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Hydrogen peroxide metabolism during peroxisome proliferation by fenofibrate

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Abstract

Fenofibrate, the hypolipidemic drug and peroxisome proliferator, was given to mice (0.23% w/w in the diet) during 1–3 weeks and enzyme activities, H_2O_2 concentration, and H_2O_2 production rate were determined. A maximal increase of 150% in liver/body weight ratio was observed after 3 weeks of treatment. Acyl-CoA oxidase, catalase and uricase activities were increased by 712%, 506% and 41% respectively by treatment with fenofibrate. Se- and non Se-glutathione peroxidase and Mn-superoxide dismutase activities were increased by 331%, 188% and 130% respectively in the liver of 2 weeks-treated mice. Cu-Zn superoxide dismutase activity was not affected by fenofibrate treatment. H_2O_2 steady-state concentration showed an increase of 89% after 2 weeks of treatment. H_2O_2 production rates, and the steady-state concentrations of the intermediates $HO^{\cdot}$, $R^{\cdot}$ and $ROO^{\cdot}$, calculated using experimental data, were higher in the liver of fenofibrate-treated mice than in control animals. According to our findings, the imbalance between H_2O_2 production and its degradation by its metabolizing enzymes during peroxisome proliferation, would result in an increased level of H_2O_2 steady-state concentration, with the resulting oxidative stress which may lead to the generation of oxidative damage and to the induction of liver carcinogenesis.

Keywords: Peroxisome proliferation; Fenofibrate; Hydrogen peroxide; Antioxidant enzyme

1. Introduction

Xenobiotics, including hypolipidemic drugs, herbicides, and phthalate-ester plasticizers, have been found to produce hepatomegaly, hepatic peroxisome proliferation, and the induction of some peroxisomal oxidizing enzymes, particularly the H_2O_2-producing acyl-CoA-oxidase, in rats and mice [1,2].

Long-term administration of peroxisome proliferators induce a very high incidence of hepatocellular carcinomas in rats and mice although they are neither mutagenic in Ames assays nor DNA damaging [3]. This led to the hypothesis that their carcinogenicity is related to biologically active products of the proliferated peroxisomes rather than to a direct chemical effect on cellular macromolecules [4].

It was proposed that the carcinogenicity of these agents may be mediated by oxidative DNA damage resulting from persistent peroxisome proliferation and increase in H_2O_2-generating peroxisomal β-oxidation enzyme system in liver [5,6].

Goel et al. [7] have shown accumulation of lipofucsin in liver during hepatocarcinogenesis by peroxisome proliferators. Lipofucsin is widely accepted as the end-product of a free radical-induced oxidative polymerization reaction involving proteins and lipids. Therefore, it seems that xenobiotic-induced peroxisome proliferation may lead to oxidation of membrane fatty acids, initiating lipid peroxidation [8,9].

In this study, we developed a model of peroxisome proliferation in mice by fenofibrate supplementation of the laboratory diet. Fenofibrate belongs to a family of hypolipidemic drugs that are commonly used to prevent human cardiovascular risks.

The aim of this work was to evaluate the H_2O_2 metabolism associated to peroxisome proliferation by fenofibrate and the levels of antioxidant enzymes.

2. Materials and methods

Animals and treatment. Female Swiss mice weighing 25–30 g were used. Fenofibrate dissolved in ethanol was

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incorporated into the rodent normal chow, and the solvent was evaporated to provide a final fenofibrate concentration of 0.23% w/w in the diet. Experimental animals were fed the fenofibrate-supplemented diet and control mice were fed normal diet subjected to the same procedure but without the drug for 1–3 weeks.

Preparation of peroxisome-rich fractions. Mice were killed by cervical dislocation after 1, 2 or 3 weeks of treatment with fenofibrate. The liver was weighed and homogeneized in a medium consisting of buffer MSTE (0.23 M mannitol, 75 mM sucrose, 10 mM Tris-HCl, 0.2 mM EDTA) (pH 7.4). Mouse liver homogenates were centrifuged at $700 \times g$ for 10 min to discard nuclei and cell debris. The supernatant (termed 'homogenate') was centrifuged at $5000 \times g$ for 10 min and the pellet was washed after the fluffy layer had been discarded. It was considered to consist of mainly heavy intact mitochondria and was termed the 'mitochondrial fraction'. The supernatant fractions were centrifuged at $12000 \times g$ for 10 min and then were washed, and the collected pellet, including the fluffy layer which consists of light mitochondria, peroxisomes, lysosomes and some microsomal membranes, was considered to be the 'peroxisome-rich fraction'. The operations were carried out at 0–2°C [10]. Protein was assayed by the method of Lowry et al., using bovine serum albumin as standard [11].

Enzyme activities. Peroxisomal acyl-CoA-oxidase activity was measured by a fluorometric assay based on the determination of H_2O_2 production, using the HRP-p-hydroxyphenylacetic acid system [12].

Catalase activity was measured in liver homogenates and peroxisomal fractions diluted 1/100, treated with Triton X-100 and incubated 3 min in a mixture reaction containing 20 mM imidazol buffer (pH 7.0), 0.1% BSA, and 1.5 mM H_2O_2. The reaction was stopped by addition of titanium IV oxysulphate in 2 N SO_4H_2. Hydrogen peroxide was determined as yellow peroxy titanium sulphate, which absorbs at 405 nm [13].

Uricase activity was measured in liver homogenates and peroxisomal fractions, by measuring oxygen consumption rate during oxidation of uric acid in a medium consisting of 50 mM phosphate buffer (pH 7.4), 100 μM uric acid, 5 μl/ml ethanol and 0.5–1.0 mg protein. A Clark electrode was used for measuring the rate of oxygen consumption. Initial oxygen content in the medium reaction is 0.22 mM [14].

Glutathione peroxidase (GPx) was measured in the homogenates following NADPH oxidation at 340 nm in the presence of reduced glutathione, glutathione reductase and tertbutyl hydroperoxide or H_2O_2 as described by Flohé and Gunzler [15]. The activity of glutathione peroxidase is expressed in U/g liver and the enzyme content is also expressed as nmol enzyme/g liver (μM).

Cu-Zn superoxide dismutase (Cu-Zn SOD) activity was determined in the homogenates by measuring the inhibition of the rate of autocatalytic adrenochrome formation in a reaction medium containing 1 mM epinephrine and 50 mM glycine-NaOH (pH 10.2) [16]. Mn-superoxide dismutase (Mn-SOD) was assayed in the mitochondrial fractions using the same method. Enzyme activity was expressed in U/g liver.

Intracellular steady-state concentration of hydrogen peroxide. Tissue slices 0.1 mm thick were incubated 10 min in 120 mM NaCl, 30 mM phosphate buffer (pH 7.4) at 30°C and at a tissue/medium ratio of 1/20. Samples of the incubation medium were diluted 1/2.5 with 100 mM phosphate buffer (pH 7.4) containing 2.8 U/ml horseradish peroxidase and 40 μM p-hydroxyphenylacetic acid as hydrogen donor and fluorescence intensity was measured at 317–414 nm. H_2O_2 concentration was determined by a

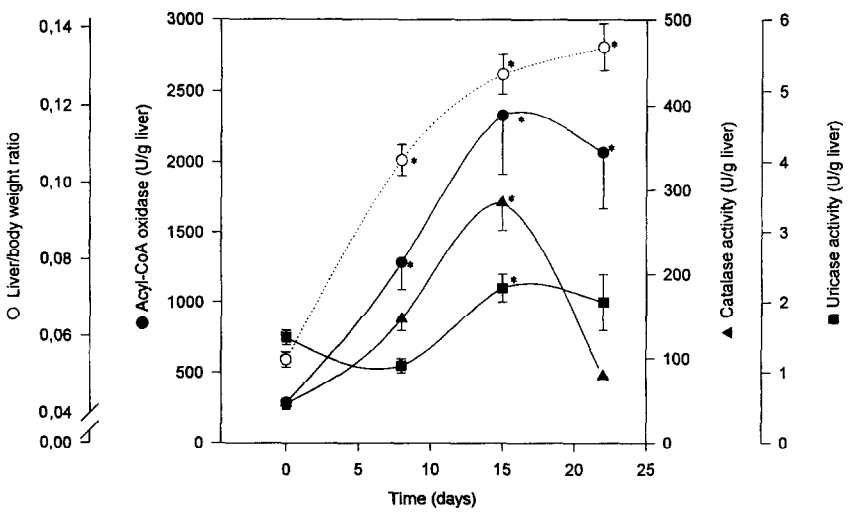

Fig. 1. Effect of the administration of fenofibrate on the wet liver/body weight ratio (○), and on acyl-CoA oxidase (●), catalase (△) and uricase (□) activities in liver homogenates. Symbols indicate mean values from 6 animals and bars indicate S.E.M. One unit of acyl-CoA oxidase activity forms 1 nmol H_2O_2/min under the assay conditions. * $P < 0.01$; ** $P < 0.05$.

standard calibration curve and calculated by substracting the value of a sample treated with 0.1 μM catalase from the value of an untreated sample [17].

Statistics. Values are expressed as mean values ± S.E.M. The significance of differences between mean values were analysed by ANOVA and Tukey test.

3. Results

Liver / body weight ratio. Wet liver/body weight ratio was increased in treated mice at all the studied times. Maximal increase of 150% was obtained after 3 weeks of treatment (Fig. 1). Water content was not affected by fenofibrate treatment (data not shown).

3.1. Enzyme activities

The administration of fenofibrate produced an induction of acyl-CoA-oxidase at all the times studied. An increase of 712% in the activity of the enzyme was observed in homogenates after 2 weeks of treatment (control value: 287 ± 34 U/g liver). One unit of the enzyme forms 1 nmol H_2O_2/min under the assay conditions (Fig. 1). Specific activity of acyl-CoA oxidase was 10- to 12-fold higher in peroxisome-rich fractions after 2 weeks of treatment (control value: 67 ± 5 U/g liver) (Table 1).

Catalase activity was measured in whole homogenates and in peroxisomal fractions of control and treated mice. Catalase activity was increased by 506% in liver homogenates of fenofibrate-treated mice after 2 weeks of treatment (control value: 47 ± 7 U/g liver) (Fig. 1). No significant changes between control and treated animals were observed in the specific catalase activity of peroxisome-rich fractions (Table 1).

Table 1
Effect of fenofibrate on the enzyme activity of the peroxisome-rich fractions of mouse liver after two weeks of treatment

Parameter	Control	Fenofibrate
Acyl-CoA oxidase (U/g liver)	67 ± 5	848 ± 145 *
Catalase (U/g liver)	5.2 ± 1.0	8.2 ± 1.2
Uricase (mU/g liver)	67 ± 9	101 ± 14
Peroxisomal protein content (mg/g liver)	8.6 ± 0.5	20.6 ± 0.3 *

Values are expressed as mean ± S.E.M. of 4–6 animals.
* $P < 0.01$.

Uricase activity was measured in whole homogenates and in peroxisomal fractions of control and treated mice. Uricase activity was increased by 41% in liver homogenates of fenofibrate-treated mice after 2 weeks of treatment (control value: 1.5 ± 0.1 U/g liver) (Fig. 1). No significant changes between control and treated animals were observed in uricase activity of peroxisomal fractions (Table 1). A slight decrease in uricase activity was observed in homogenates and in peroxisomal fractions after 8 days of treatment.

Se-glutathione peroxidase (Se-GPx) and non-Se-glutathione peroxidase (non-Se-GPx) were determined in liver homogenates of control and treated mice. Administration of fenofibrate induced increases in Se and non-Se glutathione peroxidase activities at all the times of treatment. Maximal effects were observed after 2 weeks with increases of 331% and 188% in Se-GPx and non-Se-GPx activities, respectively (control values: Se-GPx: 0.39 ± 0.02 U/g liver, non-Se-GPx: 0.25 ± 0.02 U/g liver) (Fig. 2).

Cu-Zn superoxide dismutase activity of liver homogenates was not affected by fenofibrate treatment as compared with control values (631 ± 34 U/g liver), while Mn-superoxide dismutase activity of the mitochondrial

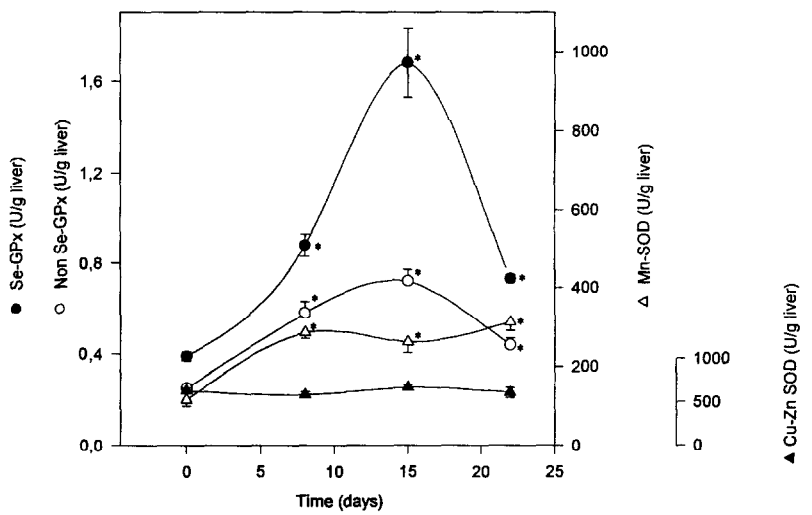

Fig. 2. Se-dependent glutathione peroxidase (Se-GPx) (●), non Se-glutathione peroxidase (non Se-Gpx) (○), Cu-Zn superoxide dismutase (Cu-Zn SOD) (△) and Mn superoxide dismutase (Mn-SOD) (▲) activities in control and fenofibrate-treated mice. Symbols indicate mean values from 6 animals and bars indicate S.E.M. * $P < 0.01$.

fractions was increased at all the times studied, with maximal increase of 171% after 3 weeks of treatment (control value: 115 ± 16 U/g liver) (Fig. 2).

H_2O_2 steady-state concentration. The intracellular H_2O_2 steady-state concentration was measured in the incubation medium of liver slices of control and fenofibrate-treated animals after reaching diffusion equilibrium. Administration of fenofibrate during 2 weeks induced an increase of 89% in H_2O_2 steady-state concentration (control value: 0.09 ± 0.01 μM) (Fig. 3).

H_2O_2 production rates. On the basis of the steady-state equations, H_2O_2 production rates were calculated using the experimental results of the determinations of H_2O_2 concentration, catalase and Se-glutathione peroxidase content (Eq. 1).

$$-d[H_2O_2]/dt = d[H_2O_2]/dt = k_{cat}[cat][H_2O_2] + k_{GP}[GP][H_2O_2] \quad \text{(Eq. 1)}$$

Fenofibrate treatment produced a 10-fold increase in the calculated H_2O_2 production rate ($d[H_2O_2]/dt$ calculated), two weeks after treatment (Table 2). This increase was similar to the one obtained from comparing H_2O_2 production rates corresponding to the measured activity of the enzyme acyl CoA-oxidase ($d[H_2O_2]/dt$ measured) (Table 2).

Lipid peroxidation chain reaction intermediates concentration. Concentrations of the species hydroxyl radical ($HO^{\cdot}$), alkyl radical ($R^{\cdot}$) and peroxyl radical ($ROO^{\cdot}$), were calculated by the double steady-state approach and the use of published rate constants [18–20]. Experimental data of H_2O_2 concentrations obtained for control and 15-days-treated animals were used for these calculations. $HO^{\cdot}$ and $R^{\cdot}$ steady-state concentrations were increased by 90%, and $ROO^{\cdot}$ concentration was increased by 37% in the liver of fenofibrate-treated mice, as compared with control animals (Table 2).

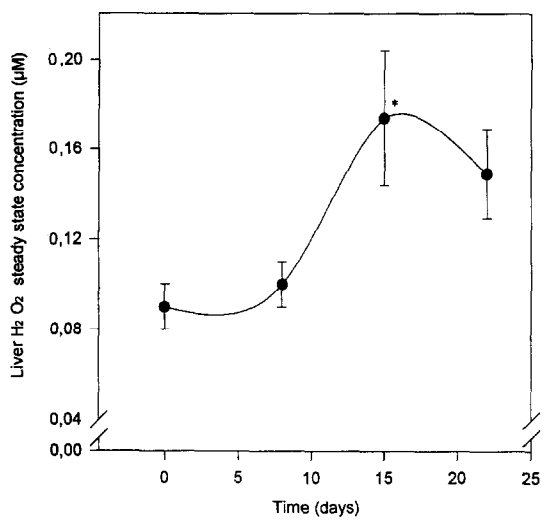

Fig. 3. Effect of fenofibrate treatment on liver H_2O_2 steady-state concentration. Symbols indicate mean values from 6 animals and bars indicate S.E.M. * $P < 0.01$.

Table 2
H_2O_2 production rates and lipid peroxidation chain reaction intermediates concentrations in the liver of mice after 15 days of treatment with fenofibrate

Parameter	Time of treatment (days)	
	0	15
$[H_2O_2]$ (μM)	0.09	0.17
[catalase] (μM)	1.20	7.26
[Se-GPx] (μM)	0.02	0.09
$d[H_2O_2]/dt$ (μM.s^{-1}) (measured)	1.1	14.1
$d[H_2O_2]/dt$ (μM.s^{-1}) (calculated)	5.2	58.6
[$HO^{\cdot}$] (aM) (calculated)	4.5	8.5
[$R^{\cdot}$] (fM) (calculated)	0.9	1.7
[$ROO^{\cdot}$] (nM) (calculated)	0.67	0.92

4. Discussion

Peroxisome proliferators are a group of structurally diverse compounds that produce similar changes in experimental animals. These responses occur primarily in hepatocytes and include enlargement of the liver, increases in relative content of peroxisomes and smooth endoplasmic reticulum, 10-fold or greater induction of fatty acid β-oxidation, carnitine acetyltransferase, and lauric acid ω-hydroxylation activities, and hypolipidemia [21]. The hepatomegaly arises from a combination of cellular hypertrophy and hyperplasia [22,23]. Morphometric analysis of liver sections from rats treated with peroxisome proliferators demonstrated 7- to 10-fold increases in relative peroxisomal volume, which arise from an increase in both the volume and number of individual peroxisomes [22,24]. In our study, the administration of the hypolipidemic fenofibrate induces hepatomegaly in mice, with an important increase in the liver/body weight ratio. Liver water content was not affected, indicating that the increase in liver weight observed was not due to cellular edema.

Hypolipidemic agents, such as clofibrate, nafenopin and tibric acid are known to produce an induction of the peroxisomal β-oxidation enzymes and therefore an increased H_2O_2 production rate [23,25]. It has also been described that administration of peroxisome proliferators such as clofibrate, bezafibrate and di(2-ethylhexyl) phtalate, produces an increase of 2- to 3-fold in rat liver catalase activity [8].

In our study, fenofibrate administration induced a 10- to 12-fold increase in acyl CoA-oxidase activity of peroxisomal fractions as compared with control animals, after the second week of treatment. A 5-fold increase in catalase activity of liver homogenates was observed after 2 weeks of treatment with fenofibrate. Specific catalase activity of the peroxisomal fractions remained constant; apparently the increase in catalase activity observed in the whole

homogenates would be a consequence of the increased number of peroxisomes. The lack of increase in catalase activity of peroxisomal fractions could, at least in part, be due to the leakage of catalase during fractionation. Beaufay et al. [26] suggested earlier that during the homogenization and fractionation of normal rat liver and centrifugation procedures, there is some catalase release from peroxisomes.

Uricase activity of the homogenates was also increased by fenofibrate treatment but to a lesser extent than catalase. The slight decrease in uricase activity observed during the first week of treatment suggests that there might be a proportion of the proliferated peroxisome population which are totally or partially deficient in uricase. Hess et al. [27] had shown electron micrographs studies of livers following treatment with clofibrate in which there was an accumulation of microbodies deficient in cores.

Cu-Zn superoxide dismutase was not affected by fenofibrate treatment, probably because it is not an enzyme directly related to H_2O_2 metabolism. However, an increase in Mn-superoxide dismutase activity was observed in fenofibrate-treated mice, suggesting the possibility of mitochondrial proliferation, as described by Gear et al. in clofibrate-treated rats [28]. Changes within the mitochondrial population have been reported by Meijer et al., with increases in the number of mitochondrial profiles and in the surface/volume ratio [29].

Acyl-CoA oxidase is a FAD-containing enzyme that catalyses the first oxidation step of peroxisomal β-oxidation with reduction of molecular oxygen to hydrogen peroxide [30]. The increase in H_2O_2 concentration observed in our model of peroxisome proliferation, reflects a high production of this species at peroxisomal level and occurs as a consequence of the induction of the acyl-CoA oxidase system. The induction observed in glutathione peroxidase could occur as a response to the increased steady-state concentration of H_2O_2.

Fenofibrate treatment for 2 weeks produced an increase in the H_2O_2 production rate obtained from calculations, and in H_2O_2 production rate corresponding to the measured activity of the peroxisomal acyl-CoA oxidase activity (Table 2). H_2O_2 production rates obtained from calculations ($d[H_2O_2]/dt$ calculated) were higher than $d[H_2O_2]/dt$ measured, because they represent total cellular H_2O_2 production.

The high production rate of H_2O_2 during peroxisome proliferation leads to a situation of oxidative stress, which is characterized by an increased H_2O_2 steady-state concentration in hepatic tissue. In the presence of iron traces, H_2O_2 can form hydroxyl radical by Fenton type reaction, which in turn will attack polyunsaturated fatty acids, initiating lipid peroxidation process. Steady-state concentration of the intermediates $HO^{\cdot}$, $R^{\cdot}$ and $ROO^{\cdot}$ are higher in fenofibrate-treated mice than in control mice, suggesting that in vivo lipid peroxidation processes might be stimulated in this model of peroxisome proliferation.

The oxidative stress hypothesis has been advanced to explain how peroxisome proliferation may result in hepatocellular carcinomas. The enhanced H_2O_2 production overcomes the ability of peroxisomal catalase and other H_2O_2-degrading enzymes, and results in a slow accumulation of oxidative damage to the genome [2]. This hypothesis is supported by evidence of in vivo and in vitro damage to DNA associated with proliferated peroxisomes [5,31], increased production of hydroxyl radical by proliferated peroxisomes [32] and a close association between lipofuscin accumulation and carcinogenesis of peroxisome proliferators [33,34].

Treatment with fenofibrate leads to an increase in H_2O_2 production rate, that cannot be completely metabolized by catalase and diffuses outside the peroxisomes, resulting in an increased H_2O_2 steady-state concentration. H_2O_2 can produce oxidative damage to DNA and the activation of oncogene(s) may lead to the generation of hepatocarcinogenesis [7].

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References

[1] Palma, J.M., Garrido, M., Rodríguez-García, M.I. and del Río, L.A. (1991) Arch. Biochem. Biophys. 287, 68–74.
[2] Moody, D.E., Reddy, J.K., Lake, B.G., Popp, J.A. and Reese, D.H. (1991) Fundam. Appl. Toxicol. 16, 233–248.
[3] Abdellatif, A.G., Preat, V., Vamecq, J., Nilsson, R. and Roberfroid, M. (1990) Carcinogenesis 11, 1899–1902.
[4] Tomaszewski, K.E., Heindel, S.W., Jenkins, W.L. and Melnik, R.L. (1990) Toxicology 65, 49–60.
[5] Kasai, H., Okadi, Y., Nishimura, S., Rao, M.S. and Reddy, J.K. (1989) Cancer Res. 49, 2603–2605.
[6] Reddy, J.K. and Lalwani, N.D. (1983) CRC Crit. Rev. Toxicol. 12, 1–58.
[7] Goel, S.K., Lalwani, N.D. and Reddy, J.K. (1986) Cancer Res. 46, 1324–1330.
[8] Tamura, H., Iida, T., Watanabe, T. and Suga, T. (1990) Carcinogenesis 11, 445–450.
[9] Grasso, P. (1985) Biochem. Soc. Trans. 13, 861–862.
[10] Boveris, A., Oshino, N. and Chance, B. (1972) Biochem. J. 128, 617–627.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Poosch, M.S. and Yamazaki, R.K. (1986) Biochim. Biophys. Acta 884, 585–593.
[13] Badhuin, B.P., Beaufay, H., Rahman-Li, Y., Sellinger, O.Z., Wattiaux, R., Jacques, P. and De Duve, C. (1964) Biochem. J. 92, 179–184.
[14] Estabrook, R.W. (1967) Meth. Enzymol. X, 41–47.
[15] Flohé, L. and Gunzler, W.A. (1984) Methods Enzymol. 105, 114–117.
[16] Misra, H.P. and Fridovich, I. (1972) J. Biol. Chem. 247, 3170–3175.
[17] González Flecha, B., Cutrín, J.C. and Boveris, A. (1993) J. Clin. Invest. 91, 456–464.

[18] Vladimirov, Y.A. and Archakov, A.I. (1972) Lipid peroxidation in biological membranes (Russian), Nauka Publishers, Moscow.
[19] Patterson, L.K. (1981) in Oxygen and Oxy-Radicals in Chemistry and Biology (M.A.J. Rodgers and E.L. Powers, eds.), pp. 89–95, Academic Press, New York, .
[20] Ivanov, I.I. (1985) J. Free Rad. Biol. and Med., 1, 247–253.
[21] Moody, D.E., Gibson, G.G., Grant, D.F., Magdalou, J. and Rao, M.S. (1992) Drug Metabol. and Dispos. 20, 779–791.
[22] Moody, D.E. and Reddy, J.K. (1976) J. Cell. Biol. 71, 768–780.
[23] Moody, D.E. and Reddy, J.K. (1978) Am. J. Pathol. 90, 435–446.
[24] Staubli, W., Schweizer, J., Sutter, J. and Weibel, E.R. (1977) J. Cell Biol. 74, 665–689.
[25] Mannaerts, G.P., Debeer, L.J., Thomas, J. and De Schepper, P.J. (1979) J. Biol. Chem. 254, 4585–4595.
[26] Beaufay, A., Jacques, P., Badhuin, P., Sellinger, O.Z., Berthet, J., De Duve, C. (1964) Biochem. J., 92, 184–205.
[27] Hess, R., Staubli, W. and Riess, W. (1965) Nature 208, 856–858.
[28] Gear, A.R.L., Albert, A.D. and Bednarek, J.M. (1974) J. Biol. Chem. 249, 6495–6504.
[29] Meijer, J., Starkerud, C., Granell, I. and Afzelius, B.A. (1991) J. Submicrosc. Cytol. Pathol. 23, 185–194.
[30] Mannaerts, G.P. and Van Veldhoven, P.P. (1993) Biochimie 75, 147–158.
[31] Fahl, W.E., Lalwani, N.D., Watanabe, T., Goel, S.K. and Reddy, J.K. (1984) Proc. Natl. Acad. Sci. USA 81, 7827–7830.
[32] Elliot, B.M., Dodd, N.J.F. and Elcombe, C.R. (1986) Carcinogenesis 7, 795–799.
[33] Conway, J.G., Tomaszewski, K.E., Olson, M.J., Cattley, R.C., Marsman, D.S. and Popp, J.A. (1989) Carcinogenesis 10, 513–519.
[34] Reddy, J.K., Lalwani, N.D., Reddy, M.K. and Qureshi, S.A. (1982) Cancer Res. 42, 259–266.