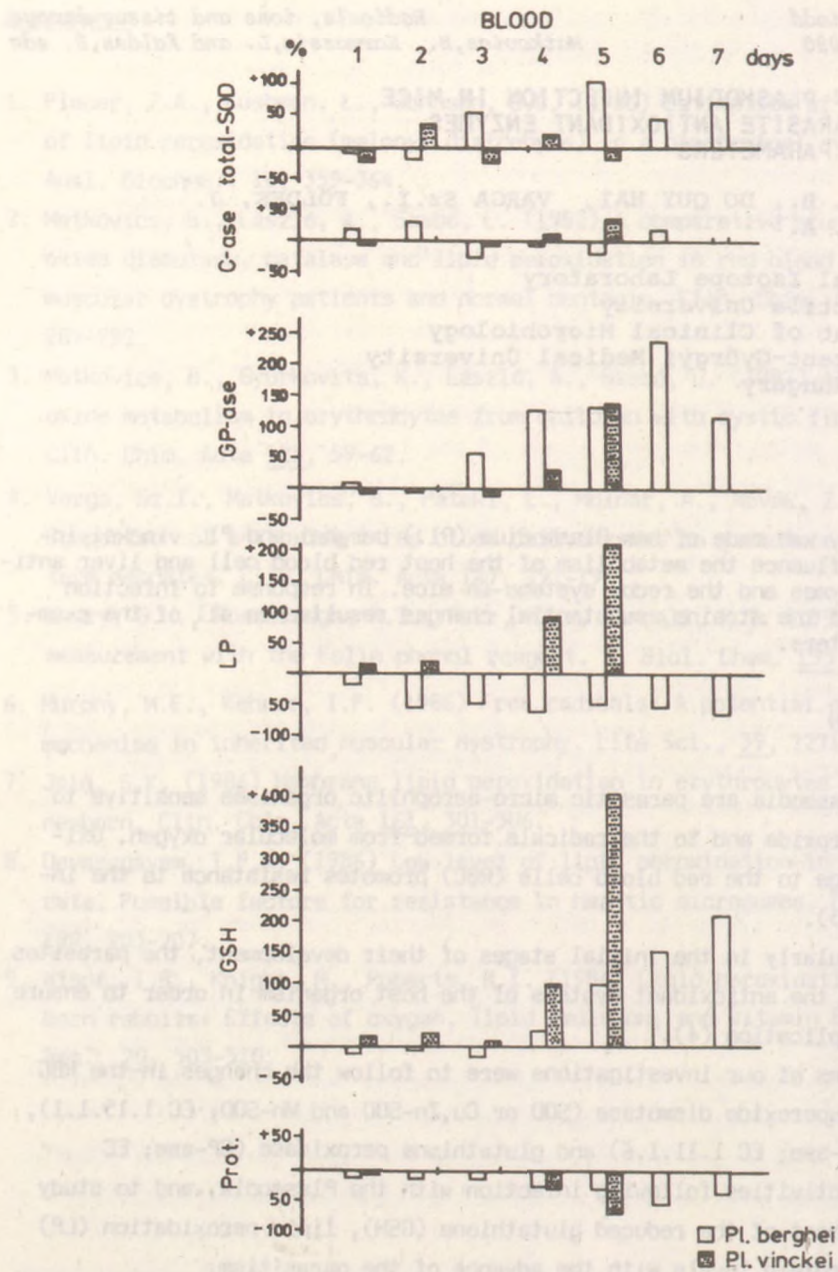


Fig. 1. Differences in RBC antioxidant enzyme activities and other parameters as percentages of control levels

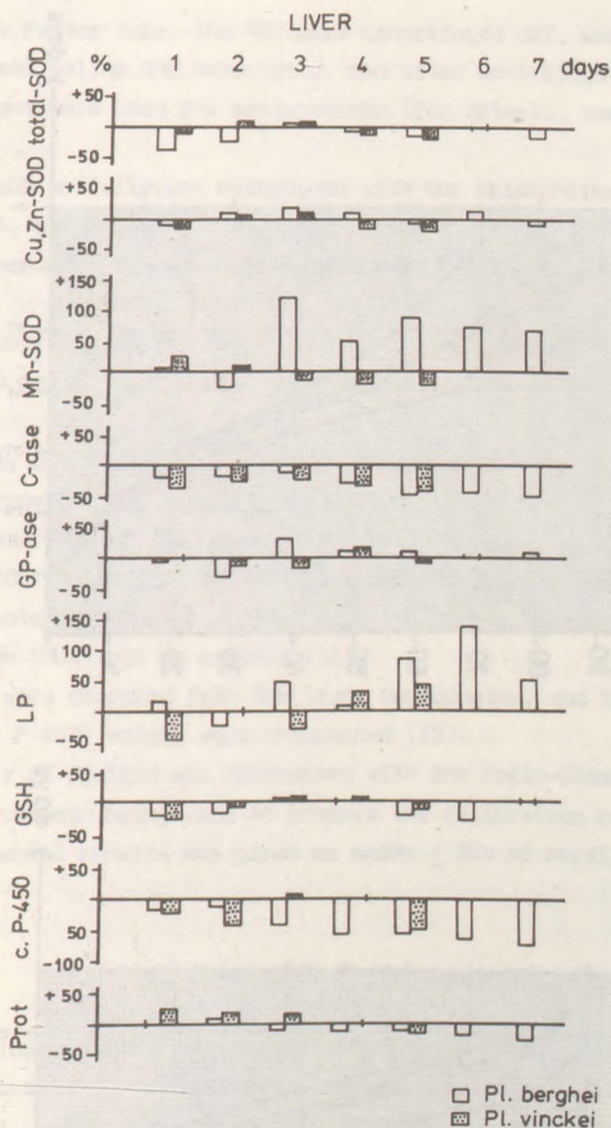


Fig. 2. Differences in liver antioxidant enzyme activities and other parameters as percentages of control levels

the same strain. Plasmodium infection was performed via i.p. injection. Each mouse received 9×10^7 *Pl. berghei* or 7.5×10^7 *Pl. vinckei* parasites. Random infected mice were killed daily. The mice lived for 7 days following *Pl. berghei*, and for 5 days following *Pl. vinckei* infection. After laparotomy, blood was taken from the caudal vein into a heparinized syringe. The whole liver was then removed and a portion of about 1 g wet weight was

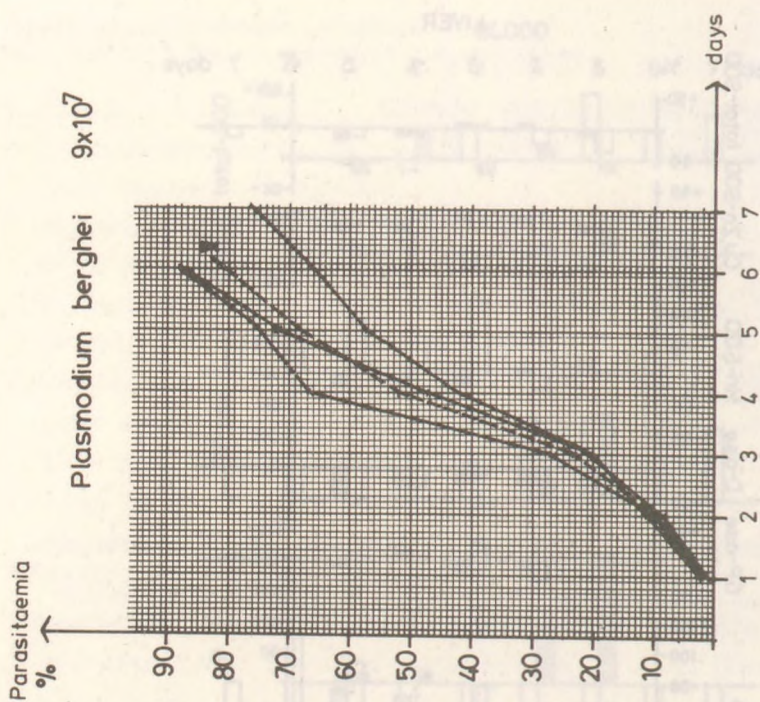
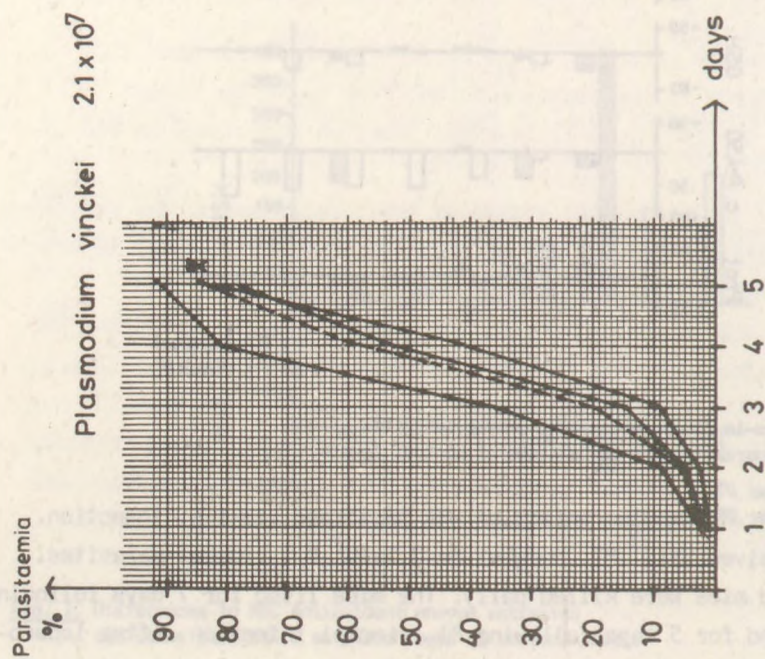


Fig. 3. Degree of the parasitaemia ($n=3$ and $\bar{x}$)

homogenized in a Potter tube. The RBC were centrifuged off, washed 3 times with physiological saline and haemolysed, and after centrifugation aliquots of the supernatant were used for measurements (for details, see Matkovics et al., 1977).

The total-SOD activity was determined with the epinephrine-adrenochrome method (5, 6). Mn-SOD was measured in the presence of KCN (7). The difference between the total-SOD and Mn-SOD values gave the Cu,Zn-SOD activity.

The C-ase activities were determined through measurement of the absorbances at 240 nm, and are given in Bergmeyer units (BU)/mg protein (5, 8).

The activity of GP-ase was determined by means of a "chemical" method, with cumene hydroperoxide as substrate (9, 10).

GSH was measured with the Ellman's reagent (10).

The LP, which is the same as the total amount of thiobarbituric acid (TBA)-active substances, was determined with TBA and is expressed in nmol malonyldialdehyde (MDA) per mg protein (11).

Microsomes were prepared from the liver homogenates, and the cytochrome P-450 (c. P-450) values were determined (12).

The quantity of protein was determined with the Folin-Ciocalteu reagent, bovine albumin being used to prepare the calibration curve (13).

The experimental results are given as means $\pm$ SEM of parallel determinations.

RESULTS

Figure 1 illustrates the percentage differences in the data relating to the RBC haemolysates, compared with those for the controls, in the cases of both parasites. Figure 2 presents the corresponding data for the liver. In both cases, the empty columns and the black columns refer to data on *Pl. berghei* and *Pl. vinckei* infection, respectively. Figure 3 depicts the extent of parasitaemia.

DISCUSSION

In most of the measurements, but mainly in the final stage of parasitism, the SOD level in the RBC was lower than in the controls after *Pl. vinckei* infection, but higher than in the controls after *Pl. berghei* infec-

tion. The situation was different in the liver, where the total -SOD and Cu, Zn-SOD activities after infection were lower than, or barely different from the levels in the control host cells; however, the activity of Mn-SOD after *Pl. berghei* infection increased together with the degree of parasitaemia. Thus, the infected cells are not well supplied in general with SOD. The same picture was found for C-ase; this was mainly the case for the liver homogenates, where a considerable C-ase activity loss could be observed as the parasitism advanced. The GP-ase activity increased together with the extent of parasitism. Following *Pl. vinckei* infection the LP began to rise early in the RBC, whereas after *Pl. berghei* infection it progressively decreased. The LP changes in the liver homogenates were smaller and opposite. Quantitative GSH measurement in the RBC is an appropriate indicator of the parasitism, but it is of much lower value in the liver.

There was a clear decrease in the liver microsomal c. P-450 level after both *Pl.* infections.

With the enhancement of the parasitism, a decrease in the amount of protein could be observed mainly in the RBC.

Other authors, using a different method, found that the total-SOD activity increased with the advance of the parasitism (14). Our results did not confirm this.

The aim of the present work was to map the variations in the anti-oxidant enzymes and in some oxidation-sensitive parameters with the advance of *Pl.* infections, in order to acquire a deeper insight into the mechanism involved in the parasitism and to obtain a model suitable for attempts at therapy. This aim proved successful in both respects.

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METHODS FOR THE INVESTIGATION OF LIPID PEROXIDATION AND THE BIOLOGICAL ANTIOXIDANT DEFENCE MECHANISM

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INTRODUCTION

The cells of the organism are subjected to the action of oxygen-derived free radicals formed during the metabolic processes of organic compounds and also during the metabolism of active oxygen species. The one-electron reaction of dioxygen leads to the generation of the superoxide radical anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) or the hydroxyl radical ($OH^{\cdot}$) and water (62). The electron donors for these reactions are compounds of the electron transport system of the cells: photosynthetic, mitochondrial and microsomal compounds (14), metal ions (29) or organic compounds such as quinones, nitroaromatic compounds or thiol compounds (47). The products of the process are dismutated by the catalytic action of superoxide dismutase, and the superoxide radicals generate H_2O_2 (24), which is decomposed by glutathione peroxidase (20) and catalase (12).

METHODS OF MEASUREMENT

1. Active oxygen species

1.1 Direct methods

Only the superoxide radical anion can be detected by direct methods, especially with pulse radiolysis (16). The other possibility is to detect the superoxide radical by using the photolysis of H_2O_2 (2). A relatively high (about 200 μM) amount of radiocals is required for detection (16). The recently developed method of ESR with rapid freezing (8) is useful in a lower (10^{-8} – 10^{-6} M) concentration range.

There are several methods for the direct detection of radicals in biological systems, e.g. flavin radical (6), quinone radical, nitroxide radical and phenothiazine radical cation formation (35).

1.2. Indirect methods

1.2.1. Detection of superoxide radical. The superoxide radical effects the reduction of cytochrome c, nitroblue tetrazolium and tetranitromethane (21), or initiates and propagates the autoxidation of epinephrine, pyrogallol, 6-hydroxydopamine (48) and hydroxalamine (43). Spin trapping is widely accepted for the detection of the superoxide radical. The spin adducts are detected especially by means of ESR. DMPO (5,5'-dimethyl-1-pyrrolyl-N-oxide) is the generally used spin trap. The problem is that DMPO-OOH is relatively unstable and is converted into DMPO-OH at a moderately high temperature (18).

1.2.2. Detection of hydroxyl radical. The direct determination of the OH[•] radical in biological samples is almost impossible, because of the relatively short half-life and the low concentration. Several indirect methods are based on the reaction of the OH[•] radical with a second chemical compound. The tryptophan absorbance change at 278 nm is a very simple system, but the accuracy is poor (37). The production of diphenols from phenols (40) and the formation of TBA-reactive products in the reaction with deoxyribose (29) are also acceptable. The oxidation of methional to ethylene followed by gas chromatography (3) is also a possibility for detection. The hydroxylation of aromatic compounds, measured by colorimetry (28) and gas/liquid chromatography (43), is also used for OH[•] radical detection. An additional method for the measurement of hydroxylated aromatic compounds is the use of liquid chromatography coupled with an electrochemical detector (41).

2. Measurement of the process of lipid peroxidation

2.1. Measurement of oxygen uptake

Oxygen uptake can be detected manometrically (58), especially at the site of unsaturated fatty acids. An aqueous emulsion must be prepared in a suitable buffer (0.025 M phosphate, pH: 6-7), immediately before use. The method is useful for fats, but also for tissue preparations (59). Oxygen uptake can also be measured in a smaller range, for instance in the liver microsomal fraction, by using a Clark electrode, because the protein content for detection is minimally 1.5-3 mg in this assay (61).

2.2. Measurement of hydroperoxide formation

The ferric thiocyanate method is based on the Fe(II)-Fe(III) conversion in the presence of oxygen radicals. The method is unsuitable for the study of peroxidation in membrane preparations, because of interference by other materials such as H_2O_2 (59). Another group is the iodometric methods. In principle, iodide is oxidised to iodine by the "active oxygen" of the peroxide, and the liberated iodine is determined. The traditional method is titrimetry with $Na_2S_2O_3$, but it requires 0.2-1.0 g of sample (49). The spectrophotometric determination of iodine is more useful, because it requires only 10-100 mg of oxidised lipids. It is important to protect the lipids from further oxidation. For this reason, all of the reagents should contain antioxidant. The excess amount of iodine must be complexed with added cadmium acetate. The absorbance is read at 350 nm. The standard is benzoyl peroxide in chloroform (50). A simple micromethod has also been described, but it requires the exclusion of oxygen by gassing-out all of the solutions with nitrogen.

The chromatographic separation of lipid hydroperoxides starts with extraction using acetone or ethanol. The acetone phase is then acidified with citric acid to pH 4.1-4.5, and extracted into chloroform. A silica plate is used for thin-layer chromatography. Development is performed with a mixture of chloroform-methanol-acetic acid-water (90:8:1:0.8 by vol.). Detection is possible with a simple modification of the ferric thiocyanate method (59). TLC with ^{14}C -fatty acids is also useful after incorporation into phospholipids. Two-dimensional TLC gives better results. The solvent system is ethyl acetate-hexane-acetic acid (75:25:2 by vol.) or chloroform-ether-methanol-acetic acid (45:45:5:2 by vol). High-pressure liquid chromatography is the best method for the separation of peroxidised products. The reversed phase column (ODS) is equilibrated with 26% acetonitrile in water at pH 3.5. A gradient from 26 to 80% acetonitrile is needed. The eluate is detected at 234 nm (13). An isocratic HPLC system may also be used with an ODS column, with 16% loading, and 100% methanol containing 0.1% ammonium sulphate as mobile phase (55). Unfortunately, it is not possible to separate the hydroperoxides from the hydroxy derivatives in this case. A new gas chromatography-mass spectrometry method has recently been described for the measurement of membrane phospholipid oxidation products. The lipids are extracted with dichloromethane, reduced with sodium borohydride and reextracted with dichloromethane. The transesterification is

carried out with the pentafluorohexyl ester. The sensitivity is about 10 ng of oxidation product on the column (56).

The amount of phospholipid peroxides can also be detected by using an "inverse glutathione peroxidase assay". The activity of glutathione peroxidase is measured under conditions where the rate is limited by the amount of peroxide substrate present (31). The modified system is phosphate buffer (pH: 7.4), reduced glutathione 0.75 mM and EDTA 1 mM (54). The enzyme converts hydroperoxides to hydroxy derivatives, producing stoichiometric amounts of oxidised glutathione, which is converted back to the reduced form of glutathione by glutathione reductase with a stoichiometric consumption of NADPH. The total change in optical density at 340 nm is caused by the consumption of NADPH and it allows calculation of the number of nmoles of lipid peroxide. The crucial point of this assay is that the phospholipid hydroperoxides are extremely poor substrates for GSH-Px (27). Therefore, an extra incubation step with phospholipase A₂ is required. A disadvantage of the enzymatic method is that the incubation required for phospholipase A₂ could possibly change the peroxide value. This problem can be solved by using phospholipid hydroperoxide glutathione peroxidase. This is specific for phospholipid hydroperoxides (53).

2.3. Measurement of lipid conjugated dienes

The measurement should be carried out with the extraction of lipids into cyclohexane or dichloromethane. Antioxidants have to be added to solvents and work is performed under a nitrogen stream. The absorption at 233 nm is due to conjugated dienes (42). Quantitative determination of conjugated diene formation can also be carried out with ¹⁴C-tetracyanomethylene is a Diels-Alder condensation (56). The amount of ¹⁴C in the Diels-Alder adducts is a measure of the conjugated diene content. The problem with this method is that it is time-consuming and more difficult than the spectrophotometric determination.

2.4. Measurement of degradation products formed during lipid peroxidation

The theoretical basis is that the lipid peroxides and/or hydroperoxides are degraded spontaneously to a large number of short-chain products especially aldehydes.

2.4.1. The thiobarbituric acid test. The method is based on the finding that 2-thiobarbituric acid in acidic medium forms a yellow-red complex with malondialdehyde and related compounds.

There are several aspects of the method which should be noted:

- (i) Trichloroacetic acid is required for the precipitation of proteins and to provide an acidic environment ($\text{pH} \leq 3.0$).
- (ii) The reaction is not specific for malondialdehyde. Some other compounds, such as hydroxyalkenals, react in the test (15).
- (iii) The composition of the unsaturated fatty acids in the sample is also important. Linoleic acid is relatively resistant to peroxidation and is not thought to produce malondialdehyde (32). Maximal malondialdehyde formation corresponds to the total depletion of arachidonic acid and decahexaenoic acid. The percentage decrease is closely correlated with the percentage of maximal malondialdehyde formation (33).
- (iv) Carbohydrates interfere with the test. It is possible to reduce the interference by heating at 80°C instead of 100°C (61).
- (v) Iron has an important effect on the TBA values. An appropriate amount of iron must be taken in the system. The best method is to remove all iron from the reagents, and 1 mM FeSO_4 is added to the trichloroacetic acid (59).
- (vi) In the case of biological fluids, the aqueous phase can be measured directly at 535 nm . With tissue homogenates, the complex must be extracted into an organic solvent, e.g. *n*-butanol (61). The butanol layer may be measured spectrophotometrically at 535 nm , or fluorimetrically, with excitation at 515 nm and emission at 555 nm (23).

2.4.2. Separation of aldehydes by HPLC. The aldehydes are complexed with 2,4-dinitrophenylhydrazine. After a TLC purification step, because of the contamination with other aldehydes, the sample is taken in the HPLC apparatus. A suitable method is to use an ODS column with methanol-water as mobile phase, using a gradient to 100% methanol. Detection at 378 nm is proposed.

2.4.3. Determination of malondialdehyde using GC. A very specific method has been described for the determination of malondialdehyde as a pyrazole derivative, synthesised with methylhydrazine and measured by means of capillary GC (52).

2.4.4. Measurement of fluorescent products. Fluorescent products are formed during the process of lipid peroxidation if the reaction takes place in the presence of compounds containing free amino groups, proteins or nucleic acids. Amino groups are nearly always present during the peroxidation of lipids in biological systems, and fluorescent products are normally obtained (11). The fluorometric determination performed in the chloroform phase after extraction, with excitation at 340-370 nm and emission at 420-470 nm. No suitable standards are available for this method.

2.4.5. Formation of ethane and pentane. This is the only possibility for the detection of lipid peroxidation in vivo in intact animals by using a non-invasive method. Volatile hydrocarbons such as ethane and pentane are produced by the peroxidation of unsaturated fatty acids (34). Exhaled gas analysis is possible using a GC or GC/MS system. The air is circulated in a closed system, first through H_2SO_4 to trap exhaled NH_3 , through KOH to trap CO_2 , and through dry ice/propanol to condense water vapour. A mixture of alkanes in nitrogen is used for the columns. The concentration of samples is also required before injection into the GC apparatus. For the calculation of ethane and pentane production, a two-compartment model must be used. One compartment is the exhalation chamber, and the other is the animal (46).

3. Enzyme assays

3.1. Catalase activity

The method is based on the decomposition of hydrogen peroxide by catalase. It can be followed by ultraviolet spectroscopy, due to the absorbance of hydrogen peroxide in this region. At 240 nm the molar extinction coefficient for H_2O_2 is $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ (5). The pH optimum is about 7.0. The hydrogen peroxide content in the assay is about 30 mM. RBC haemolysates need to be diluted to 1:100 before use and in tissue samples Triton X100 (1% w/v) is used as a detergent (1).

Some additional methods are also used for the determination of catalase activity. The H_2O_2 removal is followed by iodometric and permanganometric titration (7). The other possibility is to follow the oxygen production by using an oxygen electrode (38) or polarography (44).

3.2. Glutathione peroxidase activity

3.2.1. Fixed time assay methods. Enzyme-catalysed or spontaneous reactions of reduced glutathione (GSH) with H_2O_2 or with organic peroxides are stopped at a fixed time by the addition of strong acid, and the GSH content is measured. The GSH content can be measured by polarography. The dropping mercury electrode is used for this. The method is unsuitable for biological samples (22). The other possibility is the determination of GSH consumption by using the Ellmann reagent (5,5'-dithio-bis-2 nitrobenzoic acid). Substrates are GSH 2 mM, and tert-butyl or cumene hydroperoxide 1.5 mM, or in the case of tissue homogenates 3 mM H_2O_2 . Incubation: 5 to 10 min at 25 or 37 °C. The reaction is stopped with 0.6 M trichloroacetic acid. The consumption of GSH is measured in alkaline medium (pH: 8.3) with the Ellmann reagent, spectrophotometrically at 412 nm (36).

H_2O_2 consumption can also be determined at fixed time intervals. The reaction is stopped by acidification, unmetabolised GSH is scavenged with mercuric salts, the solution is neutralised, and the remaining H_2O_2 is reacted with scopoletin in the presence of horseradish peroxidase. The H_2O_2 - scopoletin dye is measured fluorimetrically. The method has been successfully used for evaluation of the kinetics of the enzyme, but not for routine analysis (19).

3.2.2. Continuous monitoring of oxidised glutathione formation. The basic principle of the method is that GSSG is formed during the reaction and can be reduced with an excess of glutathione reductase. The concomitant oxidation of NADPH as hydrogen donor is monitored photometrically.

Substrates are GSH 10 mM, tert-butyl hydroperoxide 12 mM or H_2O_2 1.5 mM. Glutathione reductase (2.5 U) as enzyme source and NADPH (8.4 mM) as hydrogen donor are added. The reaction is carried out in 0.1 M phosphate buffer (pH: 7.0) containing 1 mM EDTA. The NADPH loss is followed at 340 nm for 3 min as hydroperoxide-independent consumption and for 5 min additionally after the addition of the hydroperoxide (22).

In the case of liver and other tissues, tert-butyl hydroperoxide or cumene hydroperoxide can not be used because they are also substrates for glutathione-S-transferases. H_2O_2 should be used only after blocking catalase by the addition of 1 mM sodium azide. Red blood cells contain met-haemoglobin, which may cause a false reaction. Therefore, the conversion of haemoglobin to cyanomethaemoglobin prior to the assay is recommended.

3.3. Superoxide dismutase activity

3.3.1. Direct assays. It is possible to detect the O_2^- consumption spectrophotometrically at 245 nm. The loss of O_2^- means the action of superoxide dismutase in the system. The molar extinction coefficient at this wavelength is $2350 \text{ M}^{-1} \text{ cm}^{-1}$. The method can be used only in pure chemical systems (17).

3.3.2. Indirect methods

O_2^- generated chemically or enzymatically. The cytochrome c assay is the generally used system for the determination of SOD activity. The reagent mixture contains xanthine $50 \mu\text{M}$, cytochrome c $10 \mu\text{M}$ and EDTA 0.1 mM in a 50 mM phosphate buffer (pH: 7.8). Xanthine oxidase is added to give an absorbance change at 550 nm of 0.025 min^{-1} . Theoretically, it is $1.2 \mu\text{mol min}^{-1} O_2^-$ production (25).

The nitroblue tetrazolium assay is based on the reduction of nitroblue tetrazolium to nitroformazan. The assay mixture contains xanthine $50 \mu\text{M}$ and NBT $100 \mu\text{M}$ in a 50 mM carbonate buffer (pH: 10.2). Xanthine oxidase is added to give an absorbance change at 550 nm of 0.025 min^{-1} (4).

The autoxidation of epinephrine is also used for the determination of SOD activity. The reaction mixture contains $0.9 \mu\text{M}$ epinephrine in a 0.05 M carbonate buffer (pH: 10.2). The absorbance change is followed at 480 nm (39).

O_2^- generation using radiolysis. In irradiated, air-saturated aqueous formate solution at pH 7, all the primary radicals are converted into O_2^- within a short of time after the pulse. The K_{cat} value of the enzyme can be measured more precisely with this technique than with others. The only problem with this method is that a linear accelerator is required to produce a 5 MeV electron pulse.

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INHIBITORY EFFECT OF GOLD COMPLEXES
(SODIUM AUROTHIOMALATE AND AURANOFIN)
ON GLUTATHIONE PEROXIDASE ACTIVITY
IN DIFFERENT SPECIES

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SUMMARY

Two different gold compounds (sodium aurothiomalate and auranofin) were investigated as potent inhibitors of glutathione peroxidase (EC 1.11.1.9) in vitro. The sources of the enzyme were red blood cell haemolysates from different animal species (horse; cattle, goose, domestic fowl) and human subjects. The IC_{50} value was determined after a one-hour incubation with the gold compound in the presence of reduced glutathione. The inhibitory concentration was nearly the same in all species except fowl. In accordance with some earlier findings, the results suggested that Au(I) interacts with the active site of the enzyme (Se). The differences found between the species support the hypothesis that the thiol moiety of the molecules is also of importance in the effect.

INTRODUCTION

The reduction of lipid hydroperoxides plays a key role in the control of lipid peroxidation in living organisms (14). A protective effect against lipid peroxidation through the reduction of lipid hydroperoxides has been indicated for glutathione peroxidase (EC 1.11.1.9) under different conditions (3). Several compounds have been proposed as strong in vivo and in vitro inhibitors of selenium glutathione peroxidase (11). Among them, gold complexes, and especially those used in the chrysotherapy of rheumatoid arthritis (aurothioglucose and sodium aurothiomalate), were found to be effective inhibitors under both in vivo and in vitro conditions (1, 2, 5). The inhibitory effect was proposed to involve covalent binding between the selenium, as the active site of the enzyme, and gold. The thiol component of the molecules also seems to be an important factor in the effect, especially from a therapeutic point of view (10).

The aim of the present study was to detect the rate of selenium

glutathione peroxidase inhibition by using the well-known inhibitor sodium aurothiomalate and auranofin, which has not previously been investigated in this respect.

MATERIALS AND METHODS

Blood preparation: Blood was collected from four animal species (horse, cattle, goose, domestic fowl) and from healthy human volunteers. EDTA- Na_2 (0.2 M) was used as anticoagulant in a volume of 0.05 ml blood ml^{-1} . Plasma was separated from the cells within 2 hours after collection. The plasma and buffy coat were removed and the red blood cells (RBC) were washed three times with ice-cold ($+4^\circ\text{C}$) isotonic saline, and then haemolysed with a nine-fold volume of redistilled water and by freezing (-18°C) and thawing ($+25^\circ\text{C}$). The cell debris was separated by centrifugation (7000 g for 20 min at $+4^\circ\text{C}$). The protein content of the haemolysate was adjusted to 30 g/l, as measured by the method of Lowry et al. (7).

Chemicals: Sodium aurothiomalate was obtained from Sigma (St. Louis, MO) and was dissolved in physiological saline in a total volume of 0.1 ml. Auranofin was a generous gift from SmithKline Beckman (Philadelphia, PA). A stock solution was made in 50% (v/v) absolute ethanol and diluted to the appropriate concentration in physiological saline at a total volume of 0.1 ml.

Selenium glutathione peroxidase assay: The determinations were performed in triplicate. The reaction mixture, which contained 0.1 ml RBC haemolysate, 3 mM GSH in 0.05 M phosphate buffer ($\text{pH}=7.6$) and 1 mM EDTA, was incubated at 37°C for one hour with the appropriate amount of gold compound. Blank samples containing the final alcohol/water concentration in the system served for correction in the case of auranofin. The gold compounds were also incubated without RBC haemolysate, to correct for reaction of the sulphhydryl groups present in the molecules with the Ellmann reagent (false-positive reaction). The reaction was started by the addition of 6 mM cumene hydroperoxide, with incubation at 37°C for 10 minutes. The reaction was stopped with 10% (w/v) trichloroacetic acid. The protein precipitate was separated by centrifugation (2000 g for 5 min at 4°C). An aliquot of the supernatant was taken and the pH was increased to 8.3 with Tris-HCl buffer ($\text{pH}=8.9$). The residual -SH groups were measured with the

Ellmann reagent at 412 nm within 5 minutes. The activity was expressed in units: 1 U was the amount of GSH in nmoles oxidised min^{-1} at 37 °C (8).

RESULTS

The results of the present study showed that both gold compounds investigated displayed a marked inhibitory effect on the glutathione peroxidase activity. The IC_{50} value was more or less the same for all of the investigated species. The highest values were found for the avian species. The results are based on the protein contents of the RBC haemolysates, which were standardised in all samples. The IC_{50} values were calculated by linear regression analysis.

The equations were as follows:

Human: auranofin $y = -0.0118x + 1.56$ $P < 0.001$

aurothiomalate $y = -0.0148x + 1.15$ $P < 0.01$

Horse: auranofin $y = -0.0077x + 0.965$ $P < 0.001$

aurothiomalate $y = -0.017x + 0.699$ $P < 0.01$

Cattle: auranofin $y = -0.014x + 1.35$ $P < 0.001$

aurothiomalate $y = -0.024x + 1.19$ $P < 0.01$

Goose: auranofin $y = -0.0083x + 1.24$ $P < 0.001$

aurothiomalate $y = -0.020x + 1.06$ $P < 0.01$

Chicken: auranofin $y = -0.01x + 1.85$ $P < 0.001$

aurothiomalate $y = -0.02x + 1.85$ $P < 0.01$

Table 1. IC_{50} values of auranofin and sodium aurothiomalate on selenium glutathione peroxidase activity

Species	Auranofin (nmol/mg protein of RBC haemolysate)	Sodium aurothiomalate
Human	65.50	39.42
Horse	62.54	20.59
Cattle	50.00	25.80
Goose	75.42	27.55
Domestic fowl	88.95	40.37

DISCUSSION

The results revealed the marked inhibitory effect of both gold compounds on the selenium glutathione peroxidase activity. The interaction probably occurs through covalent bonding between selenium (Se^{2+}) and gold (Au^+), as proposed earlier (1). This hypothesis is supported by the results of the present study. The IC_{50} values were more or less the same for the different species. It is well known that the selenium content of the enzyme is the same for all of the investigated species (6). For this reason, it is proposed that selenium (selenocystein) is the target for the effect in this respect. The differences between the species are based on the finding that, when crude samples are used, the optimal substrate concentration may be different in different species (4, 9). In the case of a purified enzyme preparation, this difference between the species cannot be excluded. In the present study, the substrate concentration was the same in all cases for standardisation of the system, but crude samples were used instead of a purified enzyme preparation. The reason why this system was used was that in therapy the compounds react not with a purified, isolated enzyme, but with a complex of enzymes and other molecules in the cells. The main target of chrysotherapy is not the red blood cells, but in this case only the selenium glutathione peroxidase activity was measured without glutathione transferase contamination (3).

The results also supported the proposal that the target of gold compounds may be glutathione peroxidase. The inhibition of the enzyme may cause a protection of the thiol groups which are oxidised as a result of the enzyme catalysis. Penicillamine and dithiothreitol also have such an effect (13). This is supported by the result that treatment with gold-thiol compounds increased the plasma -SH level (5). The thiol moiety of the molecules could also influence the inhibitory activity. It was found that thiol protecting agents have a negative effect on the superoxide production inhibited by auranofin (11). On the other hand, thiomalic acid, as the gold-free analogue of sodium aurothiomalate, had the same effect, but only at a much higher concentration as compared to the original molecule in an in vitro system (10). This means that the binding of Au to the active site of the enzyme occurs not only to Se, but also to the cysteine residue.

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PEROXINORM INJECTION AS A POSSIBLE SOD STANDARD

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PEROXINORM injection, containing Orgotein SOD for medical use (Grunenthal), was compared with a SOD standard with an activity of 3000 U/mg, made from cattle red blood cells by SIGMA. As PEROXINORM is more readily available, we would like to use it as a SOD standard. One vial of PEROXINORM contains 4 or 8 mg SOD (depending on the packaging); the lyophilized material of the vial should be dissolved in 2 ml of sterile NaCl solution of 9 g/l concentration.

We compared the activity of PEROXINORM with that of the SIGMA standard SOD in a process based on an inhibition reaction (formazan is produced from nitrotetrazolium blue on the action of superoxide ion), developed by Nishikimi et al.

PEROXINORM produced in three different periods of time was compared with the same SIGMA SOD standard. The activities of the three PEROXINORM SOD products were on average 3150 ± 140 , 2950 ± 210 and 3300 ± 180 U/mg. The coefficients of variation were 4.45, 7.12 and 5.45%. The SIGMA product with 3000 U/mg activity costs US \$ 6.55, while PEROXINORM containing 12.533 U SOD/vial costs on average US \$ 13.

Our results indicate that PEROXINORM injection can be used as a SOD standard for measuring samples of animal origin; it is cheaper and more readily available than the SIGMA Product.

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RENAL ISCHEMIA AND REPERFUSION EFFECT CHARACTERIZED BY FREE RADICAL REACTIONS

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The effects of kidney preservation during serial investigations were examined in our department some 10 to 20 years ago. The effects of warm ischemia and cold hypothermia on structure and function were characterized. Morphological and ultrastructural alterations of the membrane structure were observed. Moreover, ionic shifts were demonstrated which similarly denoted the failure of the membrane transport.

Methodological broadening of the investigations of the tissue and organ damage led us to observe the equilibrium of the membrane system, i.e. how it changes in response to free radical action and how this can be influenced.

The present paper reports the effects of 1 and 2-hour warm ischemia, followed by 1, 2, 12 and 24-hour reperfusion. The experiments were carried out on white rats weighing 200-250 g. The animals were narcotized with an intraperitoneal injection of 0.8 ml Calypsol. Through median laparotomy, the left renal artery was prepared and temporarily occluded with a microsurgical clip. After a 1 or 2-hour ischemic period, the clip was removed and the circulation was restored.

The experimental groups:

- 1/1: 1-hour renal ischemia + 1-hour reperfusion (16 animals)
- 1/2: 1-hour renal ischemia + 2-hour reperfusion (12 animals)
- 1/3: 1-hour renal ischemia + 12-hour reperfusion (9 animals)
- 1/4: 1-hour renal ischemia + 24-hour reperfusion (11 animals)
- 2/1: 2-hour renal ischemia + 1-hour reperfusion (16 animals)
- 2/2: 2-hour renal ischemia + 2-hour reperfusion (8 animals)
- 2/3: 2-hour renal ischemia + 24-hour reperfusion (11 animals)

At the end of reperfusion, the kidneys were removed. After tissue homogenization, the tissue malondialdehyde (MDA) (as a marker of the

membrane degradation) and the reduced glutathione (GSH) content and the superoxide dismutase (SOD) activity (as markers of the endogenous scavenger state) were measured. The contralateral intact kidneys served as controls. The levels after ischemia and reperfusion were calculated as percentages of the intact values.

RESULTS

Figure 1 shows the relative MDA concentrations of the reperfused kidneys. After a 1-hour reperfusion, the MDA level is decreased. After a 2-hour reperfusion, this reduction can no longer be observed. Conversely, it is very characteristic that the values after long-term reperfusion are different. The MDA level is virtually identical with that of the controls after the 1-hour ischemia, while it is always significantly reduced after the 2-hour ischemia.

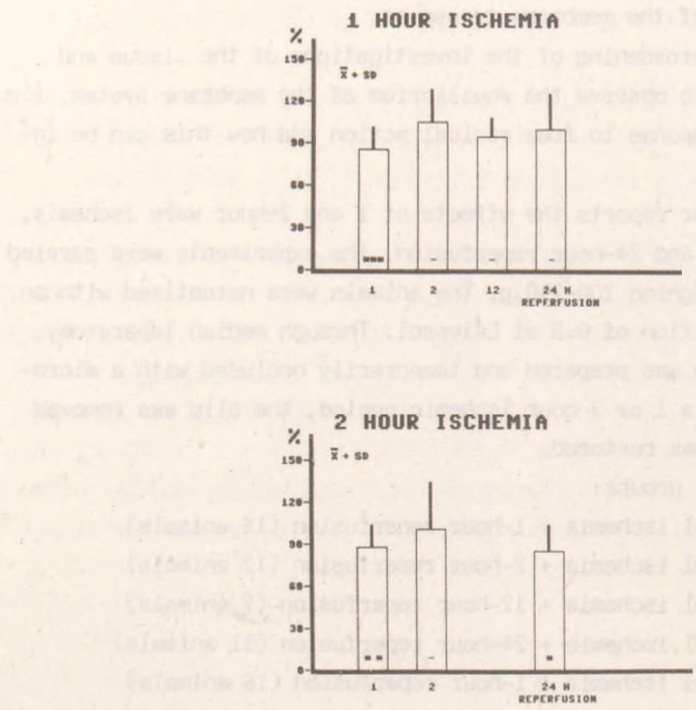


Fig. 1. Percentage changes of MDA content of reperfused kidneys in comparison with the control

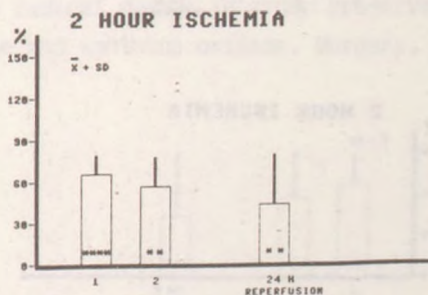
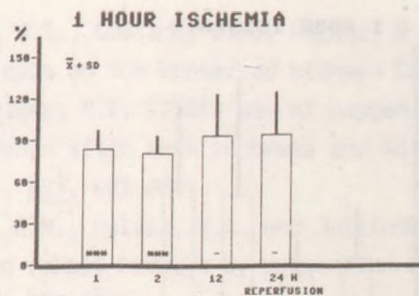


Fig. 2. Percentage changes of GSH content of reperfused kidneys in comparison with the control

Figure 2 shows the corresponding changes in the GSH concentration of reperfused renal tissue. The initial GSH reduction is not continued after the 1-hour reperfusion, or a normalization tendency is seen. After the 2-hour ischemia, the GSH decrease is always considerable and the reperfusion does not lead to a restoration.

Figure 3 shows the changes in SOD activity. Similarly to the changes in GSH, after the 1-hour ischemia the short-term reperfusion has not fully reversed the SOD decrease, but this can no longer be observed after reperfusion for 12 or 24-hours. After the 2-hour ischemia, the reperfusion cannot normalize the deranged SOD state.

The aim of the present experiments was merely to establish the effects of warm ischemia followed by reperfusion. Since it is known that the limit of ischemic tolerance of renal tissue is about 1 hour, the observed differences in the experimental groups are quite understandable. The warm ischemia produces failures in the equilibrium of the membrane system. The MDA formed from the membrane polyunsaturated fatty acids is washed away from the

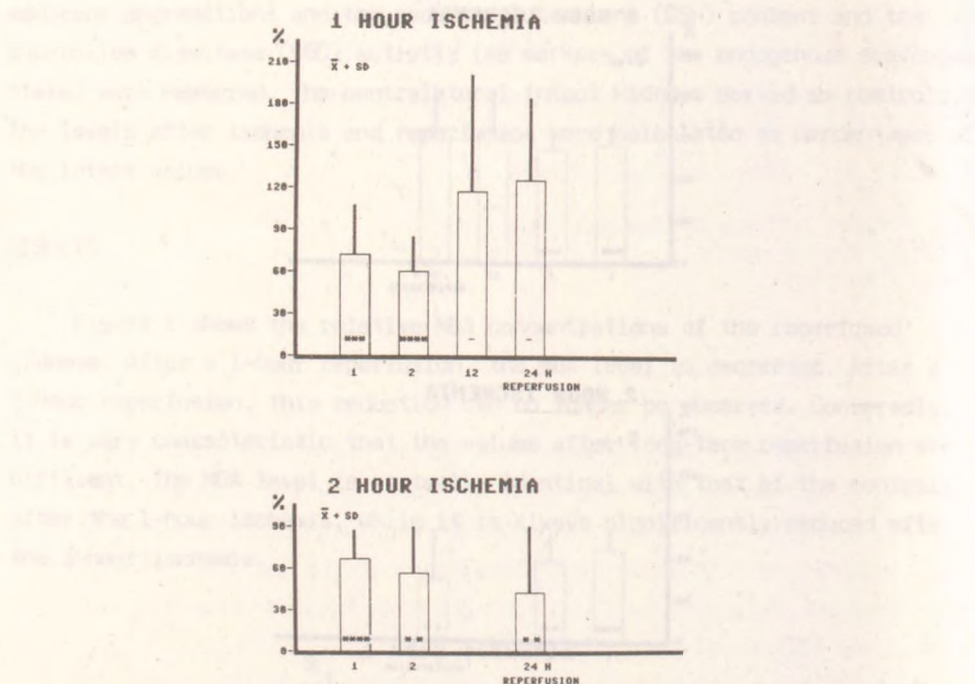


Fig. 3. Percentage changes of SOD activity of reperused kidneys in comparison with the control

tissues by the reperfusion, and this factor causes its reduction. The regeneration after the 1-hour ischemia points to the revitalization of cells and membranes, but this does not occur after the 2-hour ischemia.

This event seems to be more important as concerns the scavenger compounds: after severe ischemic tissue degradation, the reperfusion is unable to restore the scavenging involving the cellular and subcellular structure, i.e. a long-term decrease in the scavenger content indicates a definitive failure of the cell function.

Experiments to clarify further questions of ischemic, hypothermic and preserved states in renal tissue are continuing.

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EFFECTS OF WHOLE-BODY IRRADIATION ON ANTIOXIDANT ENZYMES AND LIPID PEROXIDATION OF INBRED MICE

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ABSTRACT

Mice displaying "high" (B 10 strain) and "low" (B 10.A strain) tissue SOD activities were exposed to single whole-body X-ray irradiation of various intensities. The tissue SOD activity was influenced to extents depending on the nature of the tissue. At higher radiation doses, the SOD activities increased in the tissues of the B 10.A mice and decreased in the B 10 strain. In both strains, the P-ase and C-ase activities decreased considerably following irradiation, in most cases not exceeding the lower limit of measurability. The liver and kidney LP of both mouse strains progressively rose with increase of the radiation dose. In response to a high radiation dose, the c. P-450 activity of the liver tissue decreased considerably. Smaller doses caused an elevation of the liver c. P-450. The total protein contents of irradiated mouse organs were measured and compared. In the organ homogenizates of both strains, the (Na⁺, K⁺)-ATP-ase activity decreased with increase of the radiation dose.

INTRODUCTION

In response to ionizing radiation, in aqueous solution molecular oxygen secondarily takes up one electron to form the superoxide radical anion (O₂⁻), in addition to other oxygen-containing radicals.

In our earlier studies of the locus regulating superoxide dismutase (SOD; EC 1.15.1.1) synthesis, it was demonstrated that a great difference can be observed between the inbred mouse strains B 10 and B 10.A as regards tissue SOD activity. Tissue homogenates of the B 10 strain exhibited "high", and those of the B 10.A strain "low" SOD activities. We also examined and compared the peroxidase (P-ase; EC 1.11.1.5) and catalase (C-ase; EC 1.11.1.6) activities of the tissues of the two mouse strains (1). SOD, P-ase and C-ase are in general termed antioxidant enzymes.

On the basis of those observations, we considered it interesting and important to examine in detail the effects of various single doses of whole-body X-ray irradiation on the tissue lipid peroxidation (LP) and the activities of SOD, P-ase, C-ase, cytochrome P-450 (c. P-450) and $(\text{Na}^+, \text{K}^+)\text{ATP-ase}$ in the above-mentioned mouse strains.

MATERIALS AND METHODS

The mice used for the experiments were from inbred strains. They were maintained on a normal diet under standard conditions, and received water ad libitum. All of the mice used were 3.5-month-old males.

The whole-body irradiations were performed with a TRAKIS XR-1 X-ray apparatus (Budapest, Hungary). Mice were irradiated in groups of ten. In a single exposure, they received whole-body X-irradiation ranging from 250 to 1000 R. The irradiated mice were starved for 24 hours, then sacrificed and exsanguinated, and their organs were homogenized. The ratio of the wet tissue weight (w.t.w.) and the homogenation fluid was in general 1:5 or 1:10.

SOD activity was determined on the basis of the inhibition by SOD of the spontaneous transformation epinephrine $\rightarrow$ adrenochrome at pH 10.5. The method was originally described by Misra et al. (2), and modified to our purposes (3). In the SOD measurements, the tissue was homogenized in 0.05 M K_2HPO_4 solution (pH 7.8), and after centrifugation aliquots of the supernatant were used for the measurements. Unit enzyme here was taken as the amount of enzyme which inhibited 50% of the epinephrine $\rightarrow$ adrenochrome transformation.

The guaiacol method was used for P-ase activity measurements (3). C-ase activities were determined via the rate of decrease of the H_2O_2 absorption maximum at 240 nm; the enzyme activities are given in Bergmeyer units (BU), where 1 BU is the quantity of C-ase which decomposes 1000 mg H_2O_2 in 1 min (4).

C.P-450 and b_5 values were determined by the method of Greim (5), and are given in $\mu\text{mole/protein mg}^{-1}$. A UVICORD recording spectrophotometer was employed to obtain the difference absorption curves.

For ATP-ase determinations, 0.5 ml homogenate was added to 1 ml assay medium and after incubation at 37 °C the phosphate (P_i) was determined by the method of Chen et al. (6, 7). The ATP-ase values are given in units of $\mu\text{mole P}_i \cdot \text{protein mg}^{-1} \cdot \text{h}^{-1}$.

Total tissue homogenates were used for determination of the tissue LP; the measurements were based on the thiobarbituric acid determination of malondialdehyde (MDA). The calibration curve was prepared with MDA-diethylacetate and the values obtained are expressed in nanomole MDA/g w.t.w. (8).

Protein was determined by the method of Lowry et al. (9).

The reagents employed were of the highest purity available.

It should be noted in general that the data are the averages of at least 10 parallel measurements ($n = 10$). The mean errors of the measurements are ± 5 -10%. In the calculation of the data, a PL/1 program was used on an RHO computer (Robotron, Magdeburg, GDR).

RESULTS AND DISCUSSION

Table 1 presents the changes in the SOD activities in the two strains after X-ray irradiation with 250, 500 and 1000 R. The SOD activity in the homogenates of five organs of the B 10. A strain exhibits a dose-dependent increase as a result of the irradiation, the level in several cases exceeding the SOD activity of the control by more than 100%. In contrast, the SOD activity in the supernatants of the B 10 strain organ homogenates generally displays a dose-dependent decrease. However, in contrast with the higher doses, the 250 R irradiation most often causes opposite tendencies in the two strains.

Tables 2 and 3 list the P-ase and C-ase activities in the controls and after irradiation. The variations in the P-ase activities of the spleen and pancreas display a certain radiation dose-dependence: for the homogenates of these organs in the B 10. A strain, increasing values are obtained up to a dose of 500 R, while the P-ase activities are decreased considerably in every organ homogenate by a dose of 1000 R. The liver homogenate is not P-ase active, and the comparatively low P-ase activities of the kidney (B 10 strain) and thymus are reduced to immeasurably small values by even the lowest radiation dose. The P-ase activity of the spleen decreases and then increases for the highest radiation dose, similarly to the P-ase activity of the pancreas. The P-ase activity of the thymus increases significantly as a function of the radiation dose.

With regard to the C-ase activities (Table 3) in the organs of the B 10. A strain, those of the liver, kidney, thymus and pancreas are decreased so as to be immeasurable by all of the radiation doses. The same holds for the homogenates of the liver, kidney and pancreas of the B 10

Table 1. Effects of whole body X-ray irradiation of various intensities on the SOD activities of organ homogenates of 3.5-month-old male individuals of the two mouse strains, maintained under identical conditions. SOD assayed at 480 nm; results are given as $\bar{X} \pm S$ ($n = 10$).

Organ		SOD activity, U/g w.t.w.	
		B 10.A	B 10
liver	control	2600 $\pm$ 450	9450 $\pm$ 600
	250 R	2433 $\pm$ 600	9550 $\pm$ 850
	500 R	3500 $\pm$ 400	8500 $\pm$ 750
	1000 R	4400 $\pm$ 500	6900 $\pm$ 800
kidney	control	2050 $\pm$ 100	4800 $\pm$ 460
	250 R	2070 $\pm$ 350	5200 $\pm$ 450
	500 R	2200 $\pm$ 400	3670 $\pm$ 500
	1000 R	2500 $\pm$ 340	3220 $\pm$ 355
spleen	control	650 $\pm$ 70	1350 $\pm$ 200
	250 R	645 $\pm$ 50	1530 $\pm$ 175
	500 R	775 $\pm$ 60	1220 $\pm$ 180
	1000 R	840 $\pm$ 72	1018 $\pm$ 192
thymus	control	470 $\pm$ 43	1810 $\pm$ 210
	250 R	533 $\pm$ 51	2055 $\pm$ 186
	500 R	680 $\pm$ 37	1960 $\pm$ 191
	1000 R	823 $\pm$ 42	1588 $\pm$ 205
pancreas	control	106 $\pm$ 38	900 $\pm$ 105
	250 R	166 $\pm$ 41	1045 $\pm$ 100
	500 R	292 $\pm$ 27	1165 $\pm$ 97
	1000 R	309 $\pm$ 31	1175 $\pm$ 80

strain. The activities in the spleen (B 10.A) and in the spleen and thymus (B 10) are reduced considerably by the irradiation, even in the smallest dose.

Table 4 presents the protein levels of the supernatants of the organ homogenates.

Table 5 contains the LP values measured in two large organs, the liver and kidney, as functions of the radiation dose. The c. P-450 activity changes in the liver are similarly given as a function of the radiation dose. In both strains and in both organs, the LP values rise uniformly as a function of the radiation dose. The c. P-450 displays an activity increase in the liver at 500 R in one strain (B 10.A), and at 250 R in the other (B 10). However, the liver microsomal c. P-450 activity is substantially decreased in both strains by 1000 R.

Table 2. Tissue P-ase activity values of B 10.A and B 10 strains irradiated under the conditions detailed in the experimental section; assayed at 470 nm; results are given as $\bar{X} \pm S$ (n = 10).

Organ		P-ase activity, U/g w.t.w.			
		B 10.A		B 10	
kidney	control	61 $\pm$ 20	control	85 $\pm$ 24	
	after irradi.	< 0.1	after irradi.	< 0.1	
spleen	control	1470 $\pm$ 135		1700 $\pm$ 600	
	250 R	1500 $\pm$ 142		1700 $\pm$ 450	
	500 R	2700 $\pm$ 185		1300 $\pm$ 340	
	1000 R	600 $\pm$ 100		1500 $\pm$ 635	
thymus	control	224 $\pm$ 30	control	280 $\pm$ 94	
	after irradi.	< 0.1	250 R	900 $\pm$ 145	
			500 R	900 $\pm$ 210	
			1000 R	1200 $\pm$ 200	
pancreas	control	500 $\pm$ 48		300 $\pm$ 100	
	250 R	600 $\pm$ 52		240 $\pm$ 96	
	500 R	1050 $\pm$ 180		120 $\pm$ 84	
	1000 R	450 $\pm$ 130		230 $\pm$ 90	

Table 3. C-ase activities measured under the previously described conditions at 240 nm; results are given as $\bar{X} \pm S$ (n = 10).

Organ		C-ase activity, B.U./g w.t.w.			
		B 10.A		B 10	
liver	control	1.59 $\pm$ 0.14		1.58 $\pm$ 0.15	
	after irradi.	<10 ⁻⁵		< 10 ⁻⁵	
kidney	control	1.34 $\pm$ 0.10		1.40 $\pm$ 0.13	
	after irradi.	< 10 ⁻⁵		< 10 ⁻⁵	
spleen	control	0.12 $\pm$ 0.01		0.13 $\pm$ 0.01	
	250 R	0.05 $\pm$ 0.00		0.04 $\pm$ 0.10	
	500 R	0.05 $\pm$ 0.00		0.03 $\pm$ 0.02	
	1000 R	0.04 $\pm$ 0.00		0.05 $\pm$ 0.02	
thymus	control	0.09 $\pm$ 0.01		0.10 $\pm$ 0.02	
	after irradi.	< 10 ⁻⁵	250 R	0.03 $\pm$ 0.03	
			500 R	0.04 $\pm$ 0.01	
			1000 R	0.02 $\pm$ 0.02	
pancreas	control	0.10 $\pm$ 0.01		0.12 $\pm$ 0.01	
	after irradi.	<10 ⁻⁵		<10 ⁻⁵	

Table 1. Effects of whole body X-ray irradiation of various intensities on the SOD activities of organ homogenates of 3.5-month-old male individuals of the two mouse strains, maintained under identical conditions. SOD assayed at 480 nm; results are given as $\bar{X} \pm S$ (n = 10).

Organ		SOD activity, U/g w.t.w.	
		B 10.A	B 10
liver	control	2600 $\pm$ 450	9450 $\pm$ 600
	250 R	2433 $\pm$ 600	9550 $\pm$ 850
	500 R	3500 $\pm$ 400	8500 $\pm$ 750
	1000 R	4400 $\pm$ 500	6900 $\pm$ 800
kidney	control	2050 $\pm$ 100	4800 $\pm$ 460
	250 R	2070 $\pm$ 350	5200 $\pm$ 450
	500 R	2200 $\pm$ 400	3670 $\pm$ 500
	1000 R	2500 $\pm$ 340	3220 $\pm$ 355
spleen	control	650 $\pm$ 70	1350 $\pm$ 200
	250 R	645 $\pm$ 50	1530 $\pm$ 175
	500 R	775 $\pm$ 60	1220 $\pm$ 180
	1000 R	840 $\pm$ 72	1018 $\pm$ 192
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	500 R	680 $\pm$ 37	1960 $\pm$ 191
	1000 R	823 $\pm$ 42	1588 $\pm$ 205
pancreas	control	106 $\pm$ 38	900 $\pm$ 105
	250 R	166 $\pm$ 41	1045 $\pm$ 100
	500 R	292 $\pm$ 27	1165 $\pm$ 97
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strain. The activities in the spleen (B 10.A) and in the spleen and thymus (B 10) are reduced considerably by the irradiation, even in the smallest dose.

Table 4 presents the protein levels of the supernatants of the organ homogenates.

Table 5 contains the LP values measured in two large organs, the liver and kidney, as functions of the radiation dose. The c. P-450 activity changes in the liver are similarly given as a function of the radiation dose. In both strains and in both organs, the LP values rise uniformly as a function of the radiation dose. The c. P-450 displays an activity increase in the liver at 500 R in one strain (B 10.A), and at 250 R in the other (B 10). However, the liver microsomal c. P-450 activity is substantially decreased in both strains by 1000 R.

Table 2. Tissue P-ase activity values of B 10.A and B 10 strains irradiated under the conditions detailed in the experimental section; assayed at 470 nm; results are given as $\bar{X} \pm S$ (n = 10).

Organ	P-ase activity, U/g w.t.w.			
		B 10.A		B 10
kidney	control	61 ± 20	control	85 ± 24
	after irrad.	< 0.1	after irrad.	< 0.1
spleen	control	1470 ± 135		1700 ± 600
	250 R	1500 ± 142		1700 ± 450
	500 R	2700 ± 185		1300 ± 340
	1000 R	600 ± 100		1500 ± 635
thymus	control	224 ± 30	control	280 ± 94
	after irrad.	< 0.1	250 R	900 ± 145
			500 R	900 ± 210
			1000 R	1200 ± 200
pancreas	control	500 ± 48		300 ± 100
	250 R	600 ± 52		240 ± 96
	500 R	1050 ± 180		120 ± 84
	1000 R	450 ± 130		230 ± 90

Table 3. C-ase activities measured under the previously described conditions at 240 nm; results are given as $\bar{X} \pm S$ (n = 10).

Organ	C-ase activity, B.U./g w.t.w.			
		B 10.A		B 10
liver	control	1.59 ± 0.14		1.58 ± 0.15
	after irrad.	$< 10^{-5}$		$< 10^{-5}$
kidney	control	1.34 ± 0.10		1.40 ± 0.13
	after irrad.	$< 10^{-5}$		$< 10^{-5}$
spleen	control	0.12 ± 0.01		0.13 ± 0.01
	250 R	0.05 ± 0.00		0.04 ± 0.10
	500 R	0.05 ± 0.00		0.03 ± 0.02
	1000 R	0.04 ± 0.00		0.05 ± 0.02
thymus	control	0.09 ± 0.01		0.10 ± 0.02
	after irrad.	$< 10^{-5}$	250 R	0.03 ± 0.03
			500 R	0.04 ± 0.01
			1000 R	0.02 ± 0.02
pancreas	control	0.10 ± 0.01		0.12 ± 0.01
	after irrad.	$< 10^{-5}$		$< 10^{-5}$

Table 4. Protein levels measured by the method of Lowry et al. (9), as a function of the X-ray dose used for irradiation; results assayed at 675 nm; results are given as $\bar{X} \pm S$ ($n = 10$).

Organ	Protein levels, mg/g w.t.w.			
		B 10.A		B 10
liver	control	200.0 $\pm$ 20.2		205.3 $\pm$ 19.4
	250 R	226.1 $\pm$ 23.4		172.4 $\pm$ 16.8
	500 R	300.4 $\pm$ 85.1		200.0 $\pm$ 30.0
	1000 R	240.5 $\pm$ 30.5		190.0 $\pm$ 45.0
kidney	control	172.0 $\pm$ 16.4		140.5 $\pm$ 12.3
	250 R	190.4 $\pm$ 17.8		170.8 $\pm$ 21.2
	500 R	186.5 $\pm$ 24.3		130.5 $\pm$ 19.5
	1000 R	199.4 $\pm$ 24.2		185.4 $\pm$ 21.8
spleen	control	66.8 $\pm$ 6.6		68.4 $\pm$ 6.1
	250 R	75.8 $\pm$ 9.4		67.9 $\pm$ 8.3
	500 R	60.5 $\pm$ 4.8		72.7 $\pm$ 9.4
	1000 R	67.0 $\pm$ 6.3		70.5 $\pm$ 6.9
thymus	control	61.5 $\pm$ 5.8		47.6 $\pm$ 5.2
	250 R	64.8 $\pm$ 4.7		60.4 $\pm$ 5.9
	500 R	60.9 $\pm$ 9.4		61.3 $\pm$ 4.8
	1000 R	56.7 $\pm$ 8.4		53.4 $\pm$ 5.1
pancrease	control	256.9 $\pm$ 4.8		270.4 $\pm$ 7.1
	250 R	248.6 $\pm$ 2.3		291.3 $\pm$ 3.4
	500 R	261.7 $\pm$ 4.1		200.4 $\pm$ 7.5
	1000 R	250.8 $\pm$ 2.4		245.3 $\pm$ 2.3

As concerns the liver, kidney and brain (Na^+ , K^+)-ATP-ase activities (Table 6), it was found that the kidney and brain ATP-ase in the organ homogenates of both strains exhibit a uniform decrease as a function of the radiation dose. The same holds for the liver ATP-ase activity of the B 10 strain. At higher radiation doses, a decrease is similarly characteristic of the liver homogenate of the B 10. A strain, but the liver ATP-ase is slightly increased by the 250 R dose.

Table 5. LP and c. P-450 activity changes; $\bar{X} \pm S$ (n = 10).

Organ		LP, MDA nmol/g w.t.w.	
		B 10.A	B 10
liver	control	4.0 $\pm$ 0.35	3.15 $\pm$ 0.30
	250 R	4.4 $\pm$ 0.24	3.40 $\pm$ 0.35
	500 R	4.8 $\pm$ 0.30	4.00 $\pm$ 0.38
	1000 R	5.4 $\pm$ 0.41	5.30 $\pm$ 0.42
kidney	control	3.15 $\pm$ 0.25	2.10 $\pm$ 0.20
	250 R	3.20 $\pm$ 0.20	2.25 $\pm$ 0.32
	500 R	3.34 $\pm$ 0.24	3.43 $\pm$ 0.28
	1000 R	3.45 $\pm$ 0.30	5.28 $\pm$ 0.30

Organ		c. P-450, μ mol/mg protein	
		B 10.A	B 10
liver	control	0.5398 $\pm$ 0.0545	0.5233 $\pm$ 0.0504
	250 R	0.4376 $\pm$ 0.0389	0.5652 $\pm$ 0.0398
	500 R	0.6815 $\pm$ 0.0365	0.5037 $\pm$ 0.0485
	1000 R	0.3789 $\pm$ 0.0400	0.4358 $\pm$ 0.0375

Table 6. Liver, kidney and brain (Na^+ , K^+)ATP-ase activity changes as a function of the radiation dose.

Organ		(Na^+ , K^+)ATP-ase activities micromol P_i /mg protein/hour	
		B 10.A	B 10
liver	control	12.15 $\pm$ 2.83	10.83 $\pm$ 1.56
	250 R	12.67 $\pm$ 4.73	9.77 $\pm$ 1.45
	500 R	7.11 $\pm$ 0.53	7.85 $\pm$ 0.64
	1000 R	6.61 $\pm$ 1.59	7.19 $\pm$ 0.74
kidney	control	15.15 $\pm$ 1.65	12.08 $\pm$ 1.48
	250 R	13.54 $\pm$ 1.48	12.75 $\pm$ 1.54
	500 R	10.66 $\pm$ 0.99	10.35 $\pm$ 0.96
	1000 R	8.39 $\pm$ 0.98	11.45 $\pm$ 1.12
brain	control	22.21 $\pm$ 3.84	28.04 $\pm$ 1.96
	250 R	19.18 $\pm$ 2.96	22.56 $\pm$ 4.28
	500 R	13.54 $\pm$ 2.05	18.34 $\pm$ 2.56
	1000 R	11.95 $\pm$ 2.14	16.41 $\pm$ 2.35

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IN VITRO INVESTIGATION OF HYDROXYL-SCAVENGING EFFECT OF CAVINTON^R

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ABSTRACT

The hydroxyl radical-scavenging ability of vinpocetine was studied in vitro via aromatic hydroxylation and deoxyribose degradation methods. Hydroxyl radicals were generated by the Fenton reaction. The effect of vinpocetine was compared with those of other antioxidants: mannitol, vitamin E and dimethylsulfoxide. The antioxidant capacity of vinpocetine is close to that of vitamin E, and higher than that of mannitol.

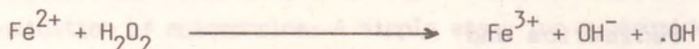
INTRODUCTION

Vinpocetine (Cavinton^R; Richter, Hungary), one of the vince alkaloids, is effective in the treatment and prophylaxis of acute and chronic hypoxic and ischemic central nervous system disorders (1, 2). During acute ischemia and reperfusion, its combination with mannitol has beneficial effects on the cerebral circulation and cell function (3, 4). The mechanism of action of vinpocetine is not fully understood. It decreases the brain vascular resistance, the viscosity of the blood and platelet aggregation, and it increases the red blood cell fluidity (5, 6).

There have been many studies to elucidate the role of free radicals in mediating tissue injury during hypoxia, and in particular during reperfusion. The oxygen free radicals leading to oxygen toxicity are the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$). Among them, the most reactive and most tissue-damaging one is $\cdot OH$, which forms in the course of interaction of O_2^- and H_2O_2 in the presence of a suitable metal ion, permanently existing in vivo (7). During the period of ischemia and reperfusion, important sources of reactive oxygen species can be the xanthine oxidase reaction, the tissue infiltration of polymorpha-

nuclear leukocytes and the autoxidation of catecholamines (8, 9). The tight control of the redox balance is critical for normal cell function, and vinpocetine may be a potential means of antioxidant therapy.

We have investigated the antioxidant capability of vinpocetine by means of the Fenton reaction in vitro:



The aromatic hydroxylation of salicylic acid and deoxyribose degradation were used for the assay of hydroxyl radicals (10, 11). In these systems we measured the scavenging of $\cdot\text{OH}$ by vinpocetine, comparing its effect with those of other well-known antioxidants: mannitol, vitamin E and dimethylsulfoxide (DMSO) (12-14). We found that the antioxidant capacity of vinpocetine is close to that of vitamin E and higher than that of mannitol.

MATERIALS AND METHODS

Reagents. Vinpocetine (Cavinton^R) was supplied by Richter, Hungary. Dimethylsulfoxide, deoxyribose and salicylic acid were purchased from Merck. Alfa-dl-tocopherol (vitamin E), thiobarbituric acid and Tween 20 were purchased from Sigma. Other reagents were from Reanal, Hungary. The reagents were dissolved in 12 mM KH_2PO_4 - KOH buffer, pH 7.4; special mention is made when some other solvent was used. The spectrofluorimetric measurements were performed with an MPF-4 Hitachi fluorescence spectrophotometer and the photometric methods with a Specord M40 photometer.

Assays. Aromatic hydroxylation: The salicylic acid and $\cdot\text{OH}$ reaction was assayed essentially as described in (11, 15). 3 ml of reaction mixture contained 2 mM salicylic acid, 1 mM H_2O_2 , 0.1 mM ferrous sulfate, 0.1 mM EDTA and free radical scavengers, the detailed concentrations of which are given in the Figures. The final concentration of KH_2PO_4 - KOH buffer pH 7.4 was 12 mM. After incubation at 37 °C for 90 min, the reaction was stopped by the addition of 0.220 ml 3 M HCl, followed by extraction with 5 ml cold ether, with mixing by vortex for 30 sec. A 4 ml aliquot of the clear organic solution was evaporated, the residue was dissolved in 0.3 ml distilled water, and 0.200 ml 10% (w/v) trichloroacetic acid (in 0.5 M HCl solution), 0.380 ml 10% (w/v) sodium tungstate (aqueous solution) and 0.380 ml 0.5% (w/v) sodium nitrite (in water) were added. After stirring, the mixture was left

to stand at room temperature for 5 min, and was then treated with 0.750 ml 0.5 M KOH solution. After 60 sec the absorbance was measured at 510 nm.

Deoxyribose degradation. The assay was carried out by a minor modification of the method in (11, 15). 3 ml of reaction mixture contained the following components, in final concentration: 2.8 mM 2-deoxy-D-ribose, 1.0 mM H_2O_2 and 0.1 mM ferrous sulfate. The KH_2PO_4 - KOH buffer final concentration was 12 mM, pH 7.4. The final concentrations of free radical scavengers are given in the Figures. The stirred reaction mixture was incubated at 37 °C for 30 min. After the addition of 0.5 ml 1% (w/v) thiobarbituric acid (in 0.05 M NaOH) and 0.5 ml 3% (w/v) trichloroacetic acid, the solution was heated at 100 °C for 10 minutes, then cooled to room temperature. The chromogen was extracted into 3 ml butan-1-ol, and its fluorescence at 553 nm was determined after excitation at 532 nm. In the case of vinpocetine, we measured the absorbance (532 nm) as well.

Mannitol degradation. 3 ml of reaction mixture contained the following materials, in final concentration: 1.7-16.5 mM mannitol, 1.0 mM H_2O_2 and 0.1 mM ferrous sulfate in 12.0 mM KH_2PO_4 - KOH buffer, pH 7.4. The procedure and the measurement were the same as the deoxyribose degradation assay.

The data are the means of the results of three separate experiments.

RESULTS

The aromatic hydroxylation of salicylic acid is a simple method for the assay of $\cdot\text{OH}$ formation in Fenton-type reactions. Mannitol could act against $\cdot\text{OH}$ in a dose-dependent manner (Fig. 1). Its concentration at 50% inhibition was 12.8 mM. With the same protocol, the reaction could be inhibited by vinpocetine (Fig. 2). Its concentration required for 50% inhibition was 0.2 mM, much lower than that of mannitol. The application of vinpocetine at higher concentration than 1.0 mM did not result in a more effective inhibition. The 50% inhibiting capacity for vitamin E could not be determined via this method, because the organic solvents applied as detergent failed to separate after the extraction procedure.

Vitamin E was dissolved in Tween-20, and a 5% (v/v) emulsion was then made in a 12 mM KH_2PO_4 - KOH buffer, pH 7.4. Deoxyribose degradation by $\cdot\text{OH}$ could be inhibited by vitamin E (Fig. 3). 50% inhibition of the reaction was observed at a final vitamin E concentration of 2.0 mM. We followed the in-

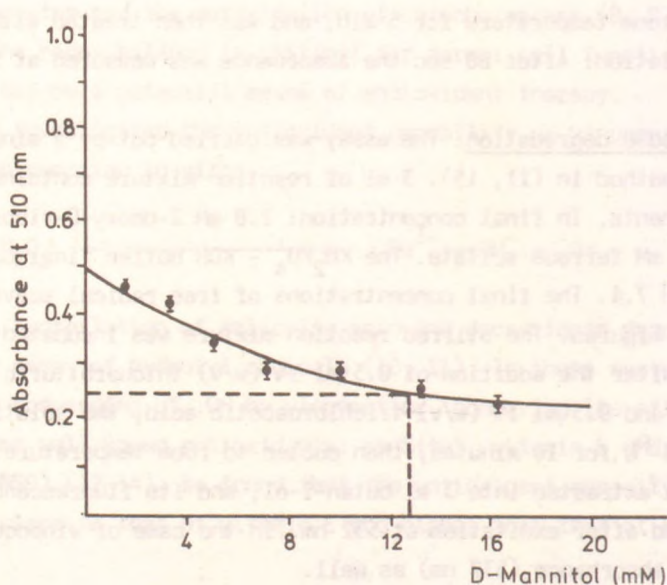


Fig. 1. Influence of mannitol on aromatic hydroxylation of salicylic acid by .OH

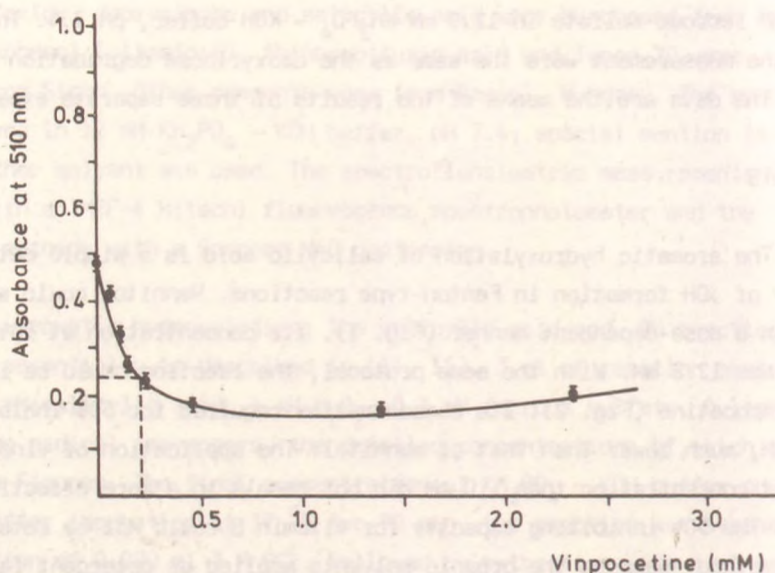


Fig. 2. Influence of vinpocetine on aromatic hydroxylation of salicylic acid by .OH

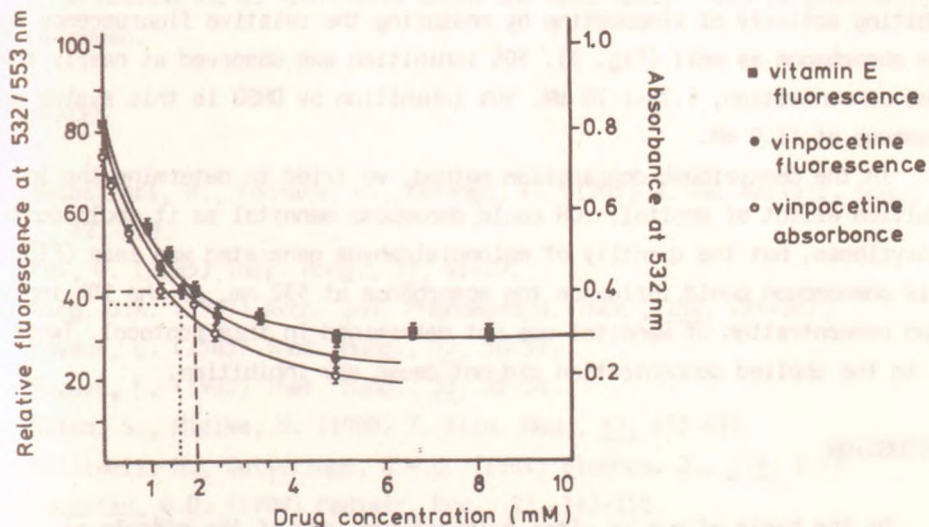


Fig. 3. Influence of vitamin E and vinpocetine on deoxyribose degradation by $\cdot\text{OH}$
 Vitamin E, fluorometric measurement
 Vinpocetine, fluorometric measurement
 Vinpocetine, photometric measurement

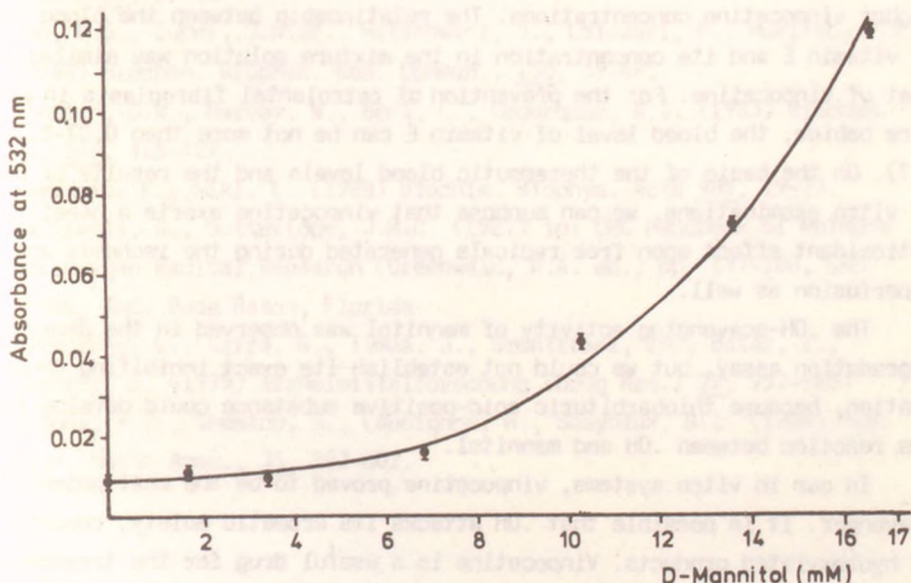


Fig. 4. Mannitol degradation by $\cdot\text{OH}$

hibiting activity of vinpocetine by measuring the relative fluorescence and the absorbance as well (Fig. 3). 50% inhibition was observed at nearly the same concentration, 1.75-1.70 mM. 50% inhibition by DMSO in this system was observed at 16.0 mM.

In the deoxyribose degradation method, we tried to determine the inhibition effect of mannitol. $\cdot\text{OH}$ could decompose mannitol as it could destroy deoxyribose, but the quantity of malondialdehyde generated was less (Fig. 4). This phenomenon could influence the absorbance at 532 nm, so the 50% inhibition concentration of mannitol was not determined in this protocol. Tween 20 in the applied concentration did not cause any inhibition.

DISCUSSION

On the basis of our in vitro examinations, one of the effects of vinpocetine may be its hydroxyl radical-scavenging ability. In our systems, its antioxidant capacity was more significant than that of other scavengers. A 0.06 mM initial plasma concentration of vinpocetine can be reached by infusion at a dosage of 1 mg/kg/25 min (16). In our in vitro systems, we used higher vinpocetine concentrations. The relationship between the blood level of vitamin E and its concentration in the mixture solution was similar to that of vinpocetine. For the prevention of retrolental fibroplasia in premature babies, the blood level of vitamin E can be not more than 0.07-0.1 mM (17). On the basis of the therapeutic blood levels and the results of our in vitro examinations, we can suppose that vinpocetine exerts a beneficial antioxidant effect upon free radicals generated during the ischemia and reperfusion as well.

The $\cdot\text{OH}$ -scavenging activity of mannitol was observed in the deoxyribose degradation assay, but we could not establish its exact inhibiting concentration, because thiobarbituric acid-positive substance could develop from the reaction between $\cdot\text{OH}$ and mannitol.

In our in vitro systems, vinpocetine proved to be the most potent $\cdot\text{OH}$ scavenger. It is possible that $\cdot\text{OH}$ attacks its aromatic moiety, resulting in hydroxylated products. Vinpocetine is a useful drug for the treatment of ischemic brain diseases, and since hydroxyl radicals are thought to be responsible for some of the tissue damage in ischemia, and especially in reperfusion, it is possible that the $\cdot\text{OH}$ -scavenging ability of vinpocetine may contribute to its beneficial effect.

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CERULOPLASMIN: AN ANTIOXIDANT FORGOTTEN?

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Ceruloplasmin (CP) is the blue-colored copper-containing α -2-globulin of mammalian plasma. Since its discovery in 1948, (10), CP has been extensively investigated to find its functions. Two basic characteristics of CP have been recognized since the protein was isolated: its high copper content and its powerful oxidase-like activity. More recently, CP has been shown to be an acute-phase protein and a strong antioxidant.

THE BIOCHEMICAL PROPERTIES OF CERULOPLASMIN

CP is an α -2-glycoprotein with a molecular mass of 135 kilodalton.

It contains 6 to 7 copper atoms per molecule. About 90 to 95 per cent of the serum copper content is bound to CP.

The amino acid sequence of the protein was determined in 1984 by Takahasi (19). It contains 1046 amino acids. Cloned cDNAs for human CP have been isolated independently by Koschinsky et al. (9) and by Yang et al. (20), and the latter group have used their clone to localize the CP gene to 3q25.

THE FUNCTIONS OF CERULOPLASMIN

CP as a copper-transporting protein

The close associations between copper and iron, direct comparisons are frequently made between the transport of iron by transferrin and the transport of copper by CP. However, CP does not act as a transport protein for copper in the same way that transferrin acts as a transport protein for iron. Copper is complexed to albumin, albumin-histidine and histidine-histidine following absorption from the gut. It is then transferred to the

liver. Once in the liver, copper is incorporated into apoCP to form the active molecule for release into the circulation. After its appearance in the circulation, CP may then serve as a copper-donor protein for the synthesis of cuproproteins in extrahepatic cells (8).

CP and iron metabolism

The release of ferrous ions liberated from ferritin into the plasma requires an oxidation step in order that iron may bind to the iron transport protein apo-transferrin (Fig. 1). A large number of studies prompted Frieden to propose a key role for CP in promoting iron binding by transferrin. This activity led to CP being designated as a ferroxidase (6).

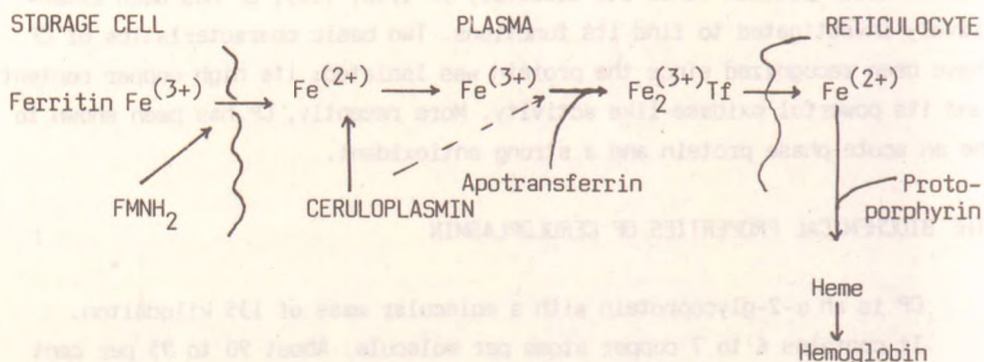


Fig. 1. The role of ceruloplasmin in iron metabolism

The antioxidant activity of ceruloplasmin

It has been known for many years that normal plasma has a powerful antioxidant activity. This function was traced to two clearly defined protein fractions. One could be identified as the iron-free portion of transferrin. The other was CP (17).

The mode of antioxidant action of CP was intensively investigated by Al-Timini and Dormandy (1).

It was proved that copper is essential for the antioxidant activity of CP.

It is well known that iron and its complexes have a free radical-generating capacity. The role of iron in the Fenton reaction is demonstrated in Fig. 2.

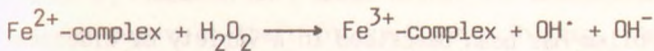


Fig. 2. Fenton reaction

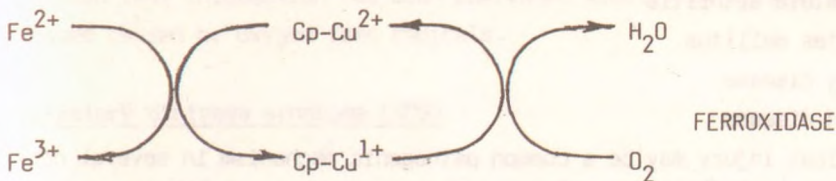


Fig. 3. Ceruloplasmin as a "ferroxidase"

Frieden et al. (6) showed that CP behaves as an oxidase not only to various organic compounds, but also to iron complexes: i.e. it is a "ferroxidase" (Fig. 3). This suggested a possible mode of action of CP as an antioxidant. If CP "immobilized" iron in the oxidized state, then the metal could not fulfil its essential catalytic role as a free radical generator. In addition to this, it has been demonstrated that CP has a superoxide dismutase (SOD)-like activity and can act as a free radical scavenger as well.

CP as an acute-phase plasma protein

A variety of pathological states have been shown to increase plasma CP levels. More than 25 years ago, it was concluded that CP was a non-specific acute-phase reactant. CP, as an acute-phase reactant, is generally thought to be induced so that its function in the host defense, most probably as an antioxidant and copper donor to tissues, can be enhanced. IL-1, which is highly elevated during inflammation and infection, is a powerful inducer of CP synthesis. It is suggested that IL-1 regulates CP gene expression at the transcriptional level, as it appears to for other acute-phase proteins (2).

CP IN VARIOUS PATHOLOGICAL CONDITIONS

An affected CP metabolism has been described in a variety of diseases:

- infection
- acute-phase reactant
- cancer
- liver diseases
- rheumatoid arthritis
- diabetes mellitus
- Menkes disease
- Wilson disease

Oxygen radical injury may be a common pathogenic mechanism in several neonatal diseases:

- retinopathy of prematurity
- bronchopulmonary dysplasia
- subependymal and intraventricular hemorrhage
- necrotizing enterocolitis
- RDS
- sepsis

The greater incidence of these disorders in preterm infants suggests that some aspect of prematurity increases susceptibility to the effects of toxic oxygen species. A possible explanation for the occurrence of these disorders in prematurity is that preterm infants may have defects in one or more of the antioxidant mechanisms. The components of this antioxidant defense system are:

- SOD
- catalase
- glutathione peroxidase (GSH-PX)
- vitamin E
- vitamin C
- uric acid
- ceruloplasmin
- apotransferrin

The intracellular antioxidants (SOD, GSH-PX, catalase) have been extensively studied in newborns (5, 12). In contrast, little attention has been paid to the possible importance of the extracellular components whose antioxidant activities depend on interaction with iron. Over 90% of the preventive antioxidant activity of plasma is associated with CP and transferrin. As preven-

tive antioxidants, these two proteins are 200 to 500 times more potent than vitamin E, compared on a molar basis. The antioxidant activities of both proteins depend on their interactions with iron, and they may therefore be called "iron-associated antioxidants" (16).

Only a few data are available on CP activity and concentration in the neonatal period. The CP activity and concentration, which are very low at birth, gradually increase, reaching the adult level by the age of 6 months.

Even less information has been published about this protein in neonatal diseases caused by oxygen free radicals.

Respiratory distress syndrome (RDS)

Neonatal RDS is a consequence of low pulmonary surfactant production. A surfactant deficiency interferes with the lung function by failing to stabilize small air spaces at end-expiration. Wasted ventilation and perfusion and some other still not clearly understood events lead to the severe clinical condition of RDS. Patients suffering from this disease generally require assisted ventilation. At higher oxygen concentrations, the natural antioxidant defenses are overwhelmed, resulting in free radical-induced tissue damage.

CP, the main plasma antioxidant enzyme, as a component of the lung epithelium lining fluid, may function as a lower respiratory tract antioxidant (15).

In 1981, Omene et al. (11) reported very low umbilical cord serum CP concentrations in preterm infants who subsequently developed RDS. Their results were confirmed by our findings (13) that serum CP activities in preterm infants without RDS were just as high as those of full-term, healthy neonates, while the patients with severe RDS had barely detectable enzyme levels (Table 1).

Antenatal corticosteroid treatment prevents RDS in preterm babies. Glucocorticoid is one of the prime agents known to stimulate surfactant synthesis in the fetal lung. Prenatal glucocorticoid administration has been shown to stimulate accelerated maturation of the intracellular antioxidant enzymes (5).

Our data demonstrated (19) that glucocorticoid given to the mother increased CP production in the fetus (see Table 1).

Table 1. Ceruloplasmin activity in premature neonates

	Full-term babies n = 28	Premature neonates						
		Steroid treatment group No.				RDS		All preterm n=38
		I n=14	II n=7	III n=17	none n=9	mild n=19	severe n=10	
Gestational age (weeks)	39.1	31.2	31.86	30.35	32.33	30.82	30.08	30.95
+ SD	1.6	2.12	2.34	2.57	2.35	1.9	2.81	2.4
Birth weight (grams)	3500	1621	1719	1472	1692	1571	1439	1572
+ SD	460	371	235	343	303	302	421	343
Ceruloplasmin activity (U/l)	12.4	12.43	6.43	2.0	13.22	6.53	1.92	6.66
+ SD	5.2	7.66	2.37	2.72	4.76	7.3	2.4	6.9
range	7-28	0-28	3-9	0-8	6-20	0-28	0-6	0-28

Intrauterine growth retardation (IUGR)

In normal conditions, maturation of the fetus correlates well with the gestational age, but in complicated pregnancy the maturational and gestational ages diverge, leading to the delivery of a small for gestational age (SGA) infant. There are several maternal and fetal conditions, most of which are chronic, associated with accelerated maturation of the fetal lung (7). SGA neonates, though low in birth weight, have no RDS and do well, in contrast with premature appropriate for gestational age (AGA) babies. Development of the fetal lung becomes highly accelerated after the 32nd week of gestation and it is dependent on the hormonal changes in late pregnancy.

We measured the serum CP activity in SGA infants and compared it with that in AGA neonates. We found a highly elevated CP activity in SGA babies (Table 2). Our data suggest that, as a consequence of the hormonal action during the chronic stress resulting in intrauterine growth retardation, the serum CP can rise and may prevent free radical-induced lung damage.

Table 2. Ceruloplasmin activity in newborns with intrauterine growth retardation

	Full-term neonates		Preterm neonates	
	AGA n=28	SGA n=14	AGA n=9	SGA n=12
Gestational age weeks $\pm$ SD	39.1 1.6	36.6 0.8	32.0 1.4	32.6 0.9
Birth weight grams $\pm$ SD	3445 475	2068 191	1716 335	1236 319
Ceruloplasmin U/l $\pm$ SD	13.5 4.8	72.4 8.6	13.2 4.8	61.0 10.4

$p < 0.001$ (AGA vs SGA Full-term)
 $p < 0.001$ (AGA vs SGA Preterm)
 $p < 0.001$ (AGA Full-term vs SGA Preterm)
 NS (AGA vs SGA Full-term)

Table 3. Ceruloplasmin activity in neonatal sepsis

Patients	Postnatal age (days)			
	1	3	8	15
1. SZ.A. ♂ Gest. age: 35 weeks Birth weight: 2200 g Dg. Congenital pneumonia	0	34	52	
2. SZ.L. ♂ Gest. age: 32 weeks Birth weight: 1400 g Dg. Bronchopneumonia	1	4	53	141
3. SZ.E. ♀ Gest. age: 31 weeks Birth weight: 1950 g Dg. Meningitis purulenta	12	67	81	
4. SZ.E. ♀ Gest. age: 35 weeks Birth weight: 2100 g Dg. Intrauterine infection	16	22	67	71

It is well-known that the CP activity rises during inflammation and infection. Four premature babies suffering from sepsis were investigated in our department. All of them had highly elevated CP activities (Table 3).

Only a few years ago, CP was described as "a protein in search of a function" (3). It now seems to have found some, and its significance should be considered not only in a few rare, inherited diseases, but during the postnatal adaptation period, as well.

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CAUSES OF CERULOPLASMIN DEFICIENCY IN SICK INFANTS

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INTRODUCTION

The serum copper (Cu) and ceruloplasmin (CP) concentrations are very low in the newborn, and even lower in premature neonates (3). It has been assumed that this is a reflection of a deficient CP protein synthesis during fetal life. In contrast with this deficient state of the newborn, pregnant women tend to have high Cu and CP concentrations, which reach three times the normal levels.

The de novo synthesis of CP takes place in the liver and depends on the availability of copper and on the effects of several factors, such as adrenal hormones, interleukins, etc. (1). It is very intriguing that, in spite of the abundance of many of the promoting factors, the CP synthesis rate seems to be low in the fetus. On the other hand, there is a possibility that the low CP levels in the newborn serum are due not to a deficient synthesis, but to defective Cu incorporation into the protein, resulting in an excess apoCP concentration (7).

Another plausible explanation for the low serum CP levels is the in vivo or in vitro degradation of the molecule. The protein is extremely susceptible to proteolytic attack (8), it can be destroyed by free radicals (9) or, after desialylation, it may be cleared up by the liver (2).

The conflicting results in the literature are based on different methodologies. The aim of the present study was to assess the involvement of the above-mentioned mechanisms in the very low enzymatic activity of CP observed in some sick premature babies.

Patient 4 was a boy from a 33-week gestation, with a birth weight of 1750 g. He had an uncomplicated early adaptation period with prolonged icterus. His starting CP_e was 22 U/l, which gradually decreased and reached zero by the second week. The Cu concentration in this sample was 8.22 $\mu\text{mol/l}$, which was considerably high for his age.

Table 1. Serum copper and ceruloplasmin concentrations in premature neonates in the early postnatal period

	Cord blood	Postnatal age (days)				
		1	3	8	15	30
Cu $\mu\text{mol/l}$	4.77	5.44	7.10	6.95	6.85	6.70
CP _e U/l	16.7	20.2	23.3	21.7	24.7	22.2
CP _c $\mu\text{mol/l}$	0.56	0.69	0.79	0.74	0.84	0.75
Cu/CP	8.48	7.93	8.96	9.42	8.15	8.89

Table 2. Serum copper and ceruloplasmin in neonates with different pathological conditions

	Cu $\mu\text{mol/l}$	CP _e U/l	CP _c $\mu\text{mol/l}$	CP _i $\mu\text{mol/l}$	CIE pattern
1. P.B. Age: <24 h Gest.age: 28 wks Birth w.: 850 g	1.2	0.0	0.0	0.0	
2. S.I. Age: <24 h Gest.age: 32 wks Birth w.: 1200 g	2.3	1.6	0.054	0.056	
3. B.A. Age: 3 days Gest.age: 32 wks Birth w.: 2160 g	5.2	2.0	0.068	0.650	
4. K.M. Age: 8 days Gest.age: 33 wks Birth w.: 1750 g	8.2	0.6	0.02	1.4	
5. Normal adult (pooled serum 50 times dil. for CIE)	16.6	135.7	3.5	ND	

MATERIALS AND METHODS

Blood samples from premature and full-term sick neonates were drawn on the day of delivery, and on the 3rd, 8th, 15th and 30th day thereafter. Informed consent was obtained from the parents.

The CP concentration (CP_i) was measured by radial immunodiffusion, using commercially available immunodiffusion plates (Heinzel GmbH, Austria).

The CP oxidase activity towards o-dianisidine as substrate (CP_e) was measured by a manual kinetic method, as described in (6).

The active CP concentration (CP_c) was calculated from the CP_e data by using a calibration curve produced with purified human CP.

Crossed immunoelectrophoresis (CIE) was performed on agarose gel according to Laurell (4), using rabbit anti-human CP serum (Behringwerke AG, West Germany).

Cu concentrations were determined by atomic absorption flame spectrophotometry.

RESULTS

Data on 37 premature neonates with an uneventful postnatal adaptation period are shown in Table 1. Their mean gestational age was 33.2 weeks and the mean birth weight was 1893 g.

Cu was within the expected normal range ($4.1-5.0 \mu\text{mol/l}$ in the newborn, increasing to $9-15 \mu\text{mol/l}$ in early infancy (see ref. 5). The CP_e was about 15-25 per cent of that in adults. The CP_c was $0.56-0.84 \mu\text{mol/l}$ ($2-4 \mu\text{mol/l}$ in adults). The average Cu to CP molar ratio was 8.35, suggesting that most of the Cu content of these examples was bound to CP. In ill neonates, the data differed from those found in "well" babies. The main clinical and laboratory results on 4 representative cases are shown in Table 2. Some prematures had an extremely low CP_e on the first day of life. Most of them later developed severe respiratory distress syndrome (RDS). In these cases (patients 1 and 2), the CP_i and Cu were hardly detectable. In other infants, e.g. patient 3, the low CP_e decreased further from the day of birth. This boy suffered from severe RDS and required assisted ventilation. By the third day his CP_e had fallen from 6 to 2 U/l, while the Cu concentration and CP_i increased.

DISCUSSION

Low Cu and CP levels were detected in the sera of premature neonates. Preterms with an uncomplicated postnatal adaptation showed parallel Cu and CP deficiencies, suggesting a low synthesis rate of the fetal liver for CP, but the protein was not different qualitatively from that found in the adult serum as judged by the Cu/CP ratio, or from the CIE patterns in cases 1 and 2. Young and Curson (10) reached a similar conclusion from investigations of the catalytic properties of neonatal CP.

In some diseased babies, the in vivo degradation of the CP molecule may have occurred. Oxygen toxicity is the most probable explanation for the postnatal decrease in the active CP levels in severe RDS.

The serum Cu is generally elevated in biliary obstruction. Desialylated CP is taken up and catabolized by the liver (2). Cu may then be utilized for the synthesis of new cuproprotein molecule or excreted in the bile. An overwhelming Cu supply due to biliary obstruction may lead to a reduced or defective CP synthesis and to the appearance of inactive, partially degraded protein in the circulation.

We assume that a CP deficiency in neonates is a consequence of a deficient synthesis by the fetal liver, and in certain diseases it is caused by in vivo degradation of the CP.

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EFFECT OF ANTIOXIDANT TREATMENT DURING HEALING OF EXPERIMENTAL MYOCARDIAL INFARCTION

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SUMMARY

The left descending coronary artery was ligated in mongrel dogs for 24 hours or for 1, 2, 3 or 4 weeks. 20 animals served as controls and 20 animals were continuously treated with the synthetic antioxidant MTDQ-DA. Lipid peroxidation and endogenous scavengers were determined in the heart tissue homogenates (ischemic and non-ischemic areas) after different ischemic periods. The results show that antioxidant treatment can diminish the harmful effects of free radical-mediated injuries during the healing process after myocardial infarction.

INTRODUCTION

Free radical-mediated reactions and their inhibition with antioxidants are well known in the acute phase of myocardial infarction (3, 9, 13, 14). However, few experimental data are available on the biochemical changes that occur during the healing of experimental regional ischemia (1, 6, 11). The importance of neutrophils in the pathophysiology and in the treatment of acute myocardial infarction has recently been revealed (4, 8). During the resolution of necrotic heart tissue, leukocytes, monocytes, macrophages and fibroblasts infiltrate the ischemic area, and the metabolically activated cellular elements can produce a large quantity of free radicals, thereby worsening the cell membrane destruction.

The aims of the present investigations were to determine (1) the lipid peroxidation activity and the endogenous scavenger state at various times after coronary ligation, and (2) the effect of antioxidant therapy during the healing of myocardial ischemia.

MATERIALS AND METHODS

40 mongrel dogs weighing 15-20 kg were premedicated with Fentanyl-Droperidol-Atropine, anesthetized with sodium barbital (20 mg/kg) and artificially ventilated with oxygen + nitrous oxide in a ratio of 1:3. Through left thoracotomy, the pericardium was opened and the left descending coronary artery (LAD) was ligated. After ECG recording (Biomedica 6-channel polygraph), the chest wall was closed.

The animals were divided into two groups. The first group contained the control animals, which did not receive antioxidant treatment. In the second group, the animals were treated with the water-soluble antioxidant MTDQ-DA (6,6'-methylene-bis-2,2-dimethyl-4-methanesulfonic acid sodium-1,2-dihydroquinoline) in a dose of 150 mg/kg body weight. The antioxidant was given in two ways: during LAD ligation, it was administered intravenously in infusion solution, and after operation oral administration was preferred daily. Dogs were sacrificed after 24 hours or 1, 2, 3 or 4 weeks. The heart was removed and tissue samples were taken from both the infarcted area and the non-ischemic part of the left ventricular wall.

Biochemical assays

The heart specimens taken from the ischemic and non-ischemic areas were washed free of blood and homogenized with an Ultra-Turrax. The lipid peroxidation was assayed by the thiobarbituric acid test, the malondialdehyde (MDA) concentration being determined spectrophotometrically at 532 nm (9). For the measurement of reduced glutathione (GSH), DTNB reagent was used with the Tietze method (12) at 412 nm. MDA and GSH levels were expressed in nmol/g wet tissue. The superoxide dismutase (SOD) activity of the supernatant was measured at 480 nm according to Misra and Fridovich (7). The activity of this enzyme was expressed as U/g initial wet tissue.

Statistical analysis

MDA and GSH levels were expressed as percentages of the normal heart tissue values (MDA 20 ± 8.5 nmol/g wet tissue, GSH 6 ± 1.8 nmol/g wet tissue) while SOD was compared with the normal level of 123 ± 8.5 U/g wet tissue. All parameters are presented as means $\pm$ SD. With Student's t-test, the differences were considered significant if $p < 0.05$.

RESULTS

In every case, the acute coronary ligation was followed by serious ischemic ECG signs. In the control group, 5 animals died because of ventricular fibrillation in the first few hours after LAD ligation. All animals treated with MTDQ-DA survived the coronary ligation.

Biochemical changes

In the control group the MDA level was decreased below the normal value only after the 24-hour ischemia. After a longer ischemic state, the lipid peroxidation activity was continuously and significantly elevated. Its peak value was $152 \pm 5.6\%$ after 4 weeks (Fig. 1). The GSH content was seriously decreased: the lowest value was $35 \pm 6.2\%$ after LAD ligation for 1 and 2 weeks. Surprisingly, slight alterations appeared in the circumflexus branch supplying the "healthy" ventricular muscle. A slight MDA elevation was observed with a peak value at 4 weeks ($125 \pm 5.4\%$); moreover, the GSH was decreased in the non-ischemic area (Fig. 1) mainly at 3 weeks ($75 \pm 8.2\%$).

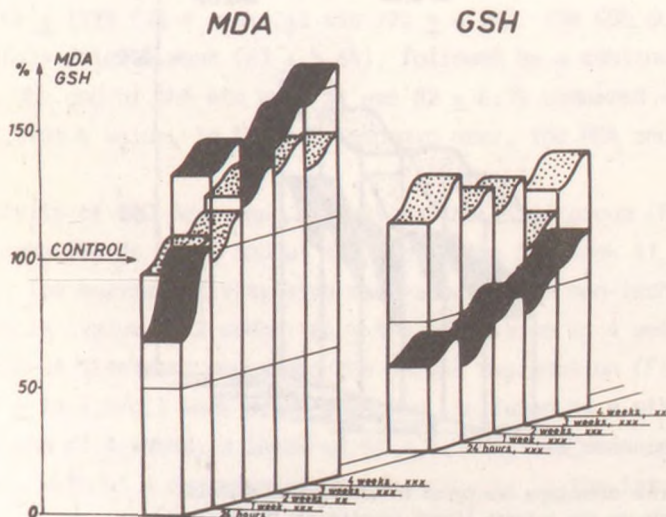


Fig. 1. In the control animals, MDA and GSH changed significantly in ischemic continuous (black slope) and in non-ischemic (dotted slope) heart tissue, depending on the duration of coronary ligation.

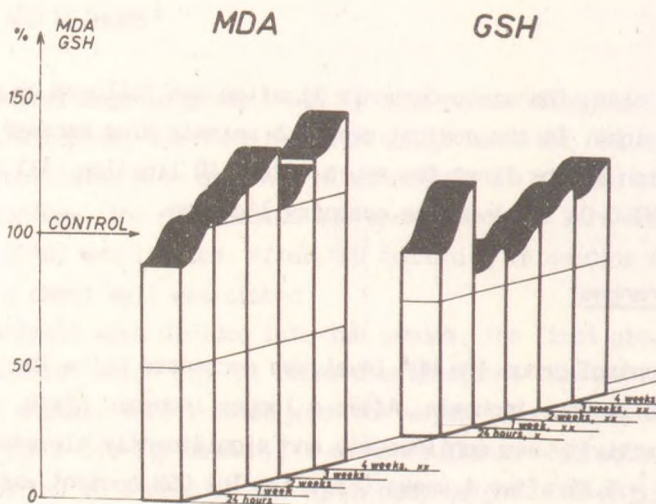


Fig. 2. After antioxidant (MTDQ-DA) treatment, only the ischemic heart tissue showed a slight depletion of GSH content and a moderate MDA increase

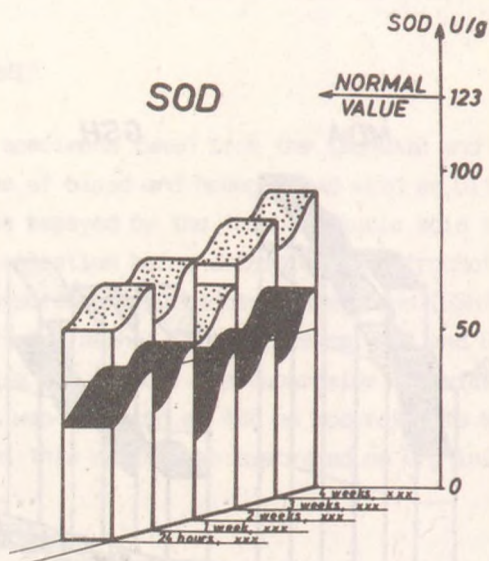


Fig. 3. Superoxide dismutase decreased in non-treated animals, seriously in the infarct tissue (continuous black slope) and moderately in the area of the circumflexus branch (dotted slope)

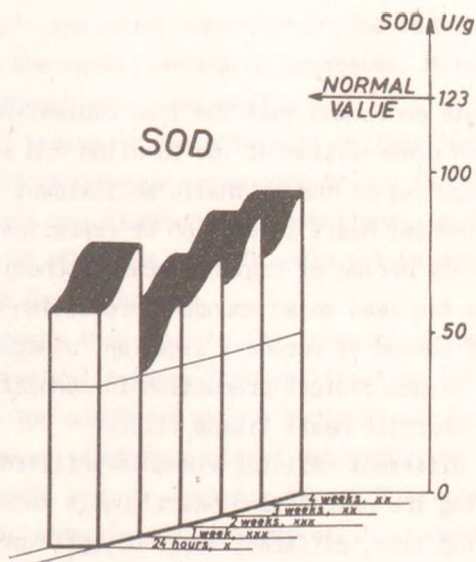


Fig. 4. Antioxidant treatment can diminish the degradation of superoxide dismutase in the infarct area without changes in the non-ischemic heart muscle

In the group receiving antioxidant treatment (Fig. 2), the MDA elevation in the ischemic area was no so high as in the control group, with changes of $106 \pm 122\%$ (at 4 weeks it was $122 \pm 6.3\%$). The GSH content was decreased mainly after 1 week ($63 \pm 5.6\%$), followed by a continuous elevation, and at the end of the 4th week it was $82 \pm 6.7\%$ compared with the normal heart tissue value. In the non-ischemic area, the MDA and GSH were normal.

The activity of SOD decreased sharply in the first group (Fig. 3) in the infarct area ($28-36$ U/g), and at the end of the 2nd week it was almost undetectable. The enzyme activity also decreased in the non-ischemic part of the heart muscle (value at 2 weeks: 63 ± 8.5 U/g, value at 4 weeks: 83 ± 10.5 U/g). MTDQ-DA treatment decreased the enzyme degradation (Fig. 4). The level was 69 ± 10.2 U/g 1 week after ligation, followed by a slight elevation. At the end of 4 weeks, a level of 84 ± 8.5 U/g was measured in the ischemic area, without a decrease in enzyme activity in the intact part.

DISCUSSION

Earlier experiments confirmed that the free radical-mediated injuries can be diminished, with preservation of the function and structure of the membrane system, through use of the synthetic antioxidant MTDQ-DA during the acute period of experimental heart infarction or reperfusion (9, 10, 13, 14, 15). During the acute period of regional heart ischemia, different pathological processes can lead to an abundant production of free radicals (2, 9). After a longer period of coronary ligation, other factors seem to be present, causing more oxygen radical production in connection with the process of healing of necrotic heart tissue (11).

Morphologically, different cellular elements infiltrate the ischemic myocardium, transforming the necrotizing heart muscle into scar tissue. Depending on the healing time, different types of cells predominate in the infarct area (1, 5). Between the first day and one week, neutrophilic leukocytes accumulate in the ischemic area and the border zone. Stimulated neutrophils produce cytotoxic free radicals (superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen). The oxygen radicals attack the membrane phospholipid structures, causing an increase in the arachidonic acid metabolism. They also promote the production of leukotrienes, prostaglandins and thromboxane at the time when the endogenous scavengers are seriously deteriorated. According to McCluskey (6), even after 3 months of coronary ligation the arachidonic acid metabolism is enhanced, together with an abundant production of free radicals. The activated neutrophils release lysosomal enzymes, leading to proteolytic disruption of the injured tissue (4).

During the second and third weeks, monocytes, fibroblasts and macrophages predominate in the ischemic area. The monocytes are capable of producing different leukotrienes, which can cause coronary vasoconstriction and enhance vascular permeability. One month after coronary ligation, the ischemic area contains fibroblasts, new capillaries, macrophages and connective tissue.

The present investigations confirm that, during the first 4 weeks after LAD ligation, a significantly elevated lipid peroxidation is present, and at the same time the endogenous scavengers are seriously deteriorated. Why these alterations also appear (but only slightly) in the non-ischemic area in the control animals is unknown. It is possible that different mediators which are produced in the ischemic area can reach the intact tissue via the

collateral circulation and cause disorders in the capillary circulation, thereby influencing the normal metabolic processes. Hemodynamic measurements to settle this hypothesis are under way.

The antioxidant treatment can eliminate neither the elevation of MDA nor the exhaustion of endogenous scavengers in the ischemic myocardium. However, it can attenuate the biochemical alterations. In the treated group, pathological levels of MDA, GSH and SOD could not be measured in the non-ischemic area of the heart tissue.

Our results suggest that, during the "repair" process after heart infarction, the free radical release from infiltrating cells must be taken into consideration. The dangerous oxygen radicals may be present mainly in the extracellular space, causing a direct deterioration of the cell membrane bilayer in the ischemic and non-ischemic areas. This process seems to be more severe than can be explained in terms of the coronary capillary flow disorders.

Exogenous antioxidant can trap the oxygen radicals arising from the different cells and/or produced by the ischemia-enhanced lipid peroxidation of polyunsaturated fatty acids. On the other hand, the beneficial effect of the exogenous scavenger material may be that it can protect the endogenous defense mechanism, and thereby preserve the natural scavenging. In this way, administration of the antioxidant MTDQ-DA is able to improve the imbalance between oxygen radical production and scavenger state during the period of healing of heart infarction.

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FREE RADICAL-INITIATED FUNCTIONAL DISTURBANCES OF PERIPHERAL NERVOUS TISSUE. A SINGLE CASE STUDY

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SUMMARY

The present study reveals the peripheral chronic neuropathy of a 47-year-old male patient, treated in our clinic for more than 7 years. From a clinical point of view, free radicals stimulated by organophosphorus ester intoxication can initiate the development of long-lasting functional and morphological disturbances in the peripheral nervous system. The history of the disorder, the development of illness, the diagnostic procedures and the therapy are described.

The absence of NAD and an energy-poor status can cause many different diseases, e.g. pellagra, alcoholism, diabetes mellitus, hypertension, and cardiac insufficiency, and can also produce porphyria (5). Organic compounds with a lipophilic character can cause porphyria, too. Thus, they alter the normal structure and function of the lipid membranes, and they influence the energy production in a negative way via a decreased NAD output and ATP synthesis.

In this study, we report the case of a 47-year-old male patient, treated in our clinic for more than seven years. On the basis of the clinically evident neurological symptoms, he was initially diagnosed as having a motoneuron disease, amyotrophic lateral sclerosis (ALS) with a progressive character. However, prolonged observation of the patient necessitated a revision of the original diagnosis. The neurological symptoms, the pathological signs and the patient's complaints were unchanged. Finally, a new investigation was indicated. A careful and exact anamnesis on the basis of detailed data in the history of his illness revealed the very important fact that up to his illness he had been working for many years in agriculture, in contact with many different organophosphorus esters with their well-known cholinesterase inhibitory activity (13) (Table 1).

Table 1

Afugan (2-(0,0-diethylthiophosphoryl)-5-methyl-6-carbethoxypyrazolo-[1,5a]-pyrimidine).
BI 58 EC (0,0-dimethyl-S-(N-methylcarbamoylmethyl)-dithiophosphate).
Dimecron 50 EC (0,0-dimethyl-0-(2-chlorodiethyl-carbamoyl-1-methylvinyl)-phosphate).
Ditrifon 50 WP (0,0-dimethyl-1-hydroxy-2,2,2-trichloroethylphosphonate).
Ekalux 25 EC (0,0-dimethyl-0-(quinoxaliny1-/2/-thiophosphate).
Flibol E (0,0-dimethyl-1-hydroxy-2,2,2-trichloroethylphosphonate).
Nogos 50 EC (0,0-dimethyl-2,2-dichlorovinylphosphate).
Phosdrin (0,0-dimethyl-0-(1-methyl-2-carbomethoxy-vinylphosphate).
Triklorfon (0,0-dimethyl-1-hydroxy-2,2,2-trichloroethylphosphate).
Ultracid 40 WP (0,0-dimethyl-S-(2-methoxy-1,3,4-thiadiazol-5-/4H/-onyl-)/4/(-methyl)dithiophosphate).
Unifos 50 EC (0,0-dimethyl-2,2-dichlorovinylphosphate).
Wofatox EC 50 and Wofatox WP (0,0-dimethyl-0-) p-nitrophenylthiophosphate .

The pathological symptoms of the patient are shown in Table 2, together with the diagnostic methods used. The vegetative functions, the sense perception and the function of the cranial nerves were unchanged. However, there was a small but significant spasticity of the skeletal muscles of the whole body, atrophy of the skeletal muscles, mainly of the right leg, with fasciculation, decreased muscle power and hemiparesis. A possible central component was excluded by using cerebral scintigraphy, investigation of CSF circulation, routine CSF diagnostics, ophthalmological tests, electroencephalography, carotid angiography, myelography of the cervical and lumbar segments of the spinal cord, and analysis of somato-sensoric evoked potentials (SEP).

The EEG and ENG measurements revealed that the signs related to a peripheral polyneuropathy. As concerns this differential diagnosis, a new investigation was performed on abnormalities in the porphyrin-haeme metabolism (2, 3, 12, 16, 17) with regard to disturbances of antioxidant enzyme activities (1, 9, 11, 14, 15) (Fig. 1).

We observed a decreased antioxidant enzyme activity and a total lack of superoxide dismutase (SOD) (Fig. 2). Pathological changes were also found in biopsy specimens of the deltoid muscle, the musculus quadriceps femoris and the sural nerve (Fig. 3). Most of the muscle fibres had a regular structure, but in both muscles, there was an evident atrophy of

Data to Porphyrin-HaemeBiosynthesis					
	I.	1987 IV.	IX.	1988 II.	Normal range
BLOOD					
Proto	Normal	Normal	Normal	Normal	5-36
$\mu\text{g}/100\text{ ml RBC.}$					
ALAD	33'58	21'14	31'03	38'31	22-44
$\times 10^{-3} \mu\text{MALA}/\text{ml RBC}/\text{min.}$					
URINE					
ALA	128'43	141'24	89'16	111'8	0-40
$\mu\text{mol}/\text{day}$					
PBG	73'92	89'20	73'92	75'54	2-16
$\mu\text{mol}/\text{day}$					
Quail. porph.	Negative	Negative	Negative	Negative	Negative
FAECES					
Proto.	227'52	Normal	Normal	Normal	12-85
$\mu\text{g}/1\text{g dry.}$					
Copro.	55'49	Normal	Normal	Normal	3-24
$\mu\text{g}/1\text{g dry.}$					
Htc %	52	53	51	55	40-54
Se Fe = gamma %	122'0	81'0	138'0	91'0	65-175
Cu = "	134'0	113'0	110'0	115'0	100-150
Zn = "	119'0	106'5	104'2	82'2	68-136

Fig. 1.

Activity of antioxidant enzymes in red blood cells during the therapy						
	I.	1987 IV.	IX.	1988 II.	IX.	Normal range
Protein	26'12	26'12	28'47	25'78	26'63	2825 $\pm$ 21
g/100 ml.						
LP	88'50	95'67	38'47	100'84	106'73	67'4 $\pm$ 159
nmol MDA/ml						
GP	0'880	1'258	1'609	2'593	2'953	2'572 $\pm$ 0'259
U/ml.						
SOD	-!	608'175	435'58	527'154	391'038	716'5 $\pm$ 92'5
U/ml.						
CAT	0'971	1'269	1'63	0'987	1'438	2'05 $\pm$ 0'68
Bergman						
U/ml.						

Fig. 2.

small bundles, also showing destruction of the normal histology, with small angular fibres (Fig. 3a). The nuclei of the atrophic fibres were increased, but only a few hypertrophic nuclei were found. These nuclei were larger, hyperchromatic and pycnic, too (Fig. 3b). In some cases, splitting phenomena and decreases in the number and diameter of the irregularly arranged myofibrils were evident. On the other hand, we did not find necrosis of the muscle fibres, fibrosis or any other cellular reactions (Fig. 3c). The

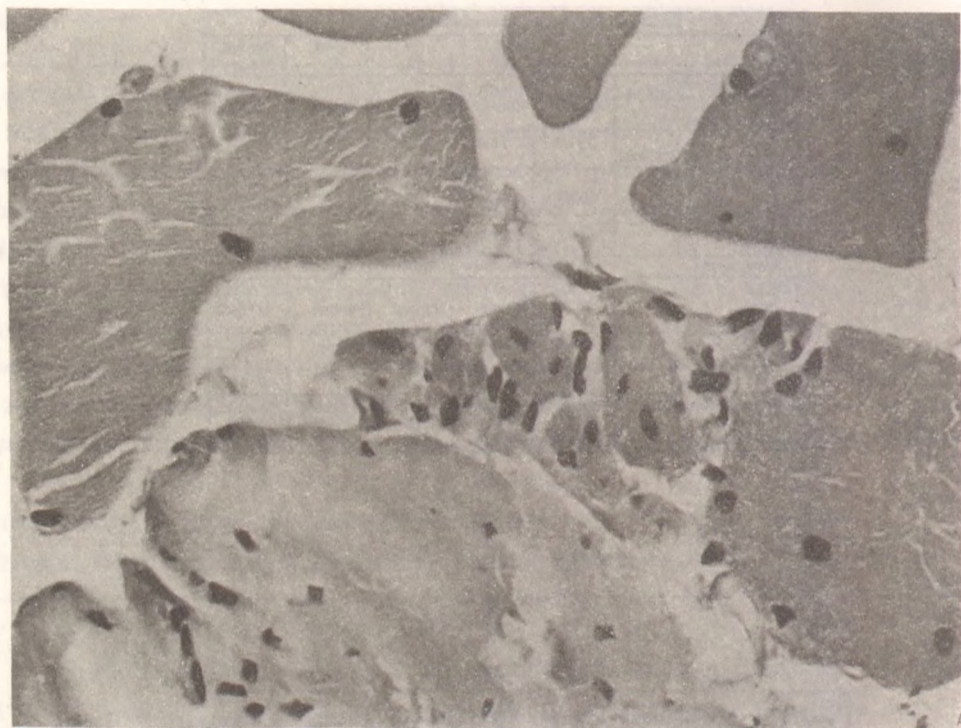




Fig. 3. Histological investigation of biopsy specimens of deltoid muscle and musculus quadriceps femoris.

- a. Typical neuropathy with small angulated fibres and myopathic lesions. HE staining, 224 x.
- b. and c. Electronmicrographs showing splitting and a decreased number of irregularly arranged myofibrils with a small diameter and with pycnic nuclei.
 b: 6000 x; the inset 1200 x.
 c: 6000 x.

enzymehistochemical investigations (succinyl-dehydrogenase, diaphorase and ATP-ase activities) demonstrated a predominance of type I fibres (73-75%) in both muscles, and approximately 60% of the atrophic fibres were of type II. No changes in the cellular ultrastructure of the muscles were found on electronmicroscopic investigation. Only in a few cases was a small degree of splitting observed in the myofibrils. In parallel with the degree of atrophy, the number of myofibrils decreased, the diameter reduced and the structure became more disorganized. Few of the myofibrils were flocculated because the z-bands were curved and they showed z-band streaming. The electronmicrographs revealed a few thin, irregularly positioned myofibrils and hyperchromatic nuclei.

The morphological findings support the diagnosis of peripheral chronic neuropathy. In the sural nerve, however, no morphological changes were detected. No were any abnormalities found in the contents of transferrin,

Table 2

Investigation	1981 Jan./June/Oct.	1983 Febr.	1984 Jan./June
Neurological symptoms	horizonto-rotatory nystagmus, decreased power of right arm, increased spasticity	areflexion of biceps, right side latent hemiparesis, fasciculation of right limbs	hypotonus of right limbs, sensory loss, fasciculation atrophy, bitemporal hemianopia
EMG	heavy denervation	denervation	peripheral nerve lesion
ENG	of right side		
SEP			
Radiology of the skull, neck, cervical and lumbar spine	cervical spondylosis		
CSP	normal	P: +, Sell.: + Cell number: 1 M/1 Ly, Prot.: 0.50 g/l	
EEG			
Ophthalmology			bitemporal hemianopia
Cerebral scintigraphy			
Carotid angiography			
Cerv. + lumb. myelography			
Internal status		normal	
Dental status			

Table 2 (cont.'d)

1985 Marc./Apr.	1986 Marc./July/Nov.	1987 Jan./Apr./Sept.	1988 Febr.
status unchanged	normal cranial nerve function, increased atrophy	status unchanged	status unchanged
status unchanged	suspected central lesion	status unchanged	status unchanged
lumbar + cervical spondylosis			
	status unchanged	status unchanged	status unchanged
	normal	normal	normal
	normal	normal	normal
	normal		normal
	normal		
		normal	
	normal		

coeruloplasmin or selenium ions in the serum and the hair, or in the microsomal enzyme activity of the blood serum.

DISCUSSION

The case history of the patient pointed to numerous signs of secondary porphyria, mostly as a consequence of intoxication with chlorinated hydrocarbons (16). In the presence of iron (II) ions, these agents can stimulate the production of peroxides and free radicals in the liver (6). This possibly causes an imbalance between produced and inactivated reactive oxygen intermediates, which leads, of course, to a massive oxidant stress of the organism, acting on different target sites, mostly located in the liver. In this organ, oxidant stress exhausts antioxidant activities and thereby induces a "chemical hypersensitivity" disorder (8). The peripheral nervous system is also extremely sensitive to such effects and responds with symptoms of neuropathy accompanied by a secondary neurogenic atrophy (4, 7).

Therapeutic treatment of the patient included the administration of Thioctazid^R, Adenyl-50, nicotinic acidamide, and different vitamins (A and B). After this treatment, the activities of antioxidant enzymes increased again and the content of serum SOD again became measurable. The morphological abnormalities were unchanged and the clinical picture improved only slowly, but the patient's complaints decreased significantly.

This report has the aim of drawing attention to the occurrence of rather uncommon diseases. However, we do not want to draw general conclusions on the basis of a single case investigation. Nevertheless, it seems very important to us that possible changes in the porphyrin-haeme synthesis and the antioxidant enzyme activities should be examined when patients suffer from atypical motoneuronal disorders of a neuropathy of unknown origin. Such investigations can establish the diagnosis, for many pharmaceuticals (10), chemical fertilizers, pesticides and organic dyes have porphyrinogenic activity and can cause oxidative stress initiated by free radicals in the organism (4). In this context, the effects of free radicals in the tissues of the peripheral nervous system are of particular importance. These tissues are highly vulnerable to these influences and react immediately with irreversible injuries after the exhaustion of the antioxidant enzyme activities.

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EFFECTS OF SH-CONTAINING RADIOPROTECTORS RELATED TO CYSTEAMINE ON SEVERAL EXPERIMENTAL ULCER-MODELS IN RATS

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INTRODUCTION

Free radicals play very important roles in the development of ulcers (1) and radiation injury (2).

Ionizing radiation damages the digestive tract in part by generating active oxygen radicals. Free radical scavenger activity provides protection against the injury of tissues by irradiation and ulcerogen processes.

The SH-containing compounds, such as derivatives of cysteamine, have got scavenger activity against various free radicals (3) and many of them have been defined as radioprotectors (4). On the other hand, it has been well-known since 1973 (5) that cysteamine induces ulcer in rats.

We set out to establish whether certain derivatives of cysteamine have ulcerogenic effects, and to examine the effects of these drugs on several experimental ulcers.

MATERIAL AND METHODS

The following drugs were used

Cysteamine HCl (MEA, mercaptoethylamine)

S,2-aminoethylisothiuroniumbromide HBr (AET)

S-2(3-aminopropylamino)ethylphosphorothioate (WR 2721)

2-(aminopropyl)amino)ethanethiol (WR 1065)

The experimental ulcer-models were as follows:

1. Shay-rat (pylorus-ligated) (5)

2. Reserpine-induced ulcer (6)

Male OFA1 (Lati, Gödöllő) albino rats weighing 180-200 gram were used.

Table 4. Parameters after drug treatment + reserpine treatment

Treatment	Ulcer index rumen/gland	Ulcer incidence in stomach	Severity incidence in duodenum
Rausedyl t mg/kg	0/60	100	- 0
+ MEA 300	the rats died with large ulcers		
+ AET 140	0/33	100	-
+ WR 2721			
100	0/30	95	extended tissue necrosis
200	0/40	100	extended tissue necrosis
300	0/30	95	extended tissue necrosis and 35% perish
+ WR 1065			
62.6	0/20	80	4 10
125	0/20	60	5 15
187.8	0/10	60	4 10

Table 5. Trends of effects of treatment

Treatment	Effects		
		Duodenum	
MEA		+	
AET		-	
WR 2721		+	
WR 1065		+	
	Secretion	Rumen/gland	Duodenum
Pylorus ligation			
+ MEA	↑	↑	+
+ AET	↓ ↓	↓↓ -	-
+ WR 2721	↑	↓ -	+
			+
+ WR 1065	↑	↑ ↓	+
			+
			+
Reserpine		↑	
+ MEA	-	↓	+
+ AET	↑		+
+ WR 2721	↑	↑	+
+ WR 1065	-	↑	+

To summarize (see Table 5), the reference drug MEA induced ulceration in the duodenum in pylorus-ligated rats, induced ulcerations in the pars glandula and increased the ulcerogenic effects of reserpine.

AET inhibited the secretion in pylorus-ligated rats, prevented the development of Shay ulcer, but enhanced the ulcerogenic effect of reserpine.

Both WR 2721 and WR 1065 increased the gastric secretion, and induced ulceration in the pars glandula and the duodenum. Both increased the ulcerogenic effect of reserpine.

DISCUSSION

It is well known that different SH compounds play a very important role in free radical scavenging (7). It is also well known that pretreatment with scavenging enzymes prevents ulceration (1). On the other hand, cysteamine, a strong free radical scavenger exerts an ulcerogenic effect. We have examined several SH-containing free radical scavenger radioprotectors, all of them cysteamine derivatives. They were given to healthy rats or to rats in which ulcers were induced in various ways.

Cysteamine was the main ulcerogenic agent in the pars duodenum. AET inhibited gastric secretion in pylorus-ligated rats. This effect was associated with protection against the formation of rumenal ulcers.

WR 2721 and its dephosphorylated derivative WR 1065 had the same effect in experimental ulcers. Both increased the secretion in pylorus ligated rats.

We concluded that the cysteamine derivatives exerted different effects on experimental ulcers, the origins of these being explained by several pathomechanisms.

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MULTIFACETED ROLE OF TRACE ELEMENTS IN THE PATHOGENESIS OF CHRONIC ARTHRITIS

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SUMMARY

A survey is presented of the literature data on the most important trace elements and their roles and possible functions in inflammatory processes, with special regard to rheumatoid arthritis.

The roles of antiphlogistic trace elements, such as copper, zinc, manganese, chromium, samarium and selenium, and of prothlogistic trace metals, such as iron, cobalt, nickel, vanadium and molybdenum, are discussed. These trace elements or metal ions regulate essential life processes, primarily via specific and stable binding to proteins, apoenzymes or amino acids. Both human and animal experiments showed that in chronic inflammation the local changes in the levels of trace elements are determinant, while the total concentration of trace elements in the whole body, due to the homeostasis, is essentially unchanged.

Some complexes of antiphlogistic trace metals, which can mimic the effects of oxygen free radical-scavenging enzymes, such as NSAID drug, plant flavonoid and propolis complexes, may be effective in the local treatment of patients suffering from chronic arthritis.

INTRODUCTION

Investigations of the biological roles of essential trace elements have led to great progress, and the enormous development in biochemistry has revealed more and more about the roles and interactions of trace elements in biochemical processes.

However, their role in pathology has not been sufficiently clarified. The main reasons for this are certain limitations in pathological research:

1. The application and extension of animal experimental models to human cases has its own limits.
2. In chronic human processes, such as autoimmune diseases and chronic inflammatory cases, nearly all other functions of the organism are modified.

At the same time, these diseases develop on the basis of a multifactorial polygenic disposition (73), but it has not been demonstrated what alterations in other functions of the diseased organism are caused by the genes which determine the genetic disposition to the disease. Thus, establishment of the roles of trace elements in human diseases would necessitate repeated measurements of trace elements in blood, tissue samples, fractionated cells, organelles, etc. over a long period, but this could not be tolerated even by "volunteer" probands.

TRACE ELEMENTS IN RHEUMATOID ARTHRITIS

The trace metal contents of blood serum and of synovial fluid from patients with rheumatoid arthritis (RA) and from normal controls (51) are listed in Tables 1 and 2.

It can be seen that the supposition a "fingerprint" diagnosis can be given through the determination of levels of trace elements in some diseases, e.g. RA (29), seems untimely. Overall, in different pathological situations, perhaps with the exception of Wilson's disease, the local changes in levels of trace elements are significant, but not the changes in their total quantity in the body as a whole.

In general, trace elements mainly enter the organism with ingested food from the natural environment, bound to organic materials such as proteins, carbohydrates, fats, flavonoids, etc. After digestion and absorption, one part of the trace elements is stably bound locally to macromolecules (enzymes or glycoproteins), while a significantly larger amount takes part in the metabolism in linkage with albumin globulin or in loose bindings with organic monomer molecules.

It is understandable that the plasma or serum levels of trace elements are not very informative in general, or the information is not sufficiently specific, e.g. the ceruloplasmin copper complex content can be elevated in different stages of inflammatory processes (74).

In RA, the trace element contents of the immune system and the joints and their accessories are of great interest. Mainly the concentrations of copper, zinc, manganese, chromium, molybdenum, cobalt and iron (as a meso-element) are examined (49, 58, 63-64, 74-77). The trace elements play similar roles in other non-rheumatoid inflammatory processes. In general, trace elements play a determining role in the production and scavenging of free radicals. However, this role is often bidirectional. The free radicals are determining in inflammatory processes (15, 26).

Table 1. Trace metal composition of blood serum (51)

Element	Source of specimens	No. of specimens containing element	Concentration (g%)			
			Mean	S.D.	Mean diff.	p ⁺
Copper	N	105	97.0	32.1	13.4	0.002
	RA	105	110.4	29.1		
Iron	N	105	131.7	64.8	68.9	0.001
	RA	102	62.8	62.0		
Aluminium	N	45	10.9	15.9	3.2	0.13
	RA	62	14.1	14.0		
Barium	N	62	6.3	6.6	7.8	0.001
	RA	103	14.1	6.8		
Manganese	N	47	2.4	3.3	1.8	0.001
	RA	82	4.2	3.5		
Nickel	N	60	4.1	5.1	3.5	0.07
	RA	81	7.6	19.3		
Cesium	N	63	6.3	4.2	4.9	0.001
	RA	97	11.2	5.2		
Tin	N	16	3.0	4.9	2.5	0.001
	RA	14	5.5	4.5		
Strontium	N	105	2.6	1.3	0.3	0.04
	RA	105	2.9	1.4		
Chromium	N	52	2.8	4.8	7.4	0.01
	RA	89	10.2	31.7		
Zinc	N	105	115.4	39.4	42.6	0.001
	RA	105	72.8	19.5		
Lead	N	87	13.1	10.2	6.8	0.001
	RA	65	6.3	6.8		
Molybdenum	N	44	3.4	4.8	3.3	0.001
	RA	76	6.7	5.6		
Cadmium	N	37	2.3	3.7	1.5	0.009
	RA	56	3.8	4.7		

N - Blood serum from 'normal' controls.

RA - Blood serum from patients with rheumatoid arthritis.

p⁺ - Probability.

Number of specimens analysed: N: n = 105 for all except Sn, where it was 81; RA: n = 105 for all except Sn, where it was 25.

Table 2. Trace metal composition of synovial fluid (51)

Element	Source of specimens	No. of specimens containing	Concentration			
			Mean	S.D.	Mean diff.	p ⁺
Copper	PM	50	27.5	16.4	57.3	0.001
	RA	50	84.8	20.7		
Iron	PM	50	29.0	28.6	29.8	0.001
	RA	50	58.8	46.3		
Aluminium	PM	31	13.7	15.0	9.2	0.001
	RA	16	4.5	8.3		
Barium	PM	30	4.1	2.4	3.0	0.001
	RA	5	1.1	1.7		
Manganese	PM	31	2.4	2.4	0.1	0.50
	RA	11	2.3	9.5		
Nickel	PM	14	1.2	2.1	0.5	0.48
	RA	2	0.7	4.8		
Cesium	PM	20	3.5	1.7	1.0	0.007
	RA	4	2.5	1.9		
Tin	PM	23	4.8	4.7	3.9	0.001
	RA	2	0.9	3.0		
Strontium ^f	PM	50	3.5	1.4	1.9	0.001
	RA	39	1.6	1.2		
Chromium	PM	16	2.3	6.5	2.2	0.021
	RA	1	0.1	0.9		
Zinc	PM	24	17.6	21.7	44.8	0.001
	RA	44	62.4	60.9		
Lead	PM	21	4.1	5.7	2.1	0.016
	RA	20	2.0	2.7		
Molybdenum	PM	9	1.0	2.1	0.9	0.003
	RA	1	0.1	0.6		
Cadmium	PM	15	2.0	3.3	1.8	0.001
	RA	2	0.2	0.6		

PM - Synovial fluid collected from patients without evidence of connective tissue disease seen at post mortem.

RA - Synovial fluid collected from patients with rheumatoid arthritis.

p⁺ - Probability.

Number of specimens analysed: PM: n = 50; RA: n = 50.

Copper

Copper is essential for cellular respiration, the production of basic material (24), bone formation, growth, vascularisation, keratosis, pigment and Hgb formation, the adequate functioning of the myocardium, the development of the brain, the nervous system and the intellectual capacity, and reproduction (75). The enzymes functioning with copper are shown in Table 3. Special mention must be made of ceruloplasmin, lysyl oxidase, ceramide-galactosyl transferase (75), cytochrome/c oxidase and SOD. The latter can function only with copper or manganese, depending on its location. As concerns RA, in a copper deficiency inflammations develop to a greater extent (43).

Table 3. Recognized Copper-Dependent Enzymes and their biochemical function (75)

Cu-dependent enzyme	Function
Cytochrome/C oxidase	Cellular respiration
Superoxide dismutase	Dismutation of superoxide anion radical
Tyrosinase	Conversion of tyrosine to Dopa
Dopamine β -hydroxylase	Conversion of dopamine to norepinephrine
Lysyl oxidase	Conversion of procollagen to tropocollagen, and of proelastin to elastin in the connective tissues
Soluble pyridoxal-dependent m monoamine oxidase (and perhaps the insoluble membrane-bound flavin-dependent monoamine oxidase)	Oxidation of catecholamines to aldehydes

Copper-histidyl-cysteine complexes have a protein stabilising effect. In a deficiency of these complexes, IgG damage occurs in the synovial fluid; it becomes self-binding, and immune complex building begins (16). This can be the initial step in RA through the stimulation of superoxide ion release from PMN leukocytes. At the same time, it is well-known that in RA the serum copper level, the ceruloplasmin level and the copper content of the

synovial fluid are enhanced, the latter to 2-3 times its normal value (9, 12, 64, 77). Copper is the only trace metal, whose level is increased in both the serum and the synovial liquid (36, 49, 50). The turnover of ceruloplasmin is increased, too. The increases in the ceruloplasmin and copper levels are correlated with the activity of the process. It is presumed that the elevated ceruloplasmin and copper levels are essentially a self-protective defensive function of the organism against the RA process (40). It is interesting that at the same time the level of SOD, which acts via copper ions, can be decreased. The administration of copper to animals reduces the severity of inflammation (33, 34, 40, 69). In the presence of copper(II) ions, thiols reduce the blastic transformation of T lymphocytes (35).

Table 4. Antiinflammatory activities of some copper complexes (75)

Compound	Carrageenin paw edema	Cotton wad granuloma	Adjuvant arthritis	Copper (%)
$\text{Cu(II)}_2(\text{acetate})_4$	A at 8	I at 100	I at 30	31.8
anthranilic acid	I at 200	NT	I at 30	
3,5-dips acid	I at 200	NT	I at 30	
$\text{Cu(II)}(\text{anthranilate})_2$	A at 8	A at 25	A at 1.20	18.9
$\text{Cu(II)}(3,5\text{-dips})_2$	A at 8	A at 5	A at 1.2	12.5
Aspirin	A at 64	A at 200 i.g.	A at 6	
$\text{Cu(II)}_2(\text{aspirinate})_4$	A at 8	A at 10	A at 1.2	15.0
D-Penicillamine	I at 200	I at 100	I at 30	
$\text{Cu(I)D-pen}(\text{H}_2\text{O})_{1.5}$	A at 8	A at 10	NT	26.7
$\text{Cu(II)}(\text{D-pen. disulfide})(\text{H}_2\text{O})_2$	A at 8	A at 25	A at 30	15.4

A = lowest active dose tested;

I = inactive;

NT = not tested.

All compounds were given by subcutaneous injection unless indicated as intragastric (i.g.); injected doses are given in milligrams per kilogram of body weight.

An ancient popular therapy was the use of a copper bracelet or copper wire around the waist (83). Chemotherapeutic compounds have been authorized as medicines in official treatment as well, if not with resounding success, e.g. Cupralene, Alcuprin, Decuprene and Permalon, a special salicyl-copper complex. Intensive clinico-pharmacological experiments are currently under

way with copper complexes of non-steroids, e.g. penicillamine, dexamethasone and other steroids, and with the copper complexes of numerous NSAIDs, e.g. diclofenac, naproxen, and niflumonic acid (75). These copper complexes mimic the O_2 or HO_2 radical-scavenging effect of CuZn-SOD (some data concerning this are given in Table 4). Sooner or later, a rich series of effective pharmaceutical products can be expected.

A prophlogistic effect of inorganic copper compounds, copper(II) salts and complexes of low stability in aqueous solution and their free Cu(II) aquo complex ions in the presence of reducing agents such as vitamin C is caused by the transformation of dioxygen into superoxide radical, singlet oxygen, hydrogen peroxide and hydroxyl radicals, and the lipid peroxidation, carbohydrate oxidation, arachidonic acid autooxidation, and protein, amino acid, nucleic acid and membrane destruction caused by these oxygen radicals (17-20, 84-85).

Like Cu(II) ions, iron(II) ions produce reactive oxygen-derived species as intermediate free radicals (22). In contrast, several Cu(II) complexes of medium stability with open structure, formed with different organic ligands such as NSAID drugs, exert significant antiphlogistic effects by scavenging superoxide radicals, oxidising iron(II) to iron(III) via their ferroxidase activity, and producing a ternary Cu-histamine-ligand complex, promoting the metabolic inactivation of histamine (57, 74-75).

Cu(II) aquo ions, Cu(II) complexes of NSAID drugs and also some non-antiphlogistics without copper decrease inflammation by inhibiting prostaglandin (PGE_2) synthase (cyclooxygenase); in this way the amount of PGE_2 in inflammation is decreased, while the level of antiphlogistic PGF_2 is enhanced (39).

Cu(II) ions usually form chelate complexes of medium stability with biologically active, antiinflammatory flavonoids in weak acid.

Zinc

Zinc is one of the most important essential trace elements. Its deficiency results in disorders of osteogenesis, growth and haematopoiesis (60). The administration of zinc accelerates wound healing and tissue regeneration (59, 82). In ulcer cruris, the zinc level is low; its elevation improves the tendency to recovery. In model inflammation, a zinc deficiency has a prophlogistic effect and a raised zinc level an antiphlogistic effect.

Autoimmune disease in mice can be improved by the administration of

zinc or histidine, and their combined intake produces an even better improvement (3). In RA, the zinc level is normal (58) or decreased, but in the synovial fluid it is significantly increased, probably as a result of the altered protein content and cytolysis. It is specific in RA that the zinc excretion with the urine is enhanced, even when the serum zinc level is normal. Multifaceted clinico-pharmacological experiments have demonstrated that different oral zinc products have real disease-modifying effects (8, 70, 71).

Zinc(II) ions increase the amount of antiphlogistic prostaglandin PGF_2 together with vitamin C, while they decrease the level of proinflammatory prostaglandin PGE_2 by inhibiting prostaglandin (PGE_2) synthase. Zinc(II) ions play a very significant role in tissue regeneration, wound healing, nucleic acid formation, protein synthesis, fibroblast proliferation, and glycine and methionine incorporation into collagenase fibres and cross-bonds in collagens.

Zinc salts in aqueous medium, i.e. their dissociated zinc(II) aquo complexes, themselves have wild antiphlogistic, astringent and wound healing effects (8, 11, 82). These effects stem from their multilateral antagonism, primarily with copper(II), iron(II) and calcium(II) ions (7).

The membrane-stabilising effect of zinc(II) ions results from the binding of zinc to the phospho groups of membrane sialoprotein lipids, and to the neighbouring -SH groups of their receptors, inhibiting their oxidation to disulfide (SS) (see copper antagonism). Thus, zinc(II) ions can reversibly stabilise the membranes of lysosomes, while they are made labile by Cu(II) ions. The lysis of lysosomes induced by copper(II) ions can only be reversed by chelators for copper(II) ions or compounds containing a thiol (-SH) group (37). Zinc(II) ions can also inhibit lysosomal enzymes: acid phosphatase, phospholipase-A and proteinase. Zinc(II) ions can also retard the formation of oxygen-derived free radicals in the membranes of erythrocytes, granulocytes and lymphocytes, in the macrophages of the reticulo-endothelial system, and in the mitochondria of liver cells. This inhibition is due to the production of $\text{Cu}_2\text{Zn}_2\text{-SOD}$, which scavenges superoxide radicals, of Zn-metallothionein, a very effective hydroxyl radical scavenger, and of the binuclear Zn-NADPH complex, which reacts more moderately than non-complexed NADPH with dioxygen to give the superoxide radical, catalysed by NADPH-oxidase (11). In this way, zinc(II) ions can effectively inhibit lipid peroxidation, i.e. the oxidation of polyunsaturated fatty acids, the main components of membranes and subcellular organelles; thus, zinc can

afford protection against the destruction of membranes and subcellular cell components (81).

As antagonists to the third messenger calcium(II) ion, zinc(II) ions inhibit the calcium-pumping $\text{Ca}^{2+}/2\text{H}^{+}$ -ATPase of the membranes, the cAMP-producing adenylylase, and protein kinase and phosphodiesterase, which are activated by calcium-calmodulin. All these enzymes take part in the second messenger processes of cells, which play an important role in inflammatory cases, in the aggregation and contraction of platelets, in serotonin release and in the histamine secretion of mastocytes.

Manganese

Manganese as an essential trace metal has a very important role in protection against inflammation and in the mucopolysaccharide synthesis of connective tissue (32). Manganese ions are constituents or activators of several enzymes, such as SOD, peroxidases and transferases, they are antagonistic to calcium ions, and they are essential for cell matrix production (88).

The mitochondrial Mn-SOD level in the granulocytes in RA is only half the normal, but that in the erythrocytes is twice the normal level (53). The turnover of manganese is decreased and can be normalised by steroid treatment (13, 14). The synovial content is normal; the serum data are uncertain. An elevated granulocyte manganese has been found in RA and other connective tissue diseases (23). During steroid therapy, the granulocyte manganese starts to return to normal levels.

Manganese(II) complexes of medium stability or dissociated Mn(II) ions exert different antiphlogistic actions in the organism. These ions can completely inhibit superoxide radical formation, catalysed by NADPH oxidase and stimulated by calcium-calmodulin (78). MnSOD and Mn(II) complexes, which mimic the SOD effect, can protect the membranes together with Cu_2Zn_2 -SOD in a compensatory way from damage caused by lipid peroxidation (27). Manganese(II) ions, like zinc(II) ions, activate different peroxidases which scavenge hydrogen peroxide, protecting the cells against the destructive action of reactive hydroxyl radicals. Manganese(II) ions inhibit the calcium pump too (2), because MnATP is produced instead of the MgATP substrate of $(\text{Ca}^{2+}, \text{Mg}^{2+})$ -ATPase; the affinity of Mn(II) ions for ATP is stronger than that of Mg(II) ions, and therefore MnATP takes part less actively than MgATP in Ca(II) transport (54).

Manganese, as a calcium antagonist, inhibits $\text{Na}^+ - \text{Ca}^{2+}$ influx and $(\text{H}^+, \text{K}^+) - \text{ATPase}$ as well. It also inhibits the arachidonic acid cascade and prostaglandin (PGE_2) formation and calcium-calmodulin-activated protein-kinase and cAMP phosphodiesterase (32).

The most important role of Mn(II) ions and their complexes is in the synthesis of mucopolysaccharides of the connective tissues. Collagenous fibre formation does not develop without the activation of glycosyltransferase, galactosyltransferase and saccharide polymerases Mn(II) ions (41).

The analgetic effect of Mn(II) complexes is also very significant; these complexes partly decrease the concentrations of lactic and pyruvic acid, and partly reduce dopamine, noradrenaline and serotonin concentrations.

The trace element content of chromatin has been examined. In every 10 nucleotides, one ion can be found. In RA, the zinc level is reduced, copper is enhanced and the manganese content is normal (30).

Selenium

Selenium is an essential trace element which to a low extent substitutes sulphur in certain amino acids.

Selenium-dependent glutathione peroxidase scavenges the harmful hydrogen peroxide and hydroxyl radical. The blood level of selenium and the activity of this enzyme decrease with the activity of acute arthritis (80).

Some enzymes also contain selenium. The administration of inorganic and organic selenium compounds together with vitamin E has been reported in inflammation cases (55).

Gold

Gold(III), used in therapy, changes the metabolism and distribution of copper, zinc and other trace elements (68). It is very important that the optimum ratio of zinc to copper in the organism is 10:1. Gold(III) can also bind to the selenium of glutathione peroxidase. Since gold therapy in RA has a very large literature, we shall not discuss this trace metal in detail.

Samarium

The antiphlogistic action of some lanthanides, e.g. Sm(III) ions, is due to their inhibition of the calcium-pumping effect (28, 72).

Chromium

Chromium(III) ions can effectively block the calcium ion channels. Cr(III) complexes induce glutathione peroxidase formation from selenoamino acids, and metallothionein formation from amino acid components. Cr(III) ions inhibit superoxide radical building by calmodulin-stimulated NADPH-oxidase, and their complexes significantly reduce NADPH and vitamin C-induced lipid peroxidation (86).

In contrast, chromate anion, as a pro-oxidant, oxidizes lipids. Chromium(III) ions, like copper(II) ions, inhibit β -glucuronidase thereby producing an antiphlogistic effect. Cr(III) ions decrease the dioxygen uptake of macrophages and their phagocytic activity and inhibit the migration of leucocytes. Cr(III) ions and complexes promote cell proliferation by enhancing RNA synthesis specifically, but not influencing DNA and protein synthesis.

PROPHLOGISTIC TRACE METALS

Iron

The essential element iron is really not a micro, but a mesoelement. However, because of its cooperation with copper and zinc, and its role in inflammatory cases, it is worth mentioning briefly as concerns its function in RA. As a prophlogistic metal, iron primarily takes part in oxygen-derived free radical formation and the activation of lipid peroxidase; in contrast, oxygen free radical production from granulocytes releases iron from ferritin (4, 6). The latter process is inhibited by different kinds of SOD (4). At the same time, iron has a local chemotactic effect on inflamed cells (6). In this way there is an active accumulation of iron in the RA tissues and in the synovial fluid through the activation of histiocytes. Ferritin is produced locally (44). The iron content of the synovial fluid significantly exceeds that of the serum (47). Iron deposits can be demonstrated histologically via Berlin blue staining (21, 67). The lipid peroxidase activity

and extent of iron accumulation are directly related to the activity of the process (62); this can be experienced in other types of arthritis too (5). The local prophlogistic effect of bivalent iron can be shown in air pouch experiments. Ferritin is produced here by macrophages in the proliferative phase of the inflammation (46, 47).

Rats suffering from Freund adjuvant arthritis were treated with desferrioxamine, which lowered their iron level and the intensity and extension of the inflammation (1).

Accordingly, it is doubtful whether iron treatment of anaemia in RA, which is usually unsuccessful, is advisable.

Cobalt

Iron and the iron group elements cobalt and nickel are prophlogistic. Both Fe(II) and Fe(III) ions, in native and artificial micro- and macro-molecular iron complexes, can result in oxygen-derived free radicals in the human organism. Nickel has the same activity, Ni(II) ions showing competitive antagonism to Fe(II), Cu(II) and calcium ions (42, 52).

Cobalt(II) ions and complexes of elements in the iron group can exert both pro- and antiphlogistic effects. Cobalt(II) ions act as promoters in "crypto-hydroxyl" radical formation (which is not readily available for hydroxyl radical scavengers) and inhibit cytochrome P-450, in this way stopping active oxygen-derived free radical formation (45, 48, 66).

Cobalt(II) ions, as calcium antagonists, block the calcium ion channels, thereby inhibiting inflammatory processes. Cobalt(II) ions diphasically first decrease the glutathione content and then increase it, which has a protective effect against hydroxyl radicals. Cobalt(II) ions inhibit intermediate phospholipid peroxide formation in membrane lipid peroxidation. At the same time, cobalt(II) ions have a stimulating effect in phagocytosis and a labilising effect on lysosomes (79).

Vanadium, molybdenum

Molybdenum, an essential trace metal, has antagonistic effects on chromium and copper; thus, molybdenum is a prophlogistic element. Molybdate ions inhibit $\text{Cu}_2\text{Zn}_2\text{SOD}$ activity and peroxidases. Xanthine oxidase, an iron and molybdenum enzyme, results in superoxide radical production.

Vanadate ions, as antagonists of manganese, inhibit Mn-SOD. Both

vanadate and molybdate inhibit the effects of ceruloplasmin and very strongly stimulate lipid peroxidation as well as NADPH oxidation by superoxide ions (31, 61, 87). These effects can be reduced with vitamin C, probably through the following reductions: $V(V) \rightarrow VO(II)$ and $Mo(VI) \rightarrow MoO_2(I)$.

Vanadate also inhibits the (Na^+, K^+) -ATPase and the calcium pump (Ca^{2+}, Mg^{2+}) -ATPase by acting on the dephosphorylating agents $(Ca^{2+}$ and $Na^+)$ forming complexes which are not suitable for phosphorylation (10, 25, 56). Vanadate ions have no effect on the phosphorylating agents $(Mg^{2+}$ and $K^+)$; moreover, the complex formation here is inhibited by Mg^{2+} and K^+ , and the inhibition is decreased by Na^+ and Ca^{2+} ions. Vanadate ions activate the Na^+/H^+ pump and Ca^{2+} influx (38). Vanadate ions stimulate adenylyl cyclase, and thus increase the intracellular cAMP concentration (65). Vanadate ions stimulate protein phosphorylation through the activation of phosphokinases. Vanadate ions inhibit the phagocytic activity of leucocytes, and reduce the numbers of leucocytes and lymphocytes. Their effect is enhanced by vitamin B_6 .

CONCLUSION

Trace elements play important roles in RA processes, mainly via SOD (Orgotein), which scavenges superoxide free radical, glutathione peroxidase, which scavenges hydrogen peroxide and hydroxyl radical, and metal-proteases. Several trace elements also play important roles in the formation of the matrix of connective tissue. Although their role in inflammation processes has not yet been explained in detail, useful results are expected that can be applied in therapy. Trace elements are believed to exert mainly local effects of great significance, and thus they may well be applied locally in therapy. Some antiphlogistic trace metal complexes, such as NSAID drug, flavonoid and propolis complexes, have been found effective in the local treatment of chronic inflammatory conditions (see following paper).

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LOCAL TREATMENT OF CHRONIC INFLAMMATORY CONDITIONS WITH PROPOLIS ENRICHED WITH ANTIPHLOGISTIC TRACE METAL IONS

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SUMMARY

Standardized propolis extracts enriched with antiphlogistic trace metal ions have been found more effective than natural propolis extracts in the treatment of inflammatory diseases, such as rheumatoid arthritis and gingivitis, and in wound healing. Both types of components of propolis extracts, the flavonoids and phenolic acid derivatives, form polynuclear, mixed ligand chelate-type complexes with trace metal ions, which can also scavenge the oxygen free radicals.

Due to the antiphlogistic trace metal complex formation, neither in the case of patients suffering from rheumatic diseases, nor in the treatment of dental patients was allergy manifested.

INTRODUCTION

The research work and application of medical plants and drugs and also some materials of animal origin for natural curative purposes began to be revived in the second half of this century. Bee glue, one of these substances, has been well known and widely used in the traditional therapy of joint diseases for thousands of years. Propolis balsam, extracted from bee glue, is also successfully applied in the treatment of rheumatoid diseases, due to its antiphlogistic effect. Propolis balsam and the ethanolic solution of propolis (12%) called PROPUR[®] are authorized in Hungary; both are produced by the Fine Chemical Cooperative in Budapest (3).

Propolis balsam extracted with 70% ethanol from bee glue in the U.K. was analysed by GC-MS, and 104 compounds were identified, including 26 compounds not previously identified in propolis (5). The main components were flavonoid aglycones, benzoic acids, substituted phenolic acids and esters, fatty acids, terpenes, beeswax, saccharides, lactones, etc. (6, 28). Nagy et al. (18-20) analysed propolis of Hungarian origin extracted

with 70% ethanol by GC-MS, and found only 87 components. 40 compounds were identical with the propolis components found in the U.K., but 47 new compounds were identified. Thus, propolis contains at least 150 components.

MATERIALS AND METHODS

The trace element contents of bee glue of Hungarian origin and PROPUR were also measured with a Jarell-Ash ICAP-9000 inductive coupled argon plasma emission spectrometer. The contents of 27 elements can be found in Table 1 (in ppm). The data show that the toxic metal (As, Cd, Hg and Pb) content is under the detection limit or very low, which meets health requirements. The antiphlogistic trace element content is between one and some hundreds ppm, i.e. very low.

The data in Table 1 also show that the total metal content of PROPUR (propolis) is 0.1 mequ/g solid. The total metal ion binding capacity of propolis (PROPUR) was measured by potentiometry or spectrophotometry in ethanol solution (2.4 mequ/g). The sum of these two values is $2.4 + 0.1 = 2.5$ mequ/g, which is the total metal ion capacity of pure, trace metal-free propolis, 4% of which is originally saturated in native propolis. This effective, small amount of natural metal ion content of propolis forms chelate-type complexes with the organic components of propolis, mainly with flavonoids, benzoic acids and phenolic acid derivatives. The composition and trace metal content can be different in each propolis sample (21). Propolis products, however, can be standardized by 100% saturation of the total metal ion-binding capacity (2.4 mequ/g) with an antiphlogistic metal ion, e.g. copper(II) ion (15, 24).

The antiphlogistic effects of these trace metals were discussed in a previous review (25). Trace metals, such as the $3d$ and $4d$ transition metal ions, e.g. copper(II) ion, and the $4f$ rare earth metal ions, e.g. samarium (III) ion, form chelate complexes with flavonoids (F1) (7-9, 11, 14, 16, 23, 26, 27) according to the following processes:

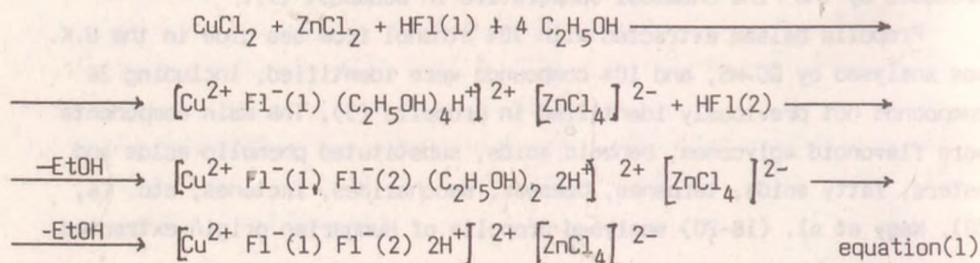


Table 1. Inorganic element contents of solid bee glue (M) and Propur (P). (D.L. = detection limit)

Element	D.L. (ppm)	M (ppm)	P (ppm)	P (equ/g)
Al	0.0025	246.2	17.98	$2.00 \cdot 10^{-6}$
As	0.050	< D.L.	< D.L.	-
B	0.004	11.0	7.976	$2.21 \cdot 10^{-6}$
Ba	0.001	28.49	0.7047	$1.02 \cdot 10^{-8}$
Ca	0.001	955.4	21.83	$1.09 \cdot 10^{-6}$
Cd	0.002	0.3237	0.0465	$8.28 \cdot 10^{-10}$
Co	0.003	< D.L.	0.1037	$3.52 \cdot 10^{-9}$
Cr	0.005	1.0495	0.8629	$4.98 \cdot 10^{-8}$
Cu	0.002	3.383	3.237	$1.03 \cdot 10^{-7}$
Fe	0.005	405.37	154.2	$8.28 \cdot 10^{-5}$
Ga	0.020	< D.L.	< D.L.	-
Hg	0.010	< D.L.	< D.L.	-
K	0.300	1141.0	465.3	$1.19 \cdot 10^{-5}$
Li	0.004	0.2708	0.0753	$1.09 \cdot 10^{-8}$
Mg	0.001	261.8	42.73	$3.52 \cdot 10^{-6}$
Mn	0.001	9.571	1.102	$4.01 \cdot 10^{-8}$
Mo	0.005	< D.L.	< D.L.	-
Na	0.010	< D.L.	< D.L.	-
Ni	0.010	0.817	0.3609	$1.23 \cdot 10^{-8}$
Pb	0.025	9.736	1.871	$1.81 \cdot 10^{-8}$
Se	0.050	< D.L.	1.235	$3.13 \cdot 10^{-8}$
Si	0.005	16.73	42.22	$3.01 \cdot 10^{-6}$
Sr	0.001	3.818	0.0728	$1.66 \cdot 10^{-9}$
Ti	0.002	5.059	0.4270	$2.67 \cdot 10^{-8}$
V	0.001	0.7102	0.2202	$8.65 \cdot 10^{-9}$
Zn	0.004	300.5	78.70	$2.41 \cdot 10^{-6}$
Cations: 0.001-0.3		< D.L. - 1141.0	< D.L. - 465.3	Sum: $1.04 \cdot 10^{-4}$

In Fig. 1, MF1 represents an unsaturated complex in which the coordination sphere of the central M (metal) cation is coordinatively saturated (coord. number 4 or 6) by further ligands, e.g. Cl^- anions (when metal chlorides are used), or solvent molecules, e.g. H_2O or $\text{C}_2\text{H}_5\text{OH}$, or it can become MF1_2 by binding another flavonoid molecule (Fig. 2). In the formulae of flavonoids (Figs 1 and 2), H atoms or HO, CH_3O or sugar-O- groups are bound to C_7 , C_3 , and C_4 , (24a).

In weak acidic medium, in which the biological effect is quite significant, the phenolic HO groups, binding to C_3 , and C_4 , of ring B, do not bind to metal ion, similarly as for salicylic and cinnamic acids and derivatives in propolis, e.g. the $(\text{Cu}^{\text{II}} (\text{C}_6\text{H}_4(\text{OH})\text{COO}^-)_2)$ complex in acidic medium ($\text{pH} = 5$). The structure of this complex can be seen in Fig. 3 (1, 13).

There are numerous different flavonoids in propolis (5-6, 18-20): $\text{F1(1)}, \text{F1(2)} \dots \text{F1(k)}, \text{F1(l)}$. The MF1 complex can also cover a complex system with mixed ligands such as $\text{MF1(1)F1(2)} \dots \text{MF1(k)F1(l)}$. In addition, because of the different aromatic components of propolis, such as benzoic acids and phenolic acid derivatives, Ar(n)Ar(O) ligands must be taken into account, too, and even polynuclear mixed anionic complexes can be produced, e.g. $\text{MF1(k)Ar(n)-MF1(l)Ar(O)}$, etc.

The most effective antiphlogistic metal-flavonoid-aromatic acid complexes are formed by copper(II) ion (24). It is practical to choose copper(II) chloride (see Eq. (1)), because sulphate ion irritates the gastrointestinal tract, while nitrate ion is toxic. The free chloride ion can be transformed by zinc(II) chloride to the complex anion $[\text{ZnCl}_4]^{2-}$. Zinc(II) salts or zinc halogenide complexes in aqueous solution have antiphlogistic, astringent and wound healing effects (24, 25, 30). The $[\text{ZnCl}_4]^{2-}$ anion also forms a zinc(II)-salicylate complex with the triethanolamine or glycol salicylate content (10% in No. II) of the ointment.

On the basis of these findings, we saturated the ethanolic solution of propolis with the antiphlogistic trace metal ions according to its total metal ion-binding capacity in the ratio required to give the desired effects (15). The material was left to stand overnight at room temperature, and next day the appropriate quantity of zinc(II) chloride was added to the solution according to the total amount of admixed trace metal chlorides: $(\text{ZnCl}_2 + \text{MCl}_2 \rightleftharpoons [\text{ZnCl}_4]^{2-} + \text{M}^{2+})$; the mixture was then again left to stand overnight. Obviously, propolis enriched with trace metal ions requires the same condition of treatment as natural propolis products (3).

Neither should be exposed to a temperature above 60-75 °C for a long time, and they must be protected from visible and UV light (storing in dark bottles), and also from alkaline effects (e.g. not to be kept in a bottle that releases alkaline substances) and from too strong acidic effects too (pH = 1).

After distillation of the alcoholic solution of metal-Propur mixture, the residue was worked into the basic ointment. This ointment is a fatty cream containing native substances and dissolves all the biologically active components of raw propolis in the oil phase, thereby promoting their absorption. The composition of the ointment base: lanoline (6.5%), bees wax (3.2%), cholesterol (1.1%), sunflower oil (11.0%), castor oil (21.3%), cetyl-stearine-alcohol (7.5%), stearic acid (2.6%), distilled water (46.9%).

We prepared four different cream (I-IV) for the local treatment of patients with chronic inflammatory conditions (15). Cream I, worked into the basic ointment, contains 1% native propolis extract, 10% triethanolamine or glycol salicylate and 1% L-ascorbic acid. Creams II, III and IV differ from cream I; instead of salicylates and 1% native propolis extract, they contain 1% metal-Propur-mixture, i.e. propolis enriched with different antiphlogistic trace metals, such as copper(II), zinc(II), etc. (15). Instead of L-ascorbic acid, cream IV contains 1% silicone oil, and cream II is without L-ascorbic acid. We prepared one cream (V) for dental application, for the treatment of gingivitis, periodontal and alveolar abscess, parodontitis and alveolar pyorrhoea. The basic ointment contains unguentum simplex (90 g), beeswax (cera alba 5.0 g) and zinc oxide (5.0 g). Cream V contains 10% propolis extract enriched with different antiphlogistic trace metal ions. Cream VI contains 10% native propolis only, without additional trace metal ions (15).

Before use of the cream, the rheumatic diseased region of the body is soaked in lukewarm (36-37 °C) water for about 10 minutes. The skin surface is dried and the cream should be applied as a thin film; it is then gently massaged into the skin by hand for one minute. After 30 minutes, the cream has already been absorbed, and the process has to be repeated. To increase the effect, the treated region of the body is suggested to be wrapped for one night. In the morning, the whole process is repeated. The treatment with cream should last for 2 weeks, applying the massage and wrapping twice a day. RA patients were mainly selected according to the localization of the disease, predominantly with hand and knee manifestations, because these regions are near the body surface and therefore amenable to local treat-

ment. Finally, the functions of these articulations could be well and reproducibly measured.

In the Physiotherapy Department of the Forest Thermal Bath in Debrecen, experimental treatment was carried out on RA patients with the above propolis creams (I-IV). The aim of these experiments was to observe the effects and possible side-effects of the creams. The treatments were performed according to the rules of drug testing on patients randomized in the order of arrival, by a semi-blind method. Before the treatment, the patients already had the results of X-ray and laboratory examinations necessary for correct establishment of the diagnosis, and rheumatological examination was also carried out (Table 2). After treatment, these examinations were repeated.

The following parameters were measured in all cases (Table 3): erythema, tenderness and pain evaluated on both graphical and visual analogous scales (VAS). The subjective parameters were scored: pain intolerable: 20, strong: 15, moderate: 10, slight: 5, without pain: 0. Circumference (in cm), grip strength (in mm HG), measured by tonometer, and drug consumption (steroid and NSAID (non-steroid anti-inflammatory drugs) were registered. Patients regularly took their other everyday medicines and disease-modifying agents (e.g. sodium aurothiomalate). Creams I-IV were applied to groups containing 10-17 patients. The numbers of patients within these groups differ because the effects of treatment could not be evaluated in all cases (noncompliance, self-medication, or arbitrary interruption of treatment). The patients were randomized in the order of arrival, and thus the distributions according to diagnosis, sex and average age were not equal in each group (Table 2).

RESULTS

The results of treatment with the four different propolis creams (I-IV) on 53 patients in four groups (I-IV) were evaluated (Table 4). The significance of the differences between the data obtained before and after treatment were calculated with the Student one-tailed t-test. Any differences between data that could be evaluated statistically were at the 5%, 1% or 0.1% level.

In group I (patients treated with cream I), circumference values were significantly reduced on both sides. Tenderness and pain values were also decreased significantly, and the improvement of functions was also signifi-

Table 2. Distribution of treated patients by diagnosis for treated groups and different sexes

Diagnosis	I (n=10)		II(n=13)		III(n=17)		IV(n=13)		Total		Overall total
	male	female	male	female	male	female	male	female	male	female	
Arthrosis genus	3	3	3	1	2	-	1	-	9	4	13
Polyarthrosis	-	-	-	2	-	3	-	3	-	8	8
Painful conditions of degenerative origin	-	1	1	2	1	4	1	6	3	13	16
St.p.traumam	-	-	1	1	1	3	1	1	3	5	8
Polyarthrititis	1	2	-	2	-	3	-	-	1	7	8
Total	4	6	5	8	4	13	3	10	16	37	53

Table 3. Numbers of parameters used to analyse treatment in the examined groups

Parameters	Numbers of parameters used				
	I	II	III	IV	Total
Tenderness	10	12	15	11	48
Pain (graphical)	10	12	16	13	51
Pain VAS (visual)	10	12	16	13	51
Consumption of medicine	8	8	7	6	29
Circumference	8	10	10	5	33
Movement in °	4	7	7	1	19
Grip strength	2	6	4	9	

Table 4. Changes in examined parameters during treatment in the examined groups

Parameters	I	II	III	IV
Tenderness	decr. ^{xx}	decr. ^{xxx}	decr. ^{xx}	decr. ^x
Pain (graphical)	decr. ^{xxx}	decr. ^{xxx}	decr. ^{xxx}	decr. ^{xxx}
Pain VAS (visual)	decr. ^{xxx}	decr. ^{xxx}	decr. ^{xxx}	decr. ^{xxx}
Circumference	decr. ^{xx}	decr. ^x	decr. ^x	decr. ^x
Movement in °	incr. ^x	incr. ^{xx}	incr. ^x	---
Consumption of medicine	11/7	8/2	7/2	8/2
Grip strength	---	incr. ^x	incr. ^x	incr. ^{xx}

^x significant at 5% level; ^{xx} significant at 1% level; ^{xxx} significant at 0.1% level

Table 5. Comparison of rate of progress in parameters for treated groups in pairs

Parameters	I/II	I/III	I/IV	II/III	II/IV	III/IV
Tenderness	NS	+ ^x	+ ^x	NS	+ ^x	NS
Pain (graphical)	+ ^{xx}	NS	NS	NS	+ ^{xx}	+ ^x
Pain VAS (visual)	+ ^x	NS	+ ^x	- ^x	+ ^x	+ ^{xx}
Consumption of medicine	+ ^{xx}	+ ^{xx}	+ ^{xx}	NS	NS	NS
Circumference	NS	NS	+ ^x	NS	+ ^x	NS
Movement in °	NS	NS	— —	NS	— — —	— — —
Grip strength	— — —	— — —	— —	NS	- ^x	NS

+ = denominator better

- = numerator better

NS = not significant

x = significant at 5% level

xx = significant at 1% level

xxx = significant at 0.1% level

— — = no information available

cant. In group II (patients treated with cream II), the circumference values were significantly reduced only on the right side. Some differences observed between the two sides probably stem from the low number of patients and do not have any clinical relevance. Tenderness and pain were significantly reduced, while grip strength was significantly increased bilaterally.

In group III (patients treated with cream III), the circumference values were significantly reduced on the right side. The subjective parameters were decreased, and the functions were significantly increased. Grip strength values were significantly increased only on the left side. In group IV (patients treated with cream IV), all the parameters were favourably changed at significant levels.

In the second step, we examined the differences in the therapeutic effects obtained with the four creams. We calculated separately the levels of significance of the different clinical parameters, with the four groups paired in all combinations. The comparison showed different results for different parameters (Table 5).

The effect of cream II (group II in Table 4) significantly surpassed that of cream I in the relief of pain and the reduction of NSAID and steroid consumption. The effect of cream III (group III in Table 4) also surpassed

that of cream I in reducing tenderness and drug consumption. The effect of cream IV (group IV in Table 4) again surpassed that of cream I in most parameters. Significant lowering of the tenderness and circumference values, alleviation of pain, and reduction of drug consumption were found. Cream II was more effective in relieving pain than cream III, as measured via VAS. The effect of cream IV surpassed that of cream II in lowering the tenderness and circumference values and in the alleviation of pain, but the grip strength was greatly increased by cream II. Only one parameter differed in the effects of creams III and IV: the alleviation of pain was more effective with cream IV.

Cream V was compared with cream VI in gingivitis and dental surgery in the case of 450 patients (15, 17). The alleviation or cessation of gingivitis and oral wound healing were found to occur about twice as quickly with cream V as with cream VI without trace metal ions.

DISCUSSION

It can be stated that all the different creams are effective in inflammatory states, and consequently propolis is one of the most important components in the adequately prepared basic ointment. The statistical evaluation of the results clearly reveals (see Table 5 and RESULTS) that creams II-V, containing trace metal ions, produce a significantly greater effect than creams without additional trace metal ions.

Some of the flavonoids (e.g. guercetin, myricetin, rhamnetin, morin, naringenin, apigenin, catechin and kaempferol) in propolis can themselves strongly scavenge the harmful oxygen-derived species, such as singlet oxygen (29), superoxide ion (22), hydrogen peroxide and hydroxide radical (12). Flavonoids are also inhibitors of several enzymes with a prophlogistic effect or playing a role in inflammatory processes (2).

In our creams, the propolis enriched with antiphlogistic trace metal ions forms a polynuclear and polyanionic chelate-type complex system, which is standardized for its metal ion content (15). During binding of the metal ions, e.g. copper(II) ions, hydrogen ions are released, which can bind to either the most basic atom of a coordinated flavonoid molecule and/or aromatic phenolic acid derivative or to the coordinated solvent molecules [Eq. (1)]. Both complex ion systems and complex compounds obtained by removing the solvent molecules are neutral. They can be worked into hydrophobic creams, thereby easily crossing cell membranes. After this, for

example, the copper(II)-flavonoid-substituted phenolic acid-zinc-chloro-complex can easily dissociate, partly to zinc(II) ions, which can exert a physiological antiphlogistic, wound healing effect (24, 30), and partly to a copper(II) complex, which can react with the amino acids, or polypeptide/protein/components of the cells.

Some copper(II) complexes of flavonoids and phenolic acid derivatives have been observed to scavenge oxygen free radicals (24).

Gyul'khandanyan (4) studied the hyperfine structure of EPR spectra of natural propolis extracts from Yerevan with $g = 2.2$ and 4.2 and spectroscopic factor 2.01 . The signal with $g = 2.2$ is characteristic of square bipyramidal copper(II) complexes in the aqueous alcoholic extract, while the signal with $g \sim 4.2$ can be related to dimeric copper or iron complexes, and the signal with a g factor of 2.01 is probably related to semiquinone free radicals. Superoxide dismutase activity was observed for all propolis extracts.

Recent research results (10) proved that the 1,1-dimethylallyl-caffeic acid ester component of propolis has the strongest allergic effect. This unfavourable effect can be eliminated by metal complex formation. Thus, we can understand that allergy was not observed, either in the treatment of patients with RA and other joint diseases, or in that of patients with gingivitis or in oral wound healing (450 patients) (17). Examinations by iontophoresis with propolis enriched with antiphlogistic trace metal ions, mainly in rheumatism of the soft parts of the body, are in progress.

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LIPID PEROXIDE PRODUCTION IN RAT LIVER MICROSOMES
AFTER TREATMENT WITH ENZYME INDUCER COMPOUNDS

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The interrelations of cytochrome P-450 induction and the intracellular lipid peroxidation are still a matter of controversy. Lipid peroxides might have a central role as endogenous inducers in the control of P-450 induction. Lipid peroxides cause the destruction of intracellular membranes, and the P-450 content of the liver has been shown to be particularly sensitive to lipid peroxidation. It was found that P-450 itself produces reactive oxygen radicals and may contribute to the initiation of lipid peroxidation. The lipid peroxidation in vivo has been proposed to be a mechanism responsible for the development of fatty liver, which can progress to cell necrosis. P-450 is responsible for most of the peroxidase activity in the microsomes.

In this study, male rats were treated with phenobarbital, m-trifluoromethyl-a-ethylbenzhydrol, 2-methyl-1,2-di-3-pyridyl-1-propanone, spironolactone, toluene, β -naphthoflavone and benzo(a)pyrene. Total cytochrome P-450 and metyrapone binding P-450 were assayed. The lipid peroxide generating activity and malondialdehyde (TBA-reacting substances) content were measured.

Treatment with all of the inducers used resulted in an increase in the microsomal contents of cytochrome P-450 and of total heme. The microsomes from phenobarbital, m-trifluoromethyl(a)ethylbenzhydrol, spironolactone, 2-methyl-1,2-di-3-pyridyl-1-propanone or toluene-treated rats contained more peroxidizing activity than the microsomes from untreated rats or from beta-naphthoflavone or benzo(a)pyrene-treated animals.

The inducers investigated did not influence or reduce the malondialdehyde reactive substance content of liver samples (Table 1).

The few data published on the inducibility of lipid peroxidation by P-450 inducers are contradictory. It was an unexpected result of our study

Table 1. Effects of several enzyme inducing compounds on the cytochrome P-450 levels, lipid peroxide content and lipid peroxide production (per cent of controls)

	P-450(CO)	P-450(MP)	LP _{cont}	LP _{prod}
PB	220	193	75	1675
RGH-3332	215	234	79	2107
SNL	110	177	98	1450
SU-4885	186	230	87	1661
toluene	198	218	107	1279
BNF	145	122	102	108
BP	195	134	88	177

P-450(CO) - total cytochrome P-450, carbon monoxide binding

P-450(MP) - metyrapone binding cytochrome P-450

LP_{cont} - lipid peroxide content

LP_{prod} - lipid peroxide generating activity

PB - phenobarbital; RGH-3332 - m-trifluoromethyl(a)ethyl-benzhydrol; SNL - spironolactone; SU-4885 - 2-methyl-1,2-di-3-pyridyl-1-propanone; BNF - beta-naphthoflavone; BP - benzo(a)pyrene

that the ("phenobarbital type") inducers strongly enhanced the lipid peroxide generating capacity without an increase of the lipid peroxide content. The present data suggest that the activity of the defense systems is also increased due to inducer effects. We can hypothesize that the interactions of various components of the intracellular structures may be efficiently protective in vivo in a broad range of functional requirements.

OXYGEN FREE RADICALS IN MYOCARDIAL ISCHEMIC STATES

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During the past decade, research in biology and medicine has yielded an immense number of data relating to the role of oxygen free radicals. However, despite the enormous quantity of experimental and clinical details, the principal question cannot be regarded as settled. The dilemma is well outlined by the remark of Opie that the oxygen free radicals are "red herrings", i.e. irrelevant factors which distract attention from the subject in question.

Although this remark has been disproved by the research results, it is present in the minds of all investigators, suggesting that the role of lipid peroxidation contra antioxidants should be reconsidered continuously.

The present paper aims to clarify only three main points on the basis of heart ischemic experiments.

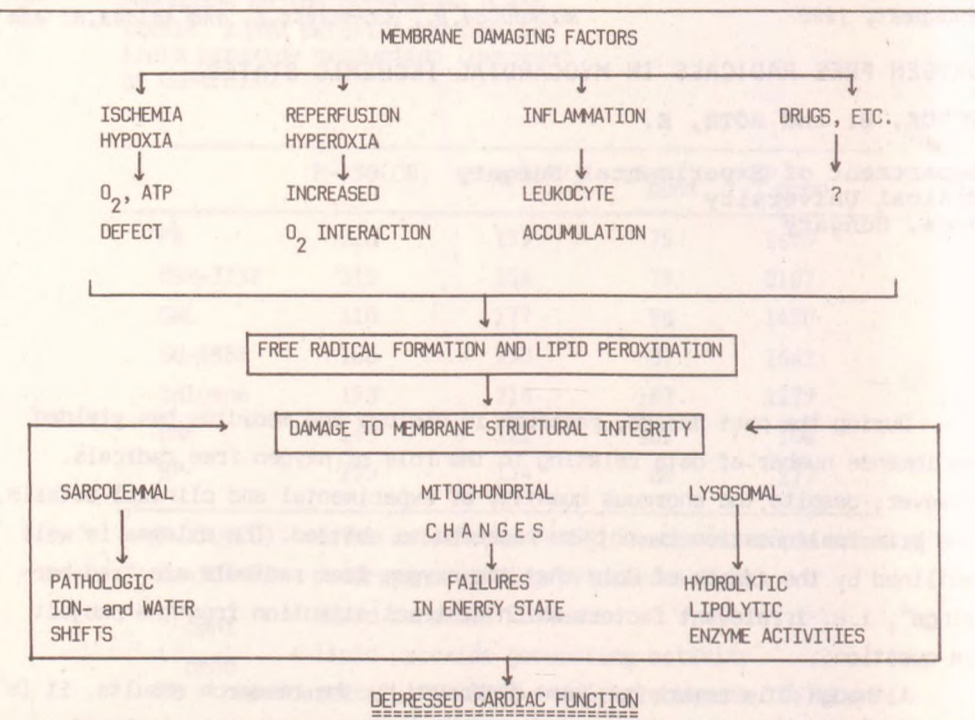
(i) How are the oxygen free radical-induced deteriorations to be interpreted?

(ii) What interdependences are to be found in the background of the structural and functional alterations?

(iii) What effects are to be expected from antioxidants?

The oxygen free radicals are continuously produced in aerobic organisms, even physiologically. Their effects, however, are blocked by ubiquitous defending compounds, different enzymes, vitamins and endogenous thiols in statu nascendi. Pathologically, as a consequence of the overproduction of oxygen free radicals, the exhaustion of the defending compounds, or a combination of these two factors, the equilibrium of the cell and membrane system breaks down. The deterioration process comes into action and, depending on the quantity and time factors, the overall cell degradations lead to the structural and functional deterioration of certain organs.

Table 1



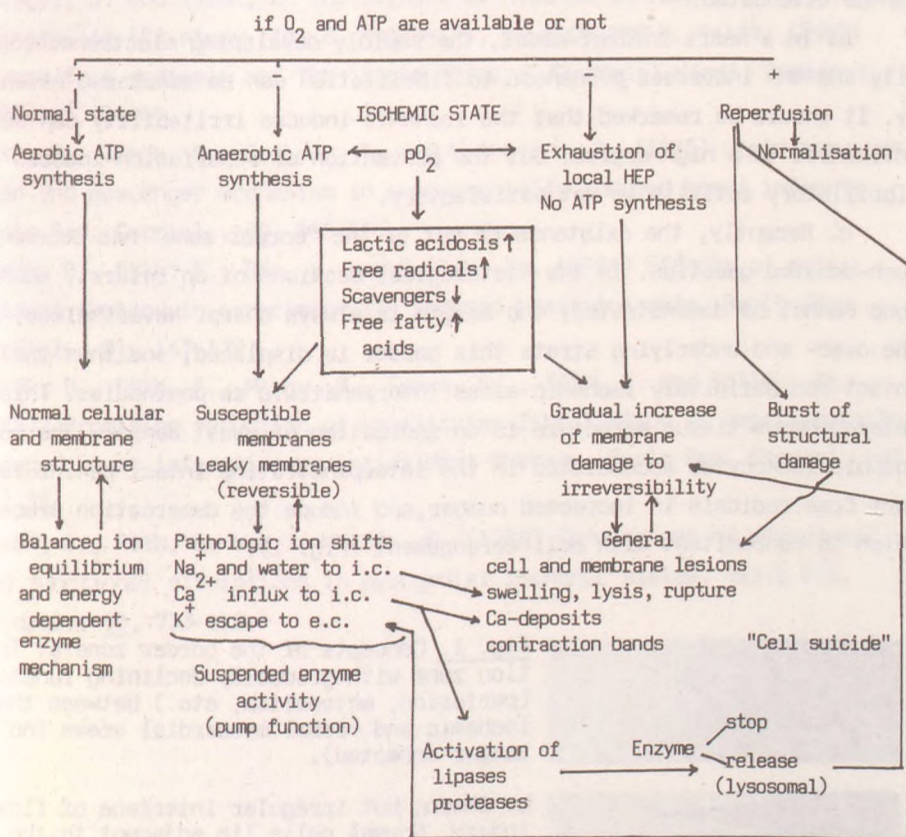
The world-wide discussion as to what the primary cell damaging factor is seems a little academic, because it is a matter of a train of causes and effects. In our opinion, in this concatenation the appearance of oxygen free radicals accelerates and exacerbates the degradation process, or rather participates in the vicious circle. Table 1 indicates that, from a practical point of view, hyperoxygenation, hypoxia-ischemia, the activation of polymorphic nuclear leukocytes in inflammation reactions, and biosynthesis-blocking drugs are all factors leading to membrane damage, and hence to cell, tissue and organ derangements.

In our view, the deteriorations at cellular and subcellular levels cannot be termed structural, biochemical or functional; they can only be explained according to the principle of structural-functional unity. Table 2 displays only the most important changes in the case of ischemia. These main processes always occur; certain factors can ensure reversibility.

Besides numerous endogenous defending compounds, many artificial scavengers are known to be agents with good effects. We analyzed the effects of ischemic factors on the scavenger state, hoping to gain knowledge which

Table 2

INTERRELATIONS OF MYOCARDIAL STRUCTURE AND FUNCTION



Note: The most important ischemic consequences are seen. Normothermia and reperfusion may accelerate the alterations; hypothermia, cardioplegia, Ca^{2+} -blockers and free radical scavengers may delay the changes.

would point to the process and its mechanism. We also attempted to eliminate well-known unfavorable effects through the application of a special artificial scavenger (MTDQ-DA) in vitro and on a heart infarct model in vivo.

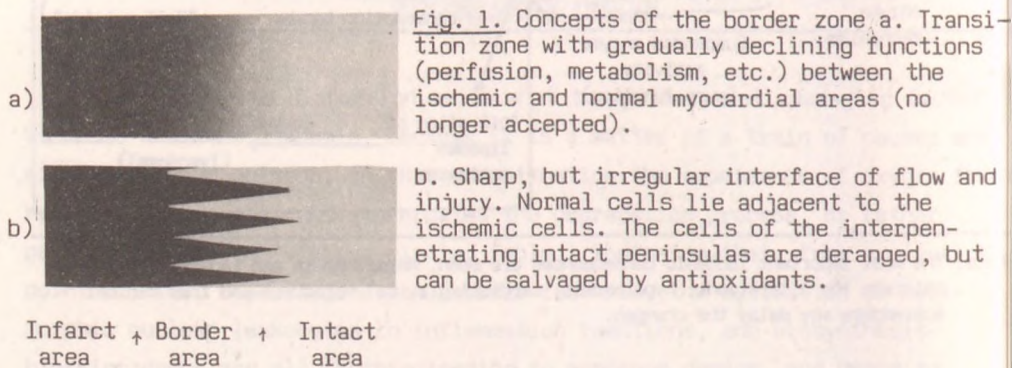
In this context, three observations should be mentioned:

a. In a hydroxyl radical generated system in vitro, the EPR technique demonstrates that MTDQ-DA displays its scavenger capacity quickly. The analysis of this MTDQ-DA effect in combination with GSH is very interesting. MTDQ-DA scavenging can be decreased or increased via the increase or decrease of the GSH concentration. This means biologically that GSH readily participates in the elimination of an ongoing deterioration process; never-

theless, because of its rapid exhaustion, the artificial scavenger may have a very advantageous effect, and thus the development of a fast derangement can be attenuated.

b. In a heart infarct model, the rapidly developing electroheterogeneity and the increased proneness to fibrillation can be decreased essentially. It should be remarked that the ischemia-induced irritability can be influenced to a high degree, but the prevention of reperfusion-induced fibrillatory activity is not satisfactory.

c. Recently, the existence or not of the "border zone" has become a much-debated question. In the histological sections of an infarct, such a zone cannot be demonstrated; the margin is always sharp. Nevertheless, in the over- and underlying strata this border is displaced, and thus the intact and definitely ischemic areas interpenetrate as peninsulas. This heterogeneous tissue structure is an indication of great danger. The polymorphic leukocytes accumulated in the interpenetrating intact peninsulas form free radicals in increased number, and induce the demarcation process, which is concomitant with cell derangement (Fig. 1).



Practically, it should be stated that the clinical expression "infarct size reduction" is a pipe-dream. The infarct size cannot really be reduced, but the deterioration of the intact peninsulas of the clinical border zone can possibly be hindered. The application of artificial scavengers for this purpose seems promising.

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LIPID PEROXIDATION OF EHRlich ASCITES TUMOR CELLS MITOCHONDRIA

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SUMMARY

The intensities of lipid peroxidation are compared in Ehrlich ascites cell and in liver mitochondria prepared from tumour-bearing mice. Malondialdehyde formation is negligible in intact ascites tumour mitochondria, but it is significantly increased in permeabilized ascites mitochondria and in isolated mitochondrial membranes. We suggest that the resistance against oxidative stress is a consequence of efficient protective mechanisms operating in these tumour mitochondria, and in this case the low level of polyunsaturated fatty acids cannot be the rate-limiting factor in lipid peroxidation. A study of the free radical scavenger system revealed that the GSH level was about 80% higher in ascites mitochondria than in liver mitochondria. The vitamin E content was 7-10 times higher in Ehrlich ascites cell than in liver mitochondrial membranes. The high vitamin E and glutathione levels might explain the increased resistance of tumour mitochondria against oxidative stress.

INTRODUCTION

The lipid peroxidation seems to be associated with many physiological and pathological processes. A number of tumour cell lines have a decreased susceptibility to oxidative stress in comparison with the corresponding normal tissues (1-3). A general hypothesis has been put forward that the increased rate of cell division is associated with a decreased rate of lipid peroxidation (4, 5). Earlier, it was shown that in the mitochondria of Ehrlich ascites cells (EAC) ferrous ions (6) do not induce a significant rate of malondialdehyde (MDA) formation. The decreased rate of lipid peroxidation in tumour tissues has been explained by the decreased availability of polyunsaturated fatty acids (PUFA-s) in the tumour membranes (5, 6). On the other hand, various defence systems have been described (especially in the liver and heart mitochondria) to maintain the structural and func-

tional integrity of the mitochondria under the circumstances of oxidative stress (7-10).

The following study was conducted to describe some characteristics of the free radical scavenger system in EAC mitochondria, in order to establish the factors responsible for the low rate of lipid peroxidation observed in EAC mitochondria. Liver mitochondria of the tumour-bearing mice were used for comparison.

MATERIALS AND METHODS

CD₁ male mice were used throughout the experiments. Liver and ascites mitochondria were prepared as described previously (11, 12). The mitochondrial inner membrane fraction was prepared from mitoplasts obtained by the digitonin method (13). Mitoplasts were either treated with Lubrol WX, 16 mg per mg mitochondrial protein, or disintegrated with an MSE 1000W sonic oscillator at 7 μ m amplitude. The inner membrane fraction was sedimented at 105,000xg in a Beckman L2-65 ultracentrifuge for 60 min, washed twice, and resuspended in the same buffer, containing 0.15 M KCl and 20 mM TRIS-HCl buffer, pH 7.2.

Lipid peroxidation protocols: ADP/Fe/NADPH - 2 mM ADP, 20 μ M Fe₂(SO₄)₃, 0.3 mM NADPH. ADP/Fe/ascorbate - 2 mM ADP 20 μ M Fe₂(SO₄)₃, 0.2 mM ascorbate. Cumene hydroperoxide - 0.5 mM cumene hydroperoxide. Incubation was performed in a 0.15 mM cumene hydroperoxide. Incubation was performed in a 0.15 M KCl, 20 mM TRIS-HCl buffer, at 37 °C, pH 7.2. Lipid peroxidation was estimated by measuring the rate of malondialdehyde formation (14).

The glutathione content of mitochondria was determined by the 5,5'-dithiobis(2-nitrobenzoic acid)-GSSG reductase recycling assay according to Griffith (15).

Mitochondrial samples were treated with 5% (v/v) 5-sulfosalicylic acid, and were then centrifuged in plastic vials in a Hitachi SCT 15B microfuge. The supernatant solutions were assayed. The sum of reduced and oxidized forms of glutathione was expressed in "GSH equivalents".

The glutathione reductase (EC 1.6.4.2) assay was based on the oxidation of NADPH in the presence of GSSG (16). The reaction rate was followed at 340 nm at 37 °C. Mitochondrial samples were solubilized with Triton X-100 at 0.3% (v/v) final concentration.

Glutathione peroxidase (EC 1.11.1.9) assay (17): the GSSG formed during the glutathione peroxidase reaction was instantly and continuously reduced by excess glutathione reductase, providing a constant level of GSH. The concomitant oxidation of NADPH was monitored photometrically at 340 nm, at 37 °C. The enzyme activity was calculated as described earlier (17). Mitochondrial samples were solubilized with Triton X-100 at 0.3% final concentration.

Vitamin E content was determined by an HPLC method, according to Thompson and Hatina (18). Briefly: after extraction and saponification, lipids were dissolved in hexane and injected into a silica gel column (OE-323 Labor MIM). DL- α -tocopherol was used as standard. Proteins were determined by the modified biuret method (19).

Chemicals: GSH reductase, t-butyl hydroperoxide and Lubrol WX were obtained from Sigma, St Louis; GSH and GSSG from Serva, Heidelberg; and DL- α -tocopherol from Merck, Darmstadt.

RESULTS

The lipid peroxidative capacity of the EAC mitochondria was measured. It was found that significantly less MDA was produced by the tumour cell mitochondria than by the liver mitochondria of the host animals. This effect was independent of the nature of the peroxidative stimuli (Fig.1). ADP/Fe(NADPH, ADP/Fe/ascorbate or cumene hydroperoxide elicited a similar response.

The MDA formation of the mitochondria is shown in Table 1, after permeabilization with various agents and treatments. It can be seen that the disruption of the mitochondrial integrity did not have a significant influence on the lipid peroxidative capacity of the liver mitochondria (Table 1/A). On the other hand, the increase in permeability of the EAC mitochondria led to an enhanced lipid peroxidation (Table 1/B). Treatment of the mitochondria with Lubrol WX resulted in the highest increase in MDA formation. It should be noted that Lubrol WX did not increase the intensity of basal (not stimulated) lipid peroxidation (data not shown).

Figure 2 demonstrates that the mitochondrial membranes are very sensitive to lipid peroxidation. The rate of lipid peroxidation in membranes isolated from the ascites mitochondria is 10 to 20 times higher than in intact mitochondria. In the case of the liver mitochondria, this effect is

Table 1. The effect of membrane permeabilization on ADP/Fe/NADPH-dependent lipid peroxidation of mitochondria^a

A) Liver mitochondria

	0 min	Time of incubation		
		10 min	20 min	30 min
		nmol malondialdehyde/mg protein		
Intact mitochondria	nd.	6.50	11.42	13.66
Freeze-thawing 1X	nd.	7.39	12.99	14.56
Freeze-thawing 5X	nd.	7.39	12.54	14.56
Sonication ^b	0.128	9.30	13.22	15.46
Lubrol WX 0.05% ^c	nd.	6.61	10.53	13.22

B) Ascites mitochondria

	0 min	Time of incubation		
		10 min	20 min	30 min
nmol malondialdehyde/mg protein				
Intact mitochondria	nd.	0.16	0.34	0.78
Freeze-thawing 1X	nd.	0.22	0.45	1.12
Freeze-thawing 5X	nd.	0.27	0.78	1.64
Sonication ^b	nd.	0.45	1.34	1.90
Triton X-100 0.1%	nd.	0.29	0.45	1.79
Lubrol WX 0.05% ^d	nd.	2.24	3.81	4.59

^a Mitochondria, 2 mg/ml protein, were incubated with 2.0 mM ADP, 20 μ M Fe₂(SO₄)₃ and 0.3 mM NADPH at 37 °C.

^b Mitochondria, 10 mg/ml protein, were sonicated for 20 sec x 3 with an MSE 100 W sonic oscillator, 7 μ m amplitude, at 0 °C, and were then diluted to 2 mg/ml protein concentration.

^c Detergents were added to the mitochondrial suspension (2 mg/ml protein) at 0 °C, 10 minutes before lipid peroxidation was started by adding ADP/Fe/NADPH.

^d It should be noted that Lubrol WX without ADP/Fe/NADPH was not able to induce lipid peroxidation.

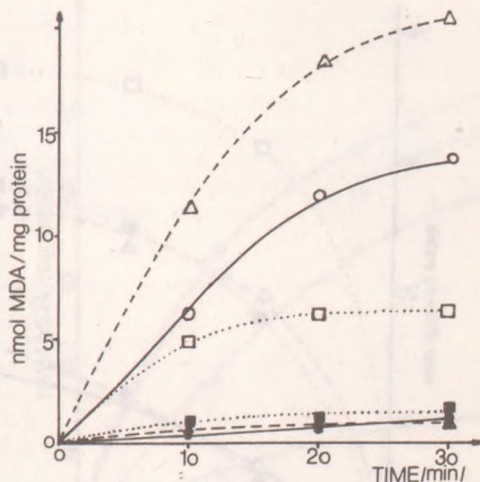


Fig. 1. Lipid peroxidation induced in liver and ascites cell mitochondria by different mechanisms.

Malondialdehyde (MDA) formation induced in liver mitochondria by (○—○) ADP/Fe/NADPH; (△---△) ADP/Fe/ascorbate; (□.....□) cumene hydroperoxide. MDA formation induced in Ehrlich ascites cell mitochondria by (●—●) ADP/Fe/NADPH; (▲---▲) ADP/Fe/ascorbate; (■.....■) cumene hydroperoxide. Incubation was performed as described in the Materials and Methods, using 2 mg/ml mitochondrial protein. Results are expressed as means of at least four experiments.

less pronounced. From these experiments, it is concluded that some parts of the protective mechanisms can be lost during the disruption of mitochondria.

To investigate the role of substrate oxidation in the protection of mitochondria against lipid peroxidation, the succinate-mediated inhibition of lipid peroxidation was also studied. Succinate inhibits lipid peroxidation both in intact mitochondria and in mitochondrial inner membranes (11). In order to inhibit lipid peroxidation, succinate must be oxidized in the respiratory chain. With teonyl-trifluoroacetone (TTFA), a specific inhibitor of succinate dehydrogenase (EC 1.3.99.1), an increased MDA formation was detected in the liver mitochondria, but lipid peroxidation was not induced in the ascites mitochondria (Fig. 3). It is also shown that TTFA was able to suspend the succinate-mediated lipid peroxidation inhibition in the liver mitochondria. The TTFA inhibition of succinate oxidase activity was higher than 92 per cent at 10^{-5} M concentration (data not shown). In isolated inner membranes of mouse liver mitochondria, the succinate inhibition of lipid peroxidation was about 50 per cent, but in the ascites mitochondrial membranes the inhibition was insignificant (data not shown).

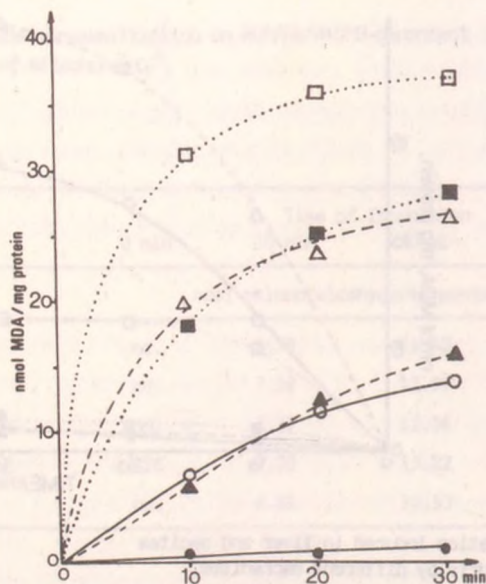


Fig. 2. Lipid peroxidation of mitochondrial inner membranes

Malondialdehyde (MDA) formation was induced in the inner membrane (1 mg/ml protein) of liver mitochondria by (Δ --- Δ) ADP/Fe/NADPH; ($\square$ $\square$) ADP/Fe/ascorbate, or in the inner membrane of Ehrlich ascites cell (EAC) mitochondria by ($\blacktriangle$ --- $\blacktriangle$) ADP/Fe/NADPH; ($\blacksquare$ $\blacksquare$) ADP/Fe/ascorbate. The ADP/Fe/NADPH-induced lipid peroxidations of intact liver ($\circ$ — $\circ$) and EAC mitochondria ($\bullet$ — $\bullet$) are shown for reference (2 mg/ml protein). Results are expressed as means of at least four experiments.

Table 2. Glutathione content and glutathione-dependent enzyme activities of liver and ascites mitochondria

GSH+GSSG was measured by the cycling reduction assay of Griffith (11,15). GSH reductase was followed by the NADPH consumption at 340 nm (12). Total GSH peroxidase (GSH-PX) activity (non-selenium and selenium-dependent GSH-PX) was determined by using t-butyl hydroperoxide as a substrate according to Flohe (13). Results are expressed as means $\pm$ S.E. (n = 5).

	GSH ^a (nmol/mg protein)	GSH peroxidase ^b (nmol/mg protein)	GSH reductase ^b (nmol/mg protein)
Liver	3.4 $\pm$ 1.6	28.5 $\pm$ 7.0	37.5 $\pm$ 4.9
Ascites	6.1 $\pm$ 2.2	10.0 $\pm$ 2.3	10.0 $\pm$ 2.3

^a Mitochondria were treated with 5% (v/v) sulfosalicylic acid, the precipitate was sedimented at 10,000 xg and the supernatant was assayed.

^b Mitochondria were solubilized with Triton X-100 at 0.3% (v/v) final concentration.

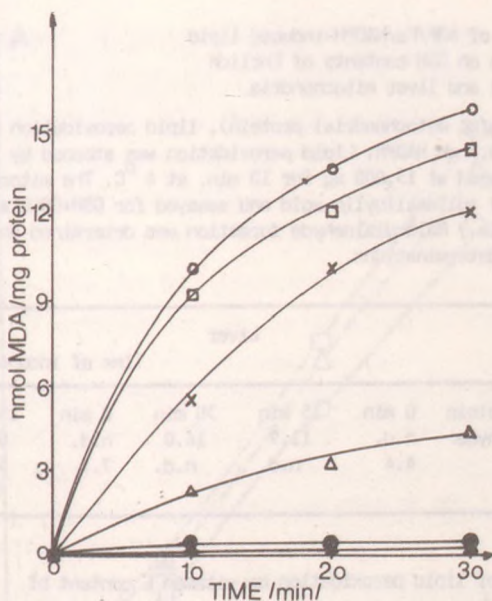


Fig. 3. The effects of succinate and tenoyl-trifluoroacetone (TTFA) on ADP/Fe/NADPH-induced lipid peroxidation of liver and EAC mitochondria

Malondialdehyde (MDA) formation was induced in liver mitochondria (X—X); liver mitochondria plus 5 mM succinate (Δ—Δ); liver mitochondria plus 10 μM TTFA (O—O); liver mitochondria plus 5 mM succinate plus 10 μM TTFA (□—□). MDA formation in EAC mitochondria (●—●); EAC mitochondria plus 10 μM TTFA (▲—▲). 2 mg/ml protein concentration was used. Results are expressed as means of at least four experiments.

To clarify whether there is a correlation between the GSH content of the mitochondria and the lipid peroxidation, the mitochondrial GSH level was measured during the course of lipid peroxidation. Table 3 shows that liver mitochondria incubated in the presence of ADP/Fe/NADPH induced increasing MDA formation and their glutathione content was rapidly lost. On the other hand, the ascites tumour mitochondria retained most of their glutathione and no lipid peroxidation was induced.

To answer the question of whether the loss of GSH could indeed increase the susceptibility of the mitochondria to lipid peroxidation, the effect of GSH on ADP/Fe/NADPH-induced lipid peroxidation in isolated mitochondrial membranes was studied. Figure 4 reveals that GSH administered in higher than 3 to 4 mM concentrations was able to decrease MDA production almost to zero level, both in EAC and in liver mitochondria. The 80 per cent difference between the GSH contents of the liver and ascites tumour mito-

Table 3. The effects of ADP/Fe/NADPH-induced lipid peroxidation on GSH contents of Ehrlich ascites cell and liver mitochondria.

In mitochondria (4 mg/ml mitochondrial protein), lipid peroxidation was induced by 2.0 mM ADP, 20 μ M Fe(SO₄)₃ and 0.3 mM NADPH. Lipid peroxidation was stopped by the addition of 2 mM EGTA. Samples were centrifuged at 15,000 xg for 10 min. at 4 °C. The mitochondrial pellet was re-suspended in 5% (v/v) sulfosalicylic acid and assayed for GSH+GSSG as described earlier (11). (n.d. = not detectable.) Malondialdehyde formation was determined on aliquots. Results are the means of triplicate determinations.

		Liver			Ascites		
		Time of incubation					
(nmol/mg protein	0 min	15 min	30 min	0 min	15 min	30 min	
malondialdehyde	n.d.	11.9	16.0	n.d.	0.25	0.7	
glutathione	4.4	n.d.	n.d.	7.7	5.5	5.2	
(GSH+GSSG)							

Table 4. The effect of lipid peroxidation on vitamin E content of mitochondria.

Lipid peroxidation was induced by ADP/Fe/NADPH as described in the Materials and Methods with 4 mg/ml mitochondrial protein. Without lipid peroxidation, the vitamin E content of EAC mitochondria decreased to 802 ng/mg protein after 30 min. of incubation at 37 °C, and the vitamin E content of liver mitochondria decreased to 79 ng/mg protein after 30 min. of incubation.

A) Ehrlich ascites cell mitochondria

	Time of incubation			
	0 min	15 min	30 min	60 min
Vitamin E	834	793	701	598
ng/mg protein				

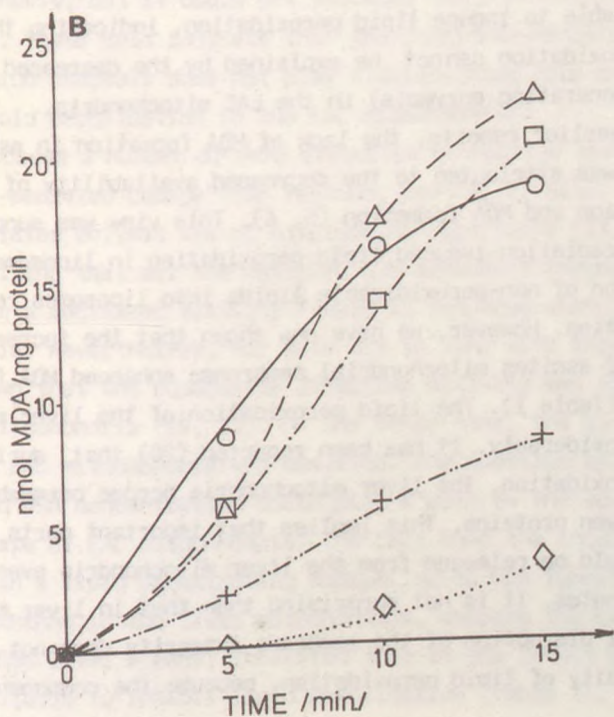
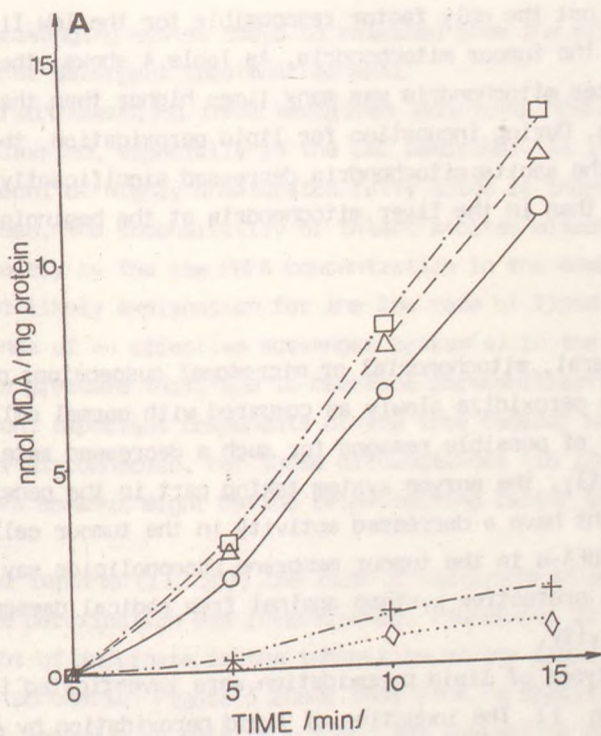
B) Liver mitochondria of tumour-bearing mice

	Time of incubation			
	0 min	10 min	20 min	30 min
Vitamin E	88	80	73	75
ng/mg protein				

Fig. 4. Lipid peroxidation of Ehrlich ascites cell (EAC) and liver inner mitochondrial membranes in the absence and presence of glutathione.

- A) EAC mitochondrial inner membrane
B) Liver mitochondrial inner membrane

Malondialdehyde formation without GSH (O—O); with 2 mM GSH (□—□); with 2 mM GSH (Δ—Δ); with 4 mM GSH (+—+); with 5 mM GSH (◇—◇). Lipid peroxidation was induced by ADP/Fe/NADPH in the presence of 1 mg/ml mitochondrial membrane protein as described in the Materials and Methods. Results are expressed as means of four experiments.



chondria is not the only factor responsible for the low lipid peroxidative activity in the tumour mitochondria. As Table 4 shows, the vitamin E content of the ascites mitochondria was many times higher than that of the liver mitochondria. During incubation for lipid peroxidation, the vitamin E content of the ascites mitochondria decreased significantly, but it was still much higher than in the liver mitochondria at the beginning of incubation.

DISCUSSION

In general, mitochondrial or microsomal suspensions prepared from cancer cells peroxidize slowly as compared with normal cells (4, 5). There are a number of possible reasons for such a decreased rate of lipid peroxidation. Firstly, the enzyme system taking part in the generation of free radicals might have a decreased activity in the tumour cells. Secondly, the content of PUFA-s in the tumour membrane phospholipids may be reduced. Thirdly, the protective systems against free radical damage may have increased activity.

Three types of lipid peroxidation were investigated in the EAC mitochondria (Fig. 1). The induction of lipid peroxidation by ADP/Fe/ascorbate and cumene hydroperoxide does not need enzymic components (20), and they were not able to induce lipid peroxidation, indicating that the lack of lipid peroxidation cannot be explained by the decreased activity of free radical-generating enzyme(s) in the EAC mitochondria.

In earlier reports, the lack of MDA formation in ascites tumour mitochondria was attributed to the decreased availability of PUFA-s for lipid peroxidation and MDA formation (5, 6). This view was supported by experiments on radiation-induced lipid peroxidation in liposomes (21). The incorporation of non-peroxidizable lipids into liposomes reduced the rate of MDA formation. However, we have now shown that the increase in the permeability of ascites mitochondrial membranes enhanced MDA formation from 2 to 10 times (Table 1). The lipid peroxidation of the liver mitochondria did not change considerably. It has been reported (20) that, during the course of lipid peroxidation, the liver mitochondria become permeable to large molecules, even proteins. This implies that important parts of the antioxidant system could be released from the liver mitochondria even after incubation for 10 minutes. It is not surprising then that in liver mitochondria the artificial disruption of the membrane integrity does not appreciably elevate the intensity of lipid peroxidation, because the components taking part in

the free radical scavenging system could be released from the mitochondria without sonication or detergent treatment as well.

When isolated mitochondrial inner membranes were used, the MDA formation was further elevated, especially in the EAC membranes. As it is unlikely that the amount of highly unsaturated fatty acids is increased during the isolation process, the insensitivity of intact ascites mitochondria cannot be explained solely by the low PUFA concentration in the membrane. Therefore, the most likely explanation for the low rate of lipid peroxidation is the existence of an effective scavenger system(s) in the ascites mitochondria. It is presumed that, due to membrane permeabilization or membrane preparation, important components of the free radical scavenger system were lost or disconnected. For these circumstances (in isolated membranes), the PUFA content might be the rate-limiting factor in lipid peroxidation.

In our earlier reports (11, 20), the role of succinate in preventing mitochondrial lipid peroxidation was investigated. Therefore, we studied the possible involvement of succinate in the inhibition of the lipid peroxidation of the EAC mitochondria. Figure 3 shows that TTFA, a specific inhibitor of succinate dehydrogenase, was able to suspend the inhibition of succinate in the liver mitochondria, but it could not increase MDA formation in the ascites mitochondria. These data indicate that the succinate-mediated lipid peroxidation inhibition probably does not play a determining role in the modulation of the lipid peroxidation in the EAC mitochondria.

Mitochondria possess a number of very effective protective systems against free radical-mediated damage (for reviews, see (8, 9, 22)). Among them, GSH and GSH-related enzymes are of outstanding importance (23). It seems somewhat surprising that all the measured GSH-dependent enzymes in the EAC mitochondria have a decreased activity (Table 2) compared with that in the liver mitochondria. Nevertheless, our data are in line with those of others, who have shown that the superoxide dismutase activity was also decreased in the EAC mitochondria (24, 25). On the other hand, the glutathione concentration in the EAC mitochondria was elevated. The question arose of whether the increased GSH concentration could play a role in the very low lipid peroxidation rate of EAC mitochondria. The fact that the incubation of the mitochondria in a lipid peroxidating medium led to MDA formation and to the loss of glutathione in the liver mitochondria, whereas the EAC mitochondria kept their GSH level steady, indicated that in the intact EAC mitochondria GSH was available to inhibit lipid peroxidation (Table 3).

The difference in lipid peroxidative capacity of liver and ascites cell inner membranes (where a glutathione-dependent protective mechanism may not operate without externally added GSH) was much less pronounced than in intact mitochondria. To prove the role of GSH in regulating the lipid peroxidation in the ascites mitochondria, glutathione in various concentrations was incubated with isolated mitochondrial membranes. The striking effect of GSH above 3 mM concentration suggests that there might be a threshold level of intramitochondrial GSH, above which the reduced form of thiol is very effective antioxidant.

From these results, we conclude that a glutathione-dependent protective mechanism should be at least partially responsible for the reduced rate of lipid peroxidation in EAC mitochondria. Moreover, it is suggested that other factors, e.g. vitamin E, or the decreased PUFA content of the membranes, may contribute to the high resistance of EAC mitochondrial membranes to peroxidative stimuli. Indeed, it was found that the vitamin E content of the tumour mitochondria was 5-7 times higher than that of the liver mitochondria (Table 4). Vitamin E can act as a chain terminator, interrupting the chain reaction of lipid peroxidation. In this step, vitamin E is itself oxidized to a tocopheryl radical, which can be reduced back to α -tocopherol by glutathione and/or ascorbate (26, 27). Thus, a positive feed-back loop of the antioxidant defence could operate in tumour mitochondrial membranes.

The data presented here might have importance as concerns an understanding of the high resistance of tumour cells against oxidative stress.

ACKNOWLEDGEMENT

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THE EFFECTS OF DINH LANG ROOT EXTRACT ON THE ACTIVITIES OF PEROXIDE METABOLISM ENZYMES IN RATS

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SUMMARY

A Dinh lang root extract (DLRE) was found to enhance the superoxide dismutase (SOD) activity in the rat striatum and cerebellum, but it failed to change the activities of glutathione peroxidase (GP) or catalase (C). DLRE increased the activities of SOD, GP and C in the rat liver.

Ginseng (*Panax ginseng* C.A. Meyer) is probably the best known medicinal plant, the root of which has been used as a restorative drug, a sedative, a psychic energizer and an agent to counter senile changes and to prolong vital force. An abrupt reduction in the natural supply of ginseng, as well as the difficulty in its cultivation, stimulated the study of other species of the Araliaceae family in an effort to find plants acting similarly to ginseng. Dinh lang (*Policias fruticosum* L.), a new medicinal plant in Vietnam, belongs in this family. Dinh lang is a small tree which has been cultivated as an ornamental plant because of its beauty. 20 to 25 cm long pieces of the stems are planted in spring or summer, and after 3 or 4 years of growing the roots are used for medicinal preparations. A Dinh lang root extract (DLRE) was found to have a pharmacological spectrum similar to that of ginseng.

Experimental and clinical studies have shown a wide range of pronounced stimulatory effects of DLRE. It increased mental and physical performances (Ngo Ung Long, 1964; Tran Ty Yen et al., 1973). Using the swimming test in mice, we found that DLRE increased performance significantly both before and after physical exhaustion. Young mice and rats treated with DLRE increased their body weight without consuming more food and water (Ngo Ung Long et al., 1973; Tran Ty Yen et al., 1973) and also showed a higher

potential to adapt themselves to unfavourable conditions (e.g. high temperature, long-term immobility, etc.) (Tran Ty Yen et al., 1984).

The present study is devoted to acquiring a better understanding of the action of DLRE. The experiment involves measurements of the activities of the peroxide metabolism enzymes in rat brain regions and rat liver.

MATERIAL AND METHODS

Preparation of DLRE and treatment of the rats

The extractum siccum of Dinh lang was made by evaporating the aqueous-alcoholic extract of the root of Dinh lang. 50 mg extractum siccum corresponded to 1 g of the dry root of this plant. The preparation was dissolved in water and injected subcutaneously (20 mg/kg body weight) once a day for 21 days. Fresh solutions were prepared daily.

Animals and preparation for biochemical analyses

Adult Wistar rats of both sexes weighing 200-250 g were used. They were housed in groups of 5 rats and kept under 12 hr light - 12 hr dark cycles in a well-ventilated animal room. Food and water were supplied ad libitum. Before the start of the treatment with DLRE, a one-week adaptation period was allowed. 24 hr after the last injection of DLRE, the rats were killed by decapitation. The striatum, cerebellum and liver were removed immediately, weighed and stored at -18°C before chemical analysis. The liver was washed free of blood with chilled saline and then dried with filter paper. Each rat brain region and the liver were homogenized in chilled saline solution (ratio of 1:10 to the wet tissue) in a Potter glass homogenizer on ice-cooling water.

Measurement of enzyme activities

Enzyme activities and protein contents were determined in the supernatants following centrifugation of the homogenates. Protein content was measured by the method of Lowry et al. (1951). Total superoxide dismutase (SOD) activity was estimated by its inhibition of the adrenaline-adrenochrome transformation, as described by Misra and Fridovich (1972) and modified by Matkovics et al. (1977). Catalase (C) activity was determined by the

spectrophotometric method of Beers and Sizer (1952). This enzyme activity was expressed in Bergmeyer units (BU) per mg protein. Glutathione peroxidase (GP) was measured by the method of Matkovics et al. (1988).

Data analysis

Student's t test was used to establish the significance of differences between groups for enzyme activities. A degree of certainty greater than 95% ($p < 0.05$) was considered significant.

RESULTS AND DISCUSSION

DLRE in the 20 mg/kg daily dosage for 21 days significantly increased the total SOD activity in the rat striatum and cerebellum of both sexes. However, it failed to change the GP and C activities as compared to the control values (Tables 1 and 2). In the rat liver, DLRE increased the activities of SOD, GP and C in both female and male rats (Table 3).

Table 1. The effects of long-term treatment with DLRE on the activities of SOD, GP and C in the rat striatum

Treatment	SOD U/mg protein	GP U/mg protein $\times 10^{-3}$	C BU/mg protein $\times 10^{-5}$
Saline, females (n = 6)	16.91 $\pm$ 0.91	29.67 $\pm$ 0.33	12.30 $\pm$ 0.70
Saline, males (n = 5)	11.20 $\pm$ 0.53	39.00 $\pm$ 2.03	23.00 $\pm$ 0.54
DLRE, females (n = 6)	26.43 $\pm$ 1.15 *	33.04 $\pm$ 0.88	17.90 $\pm$ 0.38
DLRE, males (n = 6)	21.25 $\pm$ 0.78 *	30.50 $\pm$ 0.56	13.20 $\pm$ 0.54

*P < 0.05

SOD provides a defense against oxidative damage of the tissue by inactivating superoxide radicals (Fridovich, 1979). The role of this enzyme in the central nervous system is even more important, due to its presence in a considerably high concentration in the synaptosomes, which are also rich in readily oxidizable catecholamines (Misra and Fridovich, 1972).

Dinh lang was earlier found to increase the behavioral performances in animals and men on chronic administration (Tran Ty Yen et al., 1985;

Table 2. The changes in SOD, GP and C activities in the rat cerebellum in response to DLRE

Treatment	SOD U/mg protein	GP U/mg protein $\times 10^{-3}$	C BU/mg protein $\times 10^{-5}$
Saline, females (n = 6)	11.16 $\pm$ 0.32	24.00 $\pm$ 0.28	20.60 $\pm$ 0.40
Saline, males (n = 5)	15.31 $\pm$ 0.69	32.60 $\pm$ 1.03	23.30 $\pm$ 1.20
DLRE, females (n = 6)	30.60 $\pm$ 0.80 **	37.30 $\pm$ 1.05	30.60 $\pm$ 0.80
DLRE, males (n = 6)	34.00 $\pm$ 0.36 *	41.00 $\pm$ 0.86	33.10 $\pm$ 0.86

*P < 0.05 **P < 0.01

Table 3. The effects of DLRE on the SOD, GP and C activities in the rat liver

Treatment	SOD U/mg protein	GP U/mg protein $\times 10^{-3}$	C BU/mg protein $\times 10^{-5}$
Saline, females (n = 6)	48.30 $\pm$ 1.27	28.45 $\pm$ 0.82	16.40 $\pm$ 0.37
Saline, males (n = 5)	56.15 $\pm$ 1.89	27.40 $\pm$ 0.57	17.24 $\pm$ 0.21
DLRE, females (n = 6)	74.25 $\pm$ 2.03 **	48.14 $\pm$ 0.88 *	31.30 $\pm$ 0.48 *
DLRE, males (n = 6)	82.16 $\pm$ 2.14 *	53.29 $\pm$ 0.96 *	32.20 $\pm$ 0.26 *

*P < 0.05 **P < 0.01

Tran Ty Yen et al., 1986). Recently, it was also found to increase the sexual activity and long-term memory in aged male rats and mice (Tran Ty Yen, 1988, data to be published). These behavioral changes were related to the changes in catecholamines levels in the brain (Gessa et al., 1975; Introini-Collison et al., 1986). The effects of DLRE might also be somehow related to the brain catecholaminergic system, but as yet we have no direct evidence concerning this working hypothesis.

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INTERCONNECTIONS BETWEEN THE STRUCTURE AND CHARGE
OF CRYSTALS AND LIPID PEROXIDATIVE EFFECTS
IN THE CELL-DAMAGING INFLUENCE OF QUARTZ

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INTRODUCTION

Our work in recent years led to the hypothesis that lipid peroxidative mechanisms play significant roles in the pathomechanism of silicosis, in the damage caused by SiO_2 -containing dusts to the lipoprotein membrane structure of lung tissue (1, 2).

In contrast, little is known as to how the silicogenic effect is interconnected with the structural characteristics of quartz. King dealt with this problem (3, 4), establishing that long-term exposure (120 days) resulted in no differences in silicogenic effect between the right-handed and left-handed forms of quartz.

Zaidi, in his review (5), considered that the following characteristics are responsible for the silicogenic effect:

1. the piezoelectric features of different dusts,
2. the negative surface charges of quartz,
3. the adsorptive surface capacity of quartz,
4. the crystal structure of quartz. He distinguished between the pathogenic influences of different allotropic characteristics (crystalline hydrated, amorphous, etc.).

Langer (6) reviewed the possible mechanisms of pneumoconiosis caused by quartz with following main conclusions:

1. fibrosis of the lung is elicited by "free quartz"; other important features include

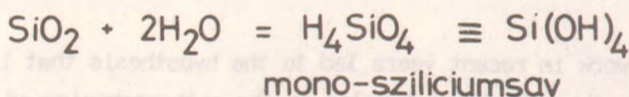
2. the negative surface charge of quartz, which is interdependent with the physico-chemical characteristics, with the geochemical and mineralogical origin of the quartz, and with other forms emerging owing to the previously mentioned circumstances,

3. differences in the biological effects of the right-handed and left-handed crystal forms, and

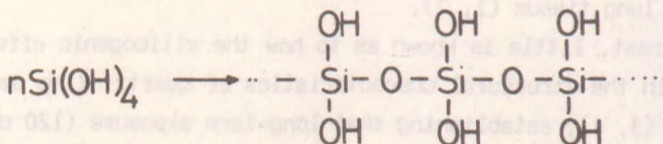
4. the influence of the magnitude of the surface and the size of the particles on the fibrosis. It should also be stressed that

5. exposure of the lung to quartz can cause the secondary exposure of other organs: the liver, kidney and spleen. This latter problem has attracted our attention.

Recently, we have analysed the role of negative surface charge. What is the mechanism by which quartz is transformed into a polymerer molecule with negative surface charge after solvation in water?



Polimerizáció:



Disszociáció:

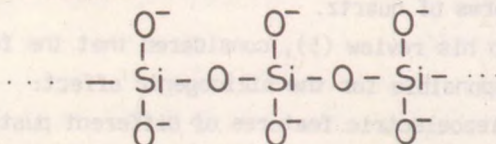


Fig. 1. Reaction sequence of product formation from intermediates, negative surface charge

As seen in Fig. 1, following the solvation of SiO_2 , the product is practically $\text{Si}(\text{OH})_4$, because it is an amphoteric molecule. Following polymerization and H^+ -disjunction in the aqueous environment, a polysilicic acid is formed, possessing negative charge, which is known in the literature as the silanol ion (7, 8). We were interested in how this process influences the lipid peroxidative effect in the lung, and whether this effect can be decreased or not.

On the basis of our experiments (2) and some data in the literature, it seemed logical to make our model investigations on the 6th day after exposure.

MATERIALS AND METHODS

From each material used, dusts with identical particle diameter were made by grinding. Under an electron microscope, the diameters of these particles ranged between 1 and 2 μm . The materials used are listed in Table 1.

Table 1.

1. Carbon (graphite of 99.999% purity)	8. DQ-12 (Essen, BRD)
2. BaTiO_3	9. Left-handed quartz (Chemical University, Institute of Mineralogy, Veszprém)
3. Corundum (Al_2O_3)	
4. Borsod coal	10. Right-handed quartz (origin as for 9)
5. Amorphous quartz (SiO_2 of 99.999% purity, PIERCE, The Netherlands synthetic)	11. DQ-12 + toluidine blue
6. Sahara sand	12. Left-handed quartz + toluidine blue
7. Si(OH)_4 , MERCK	13. DQ-12 + solution in which the lungs were washed

The dust was suspended in physiological NaCl solution, and administered intratracheally under ether narcosis in a dose of 50 mg/animal/0.5 ml. After 6 days, the rats (Wistar albino, 160-180 g body weight) were killed, and their lungs were removed and homogenized in physiological saline (1:4) with an Ultra-Turrax homogenizer. After sequential processing of the homogenate (1, 2), malondialdehyde (MDA) was measured with a spectrophotometer (Spektronom 195 + Digimom complex) at 532-600 nm.

Additional investigations were made:

1. The components of dust patterns were subjected to X-ray analysis with a microsearcher. The instrument was an ORTEC energy-dispersional micro-analyzer.

2. The differences between the crystalline and amorphous variants of quartz were analyzed with polarization optics.

3. In the case of crystalline or amorphous variants, electron diffraction analysis was made. From the ground particles, following ultrasound treatment in NaCl suspension, aliquots were applied to grids with "Formvar"

membranes. The SiO_2 particles which were then crushed by chance became analysable by electron diffraction methods. The analysis was performed under a JEOL 100 C electronmicroscope.

4. In order to neutralize the negative surface charge of the quartz, on the basis of data in the literature (10-12), the patterns were coated with materials possessing different positive charges. Two materials were selected: DQ-12, which is accepted as an international standard and which contains 80% of free SiO_2 (Essen, BRD), and the practically pure SiO_2 , containing right-handed crystalline quartz.

As coating substances, toluidine blue (11) and the supernatant of the 40,000 g fraction of the washing solution of the lung (12) were employed, the original methods being slightly modified. Both coating substances have positively charged groups which are bound practically irremovably to the surface of quartz.

RESULTS AND DISCUSSION

Here it should be emphasized that we utilize the complete lung. We should like also to stress that the examinations were made in the early phase of exposure, in order to shed light on the mechanisms of silicytic alterations.

First we present the results of the additional investigations, since the evaluation of these data might provide an explanation of the peroxidative effects in vivo.

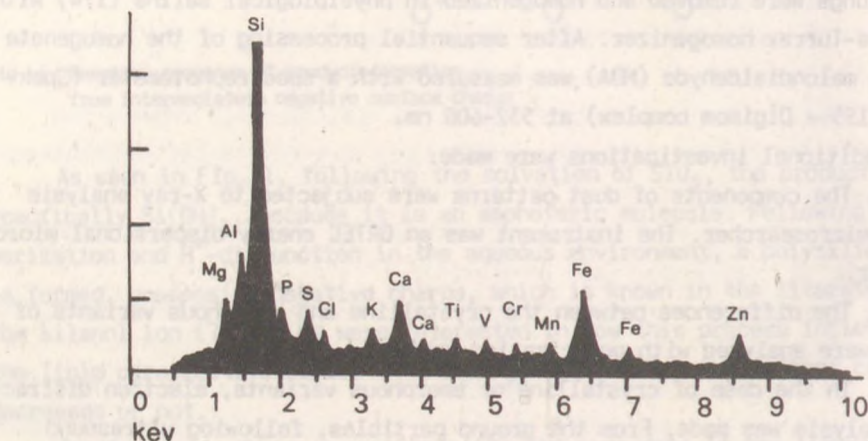


Fig. 2. Spectrum of international standard DQ-12, obtained by microsearcher analysis. Mean of 30 analyses.

1. DQ-12 and the practically pure quartz were analyzed with the microsearcher.

Robock earlier (13) analyzed this DQ-12 pattern by IR spectrophotometry, flame photometry and X-ray fluorescent techniques. The Figure reveals that DQ-12 contains mostly free quartz (with only a slight proportion of silicate dust), and practically each of the notable elements.

Figure 3 illustrates the microsearcher analysis of Borsod coal.

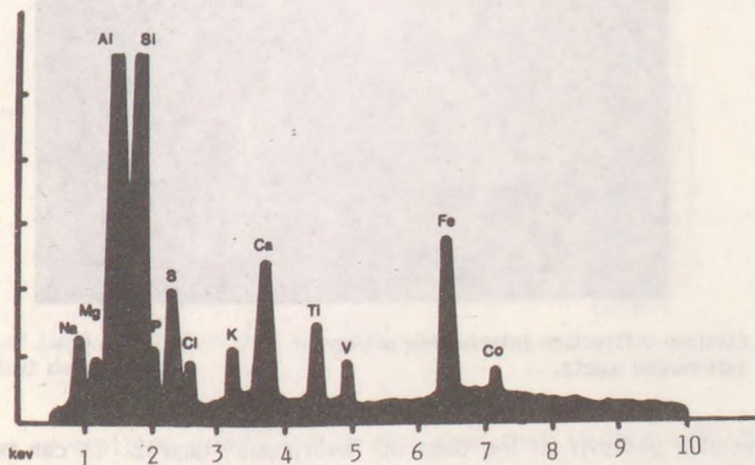


Fig. 3. Spectrum of Borsod coal pattern obtained by microsearcher analysis. Mean of 30 analyses.

It is known that in some Hungarian mines (Tatabánya, Borsod, etc.), silicosis is an unknown illness. It follows from Fig. 3 that in these mines the quartz exists practically only in the form of silicate. The free quartz content, as compared with DQ-12, is negligible.

2. Microsearcher analysis of other materials containing practically only SiO_2 (amorphous quartz, racemic left-or right-handed quartz) gave only a Si peak, without any sign of impurities, so this is not demonstrated. The limit of error was 20 ppm.

For the analysis of the crystalline or non-crystalline character of the materials (left-handed quartz, DQ-12, amorphous quartz, $\text{Si}(\text{OH})_4$), electron diffraction examinations were performed.

In Fig. 4, it is seen that the interference pattern of the retroform projection in the case of left-handed quartz shows exact identity with the picture obtained in the case of DQ-12. We did not succeed in observing the

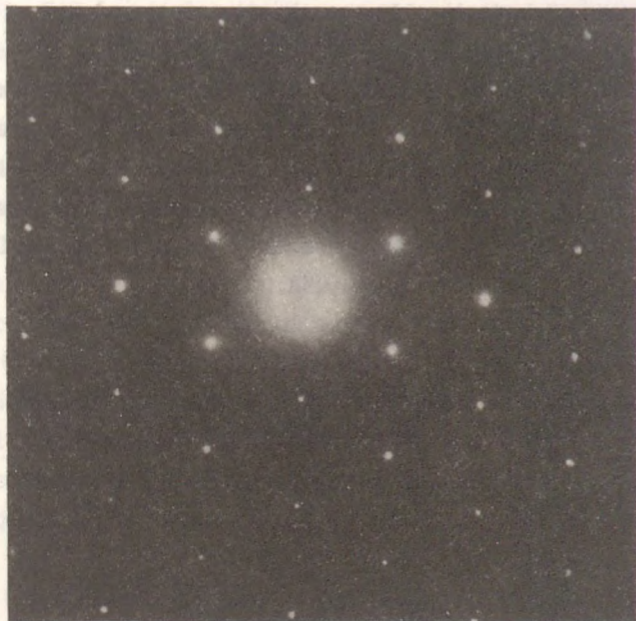


Fig. 4. Electron diffraction interference pattern of left-handed quartz.

interference pattern in the case of "amorphous" quartz. It can be excluded that microcrystalline particles might exist in this case too, but below the sensitivity limit of our method. For Si(OH)_4 the situation was the same.

3. Analysis with polarization optics demonstrated that the materials with amorphous structures are isotropic, or in other words optically inactive ones, whilst the crystalline quartz is anisotropic. However, these examinations are only of a qualitative character.

The results concerning the lipid peroxidation processes running parallel with the cell damage in vivo are demonstrated in Table 2 and Fig. 5.

The histogram is divided into three groups. The first group (carbon, BaTiO_3 , corundum) cause practically insignificant effects, so they cannot be treated as silicogenic.

The second group contains materials 9-11, which have a highly expressed lipid peroxidative influence. They are all crystalline materials containing free quartz.

The third group contains those materials which were treated with some kind of coating agents in order to decrease the cell-damaging effect. It is seen that this was successful.

From these results, it follows that the lipid peroxidative damage was

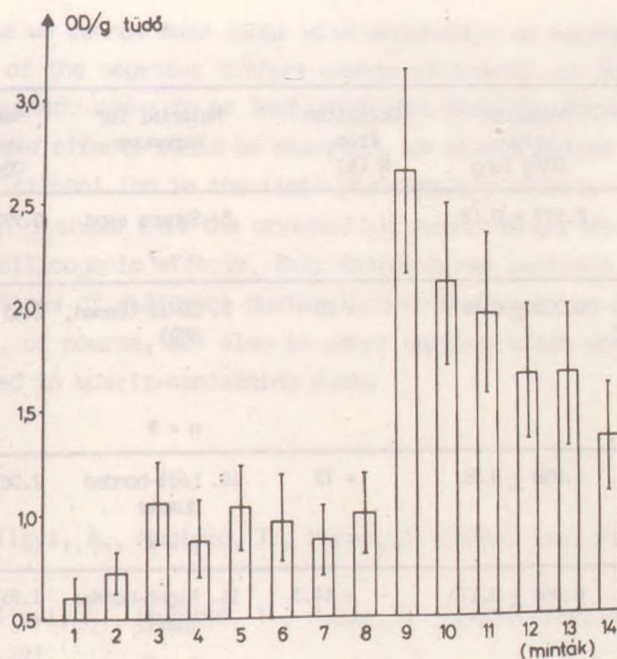


Fig. 5. Values of lipid peroxidation (MDA), in units of optical density/g lung.

caused by the crystalline quartz. It is also seen that in this type of influence there is no difference between the left-and right-handed variants. The highest effect was elicited by the DQ-12 standard dust. Though apparently less effective, there are no significant differences between the two kinds of crystalline quartz and DQ-12.

It is worth mentioning that the natural lavage agents inhibit more intensively than toluidine blue.

Certain literature data verify the inhibition of the surface charges of quartz by different natural substances. For example (14, 15), serum proteins absorb on the surface of quartz, thereby decreasing its haemolytic activity. One very interesting work (16) reported the chemical compositions of lavage solvents after washing out of the lung. The main components of these solvents are proteins and phospholipids. Since both these groups of molecules have positive charges, they are able to react with the negative charges of the silanol ions, thereby reducing the charges and activities of the silanol ions. This process might result in decreases of the peroxidative influence and cell-damaging effect.

Though the measure of lipid peroxidation decreased substantially, complete elimination was not observed, and membrane damage still occurs.

Table 2.

Material for exposure	Measured value OD/g lung	Deviation from N (%)	Material for exposure	Measured value OD/g lung	Deviation from N (%)
1. Normal (N) n = 11	0.597 $\pm$ 0.12	-	8. Sahara sand n = 7	0.992 $\pm$ 0.198	+ 65.3
2. Carbon (graphite of 99.999% purity) n = 7	0.720 $\pm$ 0.14	+ 20	9. DQ-12 (Essen, BRD) n = 9	2.65 $\pm$ 0.53	+341.6
3. BaTiO ₃ n = 7	1.050 $\pm$ 0.21	+ 75	10. Left-handed quartz n = 6	2.089 $\pm$ 0.417	+248.2
4. Corundum (Al ₂ O ₃) n = 8	0.866 $\pm$ 0.173	+ 44.3	11. Right-handed quartz n = 6	1.955 $\pm$ 0.39	+225.8
5. Borsod coal n = 4	1.032 $\pm$ 0.206	+ 72.0	12. DQ-12 + tolui- dine blue n = 6	1.667 $\pm$ 0.33	+177.8
6. Amorphous quartz (SiO ₂ of 99.999% purity, PIERCE n = 7	0.954 $\pm$ 0.19	+ 59.0	13. Left-handed quartz + toluidine blue n = 8	1.671 $\pm$ 0.33	+178.5
7. Si(OH) ₄ MERCK n = 6	0.872 $\pm$ 0.174	+ 45.3	14. DQ-12 + lavage n = 10	1.358 $\pm$ 0.271	+126.3

To summarize the results:

1. Crystalline quartz is responsible for the lipid peroxidative effect of SiO₂. Oxygen free radical intermediates, formed via a polyenzymatic process, presumably play the determinant role in the mechanism. We assumed this earlier. In our opinion, cell processes are responsible for this effect. This means, that the "natural killer" mechanism is not the only factor for which it is known that membranal NADPH oxidase forms oxygen free radicals by macrophages. To prove this mechanism in vivo is very difficult,

of course, because we cannot make lungs with absolutely no macrophages.

2. The role of the negative surface charge of quartz, at least in the cases we have examined, seems to be less important than the former mechanism. Although some effects could be observed, we cannot define the proportion played by silanol ion in the lipid peroxidative effect.

3. Since Fig. 5 shows that the crystalline quartz plays the determinant role in the silicogenic effects, this method seems suitable for toxicological examinations of different dusts. This method could be employed not only in mines, of course, but also in other working sites where the workers are exposed to quartz-containing dusts.

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INCREASED SENSITIVITY OF ERYTHROCYTES TO OXIDANT STRESS IN CHILDREN WITH CELIAC DISEASE

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SUMMARY

The erythrocyte glutathione (GSH) content in celiac children was elevated and the glutathione disulfide (GSSG) level was significantly decreased compared to the values in normal controls. The erythrocyte GSSG level and GSSG/GSH ratio in celiac children also differed from those in children with iron deficiency. In vitro acetylphenylhydrazine treatment proved the impaired glutathione stability of the erythrocytes in celiac disease and cow's milk allergy. Parallel rises in the methemoglobin and hemichrome levels of the blood cells were seen. The selenium content of the red blood cells of celiac patients was decreased. All alterations of the erythrocytes tended to normalize during the dietetic period. These data suggest a reduced protective capacity of the erythrocytes against oxidative stress in celiac disease and in cow's milk allergy in childhood.

INTRODUCTION

Gluten-induced abnormalities have been described in the small intestinal structure in celiac disease (CD), with an increased number, a higher proliferative activity and an altered distribution of the inflammatory cells of the mucosa. These cells are known to induce free radical production. Even villous atrophy, the most characteristic sign of the disease, can be assumed to be a result of these highly damaging compounds, although they may also have systemic effects.

The glutathione redox system is a sensitive indicator of in vivo oxidative processes /3/. In the present study on the presumed role of oxygen free radicals in the pathology of CD, examinations were performed on erythrocytes. The reduced (GSH) and oxidized (GSSG) glutathione contents and the oxidative derivatives of hemoglobin (methemoglobin and hemichrome) were measured to determine the sensitivity of the red blood cells to

oxidants. In another series of patients the selenium content of the erythrocytes was also investigated.

MATERIAL AND METHODS

11 children with CD on gluten challenge, 11 with CD in remission, 5 patients with cow's milk protein intolerance and 26 healthy children before minor surgical intervention were studied. As an iron deficiency influences the erythrocyte glutathione level, a second control group consisted of 11 children with nutritive iron deficiency.

The blood concentrations of GSH and GSSG were determined by a sensitive method, using glutathione reductase /1, 6/. The stability of GSH was measured by the method of Beutler /2/, with calculation of the ratio of GSH remaining after in vitro incubation of the whole blood in the presence of 5 mg/ml acetylphenylhydrazine for 1 hour.

RESULTS AND DISCUSSION

The GSH levels of the erythrocytes in the control and CD patients are presented in Fig. 1/A. The GSH content was higher in the red cells of CD patients and nutritive iron deficiency cases than in the healthy control group.

The red cell oxidized glutathione proved to be elevated in the nutritive iron deficiency group compared to the normal controls, while patients with CD exhibited markedly lower values (Fig. 1/B). The erythrocyte GSSG/GSH ratio in children with CD was also significantly decreased compared to the control groups.

As an iron deficiency is a customary supporting symptom of acute CD, the difference in GSSG values between the CD group and the nutritive iron deficiency group emphasizes the role of factors other than merely an iron deficiency.

Acute oxidant stress induces a decrease in the GSH content in the red blood cells and increases the GSSG level. If the oxidant stress is long-lasting, however, the compensation process results in an increase of GSH. This phenomenon was observed under laboratory conditions by administering methylene blue to rabbits /4/. The elevated erythrocyte GSH and the decreased GSSG level of patients with CD on gluten challenge can therefore be regarded as a consequence of permanent oxidant stress.

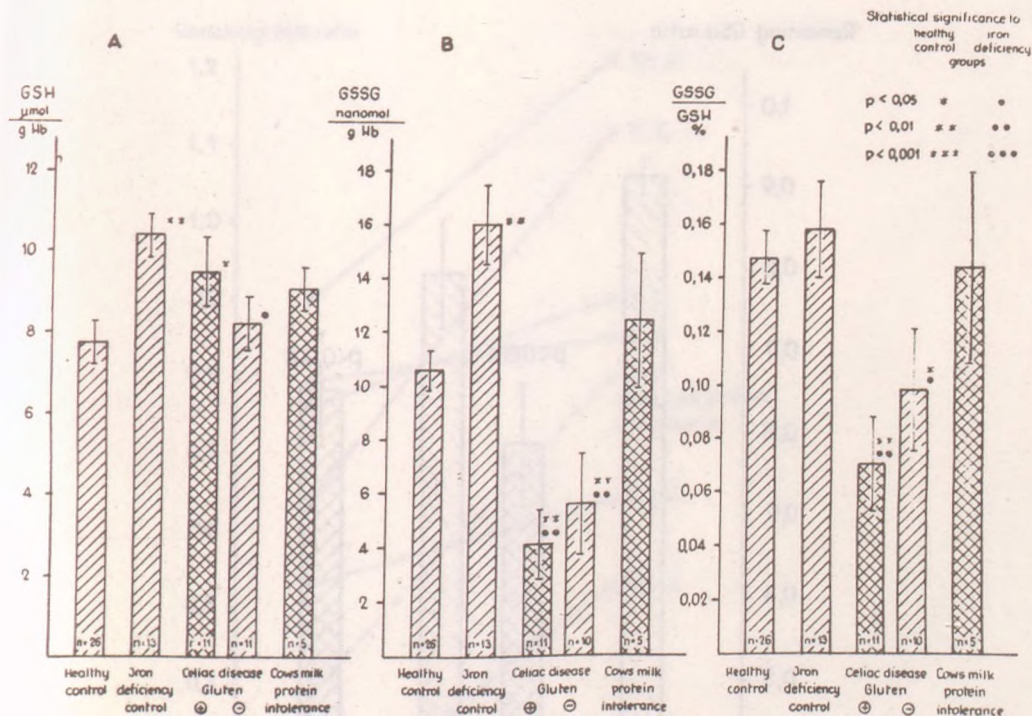


Fig. 1 Reduced and oxidized glutathione contents of erythrocytes, and their molar ratio ($\bar{x} \pm SE$)

The impaired defense of the erythrocytes against oxidative stress in CD and cow's milk allergy was proved by the *in vitro* acetylphenylhydrazine loading test of the blood, which resulted in a definite decrease of the erythrocyte GSH level as compared to the controls (Fig. 2), and an improvement during the diet (Fig. 3).

During gluten and milk challenge, the presence of unstable methemoglobin and hemichrome, measured by a sensitive method, is further evidence of a decreased erythrocyte defensive capacity against oxidative stress (Fig. 4).

The definite decrease in the erythrocyte GSSG/GSH ratio in CD patients on gluten challenge points to the decreased activity of glutathioneperoxidase, which contains selenium (Fig. 1/C).

The selenium level of the blood cells of CD patients on gluten challenge was found to be 51% lower than that in the control group: 228 ± 158 ng/g HB and 465 ± 136 ng/g Hb, respectively ($p < 0.001$) (Fig. 5). The selenium

Remaining GSH ratio

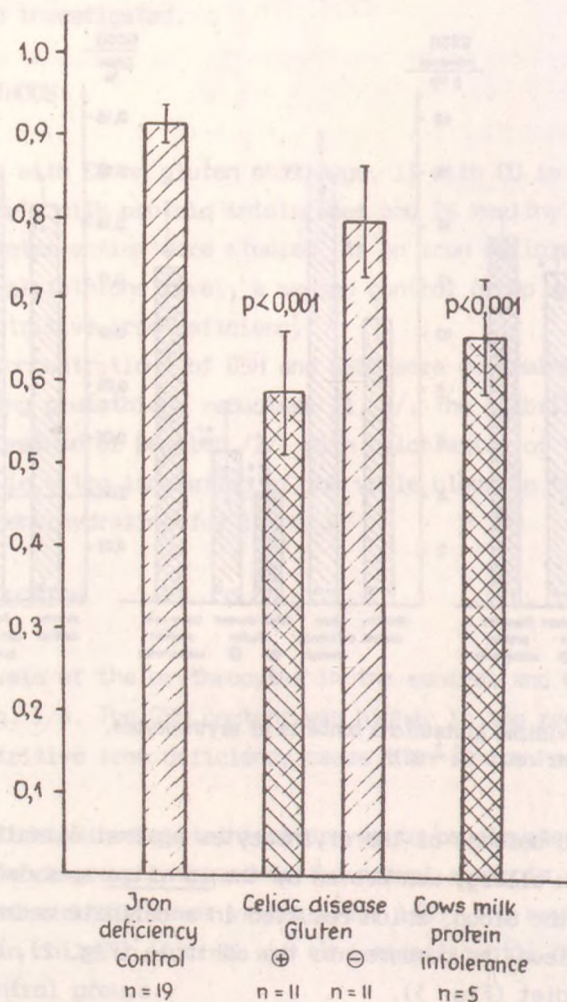


Fig. 2 Change induced in glutathione stability of red blood cells by acetylphenylhydrazine ($\bar{x} \pm SE$)

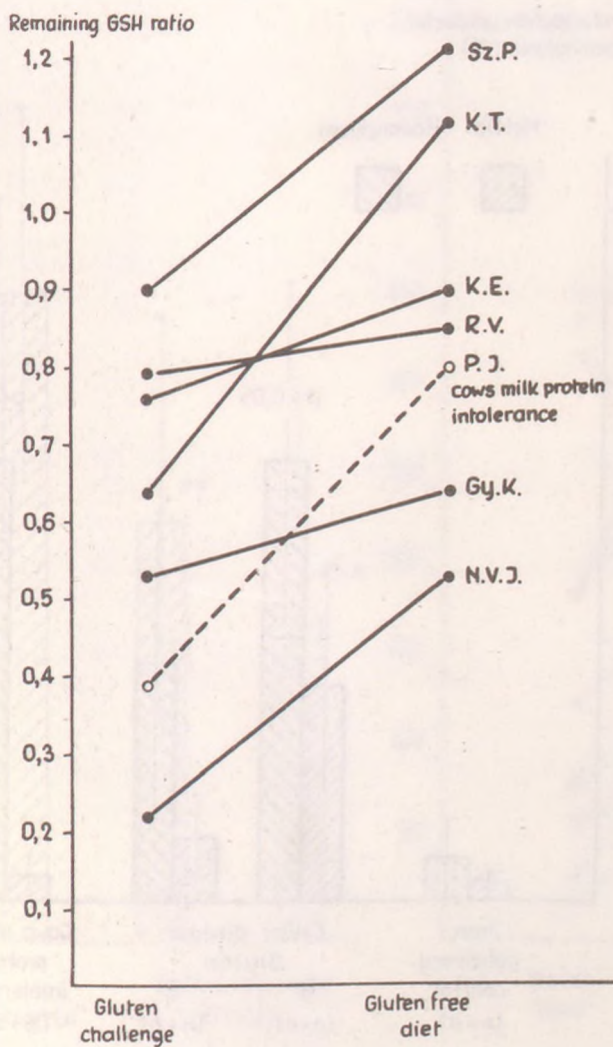


Fig. 3 Change in glutathione stability of red blood cells in celiac patients on gluten-free diet

Ratio of oxidation products
of haemoglobin (%)

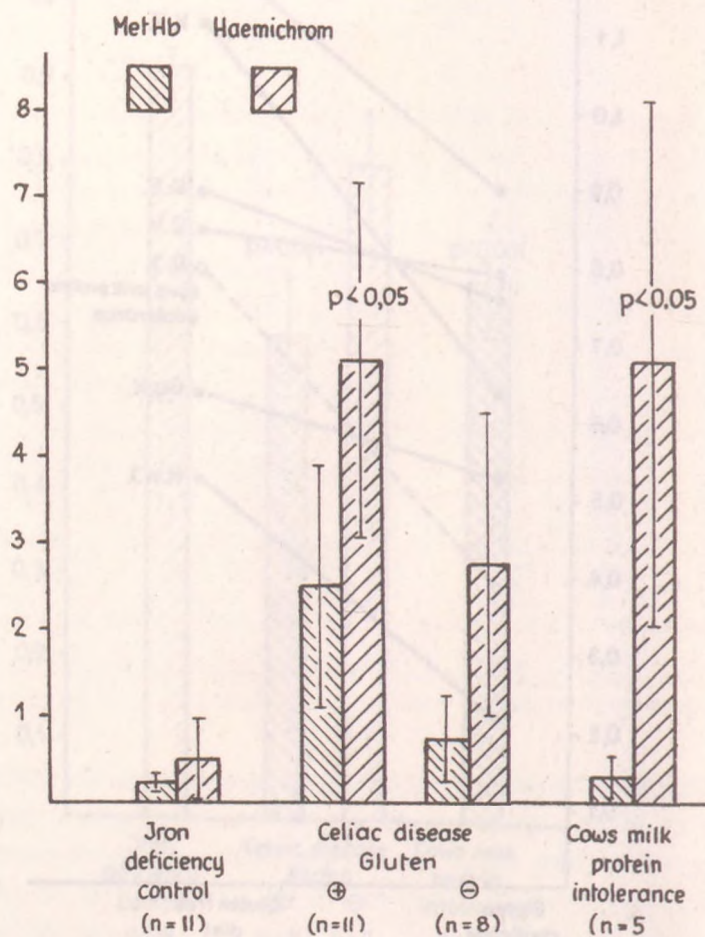


Fig. 4 Ratio of oxidation products of haemoglobin
($\bar{x} \pm SE$)

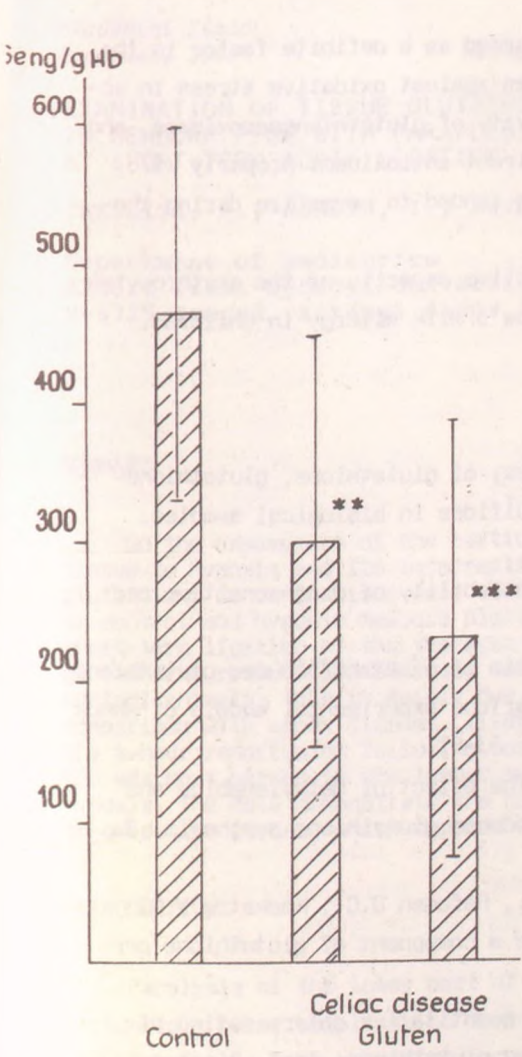


Fig. 5 Whole blood selenium content of celiac patients on gluten challenge and on gluten-free diet

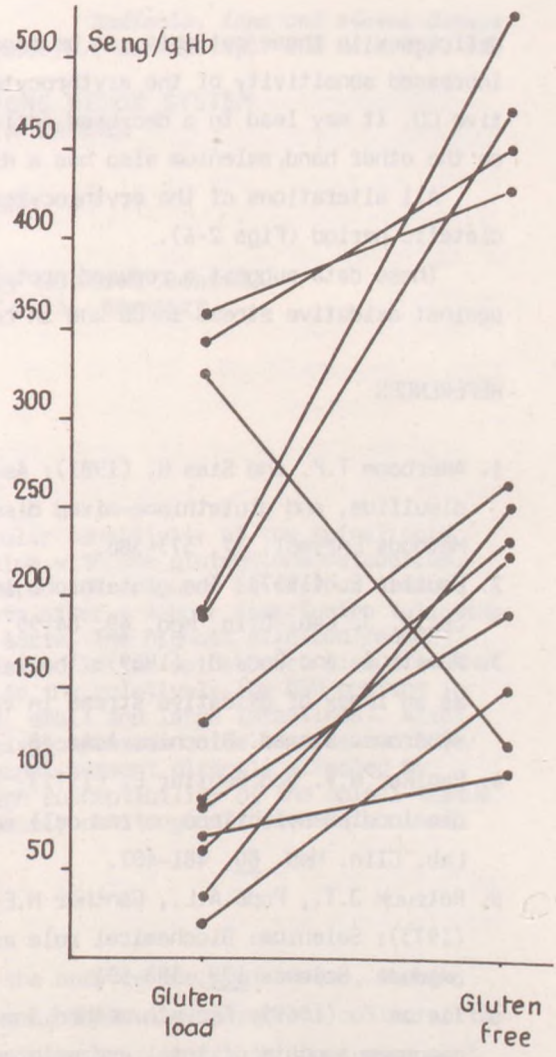


Fig. 6 Changes in whole blood selenium content of celiac patients on gluten-free diet

deficiency in these patients can be regarded as a definite factor in the increased sensitivity of the erythrocytes against oxidative stress in active CD. It may lead to a decreased activity of glutathioneperoxidase, and on the other hand, selenium also has a direct antioxidant property /5/.

All alterations of the erythrocytes tended to normalize during the dietetic period (Figs 2-6).

These data suggest a reduced protective capacity of the erythrocytes against oxidative stress in CD and in cow's milk allergy in children.

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EXAMINATION OF TISSUE GLUTATHIONE REDOX SYSTEM
IN NEWBORN PIGS WITH PARAPLEGIA CAUSED
BY SHORT-TERM AORTA LIGATION

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SUMMARY

On the assumption of the particular sensitivity of the spinal cord tissue to hypoxia and its interrelation with the glutathione metabolism, glutathione concentrations were measured and compared in various tissues of control and hypoxic newborn piglets after a 4-hour reperfusion following short-term ligation of the thoracic aorta. The highest oxidized/reduced glutathione ratio (GSSG/GSH) was measured in the spinal cord tissue in the control animals. This is mainly due to the relatively low GSH content in comparison with other tissues (liver, small and large intestines). After the 4-hour reperfusion following aorta compression, the increase in GSSG/GSH was most marked in the lumbar spinal segment directly affected by hypoxia. The data demonstrate the high susceptibility of the spinal tissue to oxidative stress, which depends mainly on its glutathione metabolism.

INTRODUCTION

Paralysis of the lower part of the body is the most severe, though fortunately rare, complication during or following operations on the aorta /2/. Paraplegia is a result of ischemic-hypoxic injury to the lumbar part of the spinal cord, but the exact causes are not known. On the assumption that oxygen free radicals might have a role in the paralysis emerging during the reperfusion period following aorta strangulation, an animal experimental model was earlier devised to investigate this question /3/.

The previous experiments proved the presence of active oxygen radicals and their damaging effect, and the glutathione system of the red blood cells was found to react most readily to oxidant stress /4/. An attempt has now been made to establish how different tissues (cervical spinal cord, lumbar spinal cord, liver, kidneys, small and large intestines) respond to oxidant stress.

MATERIAL AND METHODS

Experiments were made on 11 control and 11 operated ischemic newborn pigs; aorta strangulation lasted 36 ± 4 minutes. After reperfusion for 4 hours, the animals were killed and the above-mentioned tissues were sampled to measure the reduced (GSH) and oxidized (GSSG) glutathione concentrations by the method of Tietze /6/ and Akerboom and Sies /1/, using glutathione reductase.

RESULTS AND DISCUSSION

In the control animals, the highest GSH content was found in the liver, and the GSH levels in the small and large intestines were also higher than that in the spinal cord. The lowest GSH level was measured in the renal tissues. The GSSG concentrations were largely the same in the different tissues of the control animals, but the GSSG level in the renal tissue was somewhat lower. In consequence of these data, the molar ratio GSSG/GSH was found to be the lowest in the liver, and relatively low in both the small and large intestines. The highest GSSG/GSH was found in the spinal cord tissues, indicating a marked susceptibility of this tissue to oxidative stress.

In hypoxic animals, after a 4-hour period of reperfusion following the short-term aorta ligation, there was a definite decrease in GSH content, simultaneously with an increase in the GSSG concentration in various tissues. The ratio GSSG/GSH for the tissue samples examined was higher than that in the control animals. The most pronounced increase in GSSG/GSH was found in the lumbar segment of the spinal cord directly affected by hypoxia. This rise exceeded the result for any other tissue examined, proving the most extensive oxidative damage.

It is considered that the oxygen free radicals produced during the reperfusion period following operations on the aorta can result in more serious oxidative damage in the spinal cord than in other tissues, because of its increased susceptibility to oxidative stress, mainly due to its decreased GSH reserve.

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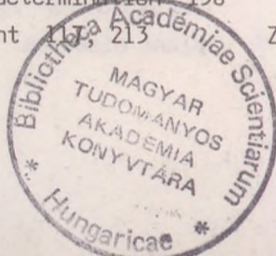
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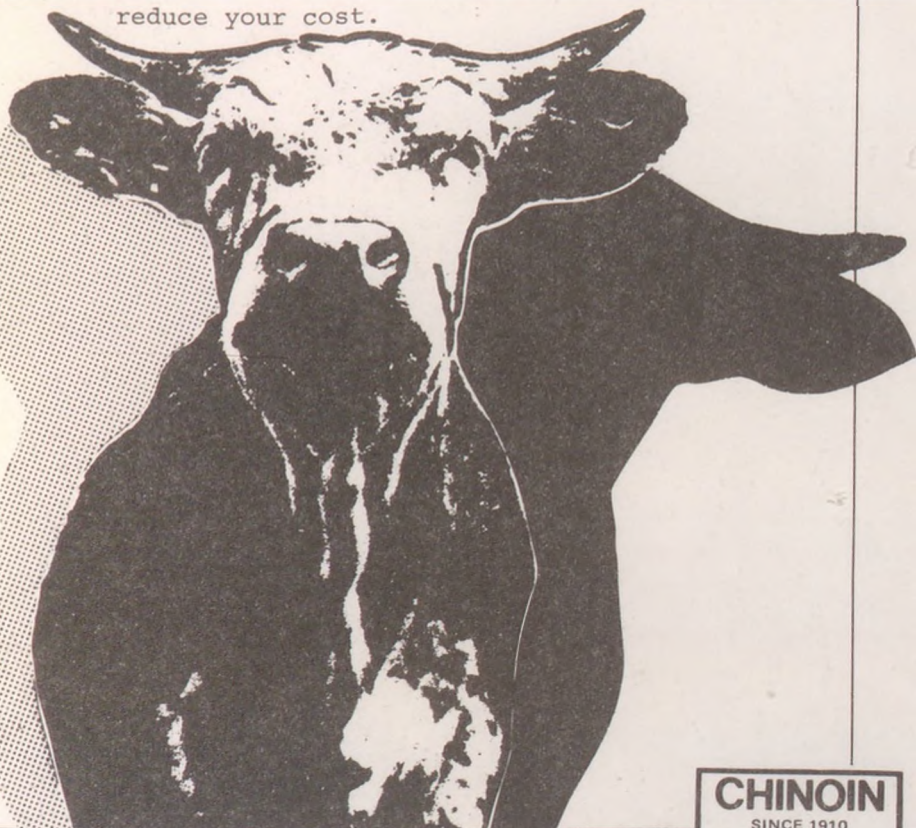
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The book contains the most important papers and poster abstracts presented at the Third Oxygen Radical Conference organized in Szeged on 12—14 January 1989. The main topics of the meeting were the physiology and pathology of oxygen radicals which take part in different human and veterinary diseases. The book also provides results on research into low and high molecular weight antioxidants (both natural and synthetic ones). Some lectures reported experimental results relating to selenium and trace metals.

The major part of the book pertains to the results of lipid peroxidation studies, including work on the mechanism, measurements and the role in different pathological events.

The book presents such a broad spectrum of information on the main topics and related fields that it will be of value for human and veterinary physicians, biologists and radiation experts, biochemists, chemists, physiologists, gerontologists, pathophysiologists and others.

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