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FERROUS ION-MEDIATED CYTOCHROME *P*-450 DEGRADATION AND LIPID PEROXIDATION IN ADRENAL CORTEX MITOCHONDRIA

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SUMMARY

The relationship between the degradation reaction of cytochrome *P*-450 and lipid peroxidation was studied utilizing bovine adrenal cortex mitochondria. The two reactions were found to be closely correlated in terms of their response to storage of the mitochondrial preparation, stimulation by Fe^{2+}, inhibition by EDTA and their initiation by cumene hydroperoxide. Both reactions were also found not to be inhibited by catalase, superoxide dismutase, 1,4-diazabicyclo-(2,2,2)-octane and alcohols, indicating that H_2O_2, superoxide, singlet oxygen and hydroxyl radicals do not participate in these reactions. Yet, diphenylamine proved to be a powerful inhibitor for both reactions, suggesting the involvement of a radical species. Cumene hydroperoxide could induce these two reactions at below 0.1 mM concentrations in the presence of molecular oxygen. The chemiluminescence observed during the Fe^{2+}-mediated lipid peroxidation reaction which was not inhibited by either superoxide dismutase or 1,4-diazabicyclo-(2,2,2)-octane, was biphasic: one was a rapid burst; and the other was a slowly increasing emission. The latter portion of the emission of light coincided with the formation of malondialdehyde. These results indicate that in adrenal cortex mitochondria the degradation of cytochrome *P*-450 is closely related to lipid peroxidation.

INTRODUCTION

The peroxidation reaction of unsaturated fatty acids of membrane phospholipids has important biological significance. As a result, microsomal and mitochondrial enzymes as well as the cytochromes are significantly destroyed [1, 2]. In addition, the respiratory control of the mitochondria is damaged [3] and lysis of the mitochondria occurs [4, 5]. Recently, in rat liver microsomes, Levin et al. [6], Jacobson et al. [7] and Vatsis et al. [8] reported that lipid peroxidation was observed along with a concomitant loss of microsomal cytochrome *P*-450, the terminal oxidase for the hydroxylation reactions. Accordingly, lipid peroxidation was found to be associated with a sharp decline in the activities of pentobarbital oxidation, acetanilide

hydroxylation, aniline hydroxylation and ethylmorphine demethylation in rat liver [7–9].

Adrenal cortex mitochondria also contain cytochrome *P*-450, a unique adrenal ferredoxin (adrenodoxin) and an NADPH-dependent diaphorase for steroid hydroxylation reactions in addition to the electron transport system for oxidative phosphorylation. Since there is no report on the degradation processes of cytochrome *P*-450 and its relationship to the lipid peroxidation reaction in adrenal cortex mitochondria, we have decided to study the reaction of Fe^{2+} - mediated lipid peroxidation associated with cytochrome *P*-450 degradation.

MATERIALS AND METHODS

$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was purchased from Baker and Adamson. EDTA and diphenylamine were obtained from Fisher. 1,4-diazabicyclo-(2,2,2)-octane and malondialdehyde bis-dimethyl acetal were from Aldrich. Superoxide dismutase, catalase (EC 1. 11. 1. 6), deoxycorticosterone, $NADP^+$, glucose 6-phosphate and its dehydrogenase (EC 1. 1. 1. 49) were purchased from Sigma. Cumene hydroperoxide was from I.C.N.-K. and K. Bovine adrenal cortical mitochondria were prepared as follows: fresh adrenals were collected at a local slaughter house and brought to the laboratory on ice within 1 h of the animal's death. Fat was carefully removed from the surface of each gland which was then bisected longitudinally. The medulla was removed and the cortex region was sliced off the capsule by the use of a scalpel. Subsequent operations were performed in the cold room or at 0 °C. The cortex was washed with 10 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM EDTA. This initial rinse medium was removed by centrifugation at 0 °C. The cortex was then suspended in the same buffer (4 ml/g wet weight) and homogenized by two rapid passes using a loose teflon homogenizer followed by one slow pass with a tight homogenizer. The homogenate was then centrifuged at $800 \times g$ for 10 min to remove cellular debris and unbroken cells. The resulting supernatant was further centrifuged at $7000 \times g$ for 30 min. The mitochondria that precipitated after centrifugation were then washed three times by 10 mM phosphate buffer (pH 7.4) containing no EDTA by centrifugation at $11000 \times g$ for 20 min. These washed mitochondria were resuspended in the same buffer, stored at 0 °C and used within 48 h.

The incubation mixtures for lipid peroxidation, cytochrome *P*-450 degradation and heme degradation are described in the figures. Standard incubations were carried out at 37 °C for 60 min in 10 mM phosphate (pH 7.4) under aerobic conditions. Malondialdehyde was freshly prepared by hydrolizing malondialdehyde bis-dimethyl acetal with 0.55 M HCl followed by neutralization with 3 M NaOH before use. Malondialdehyde content was determined by the thiobarbiturate method of Hunter et al. [10], using the molar extinction coefficient of $9 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 532 nm [4]. Cytochrome *P*-450 was determined by the method of Greim [11] immediately after incubation using a molar extinction coefficient of $91 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ for the absorptive increment between 450 and 490 nm [12]. Heme was determined by the method described by Omura and Sato [13] using a molar extinction increment of $32.4 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ between 557 and 575 nm. The assays for steroid hydroxylation were described previously [14]. Protein was determined by the biuret method using bovine serum albumin as a standard [15]. Spectrophotometric measurements were

performed in an Aminco-Chance dual wavelength spectrophotometer. Chemiluminescence measurements were carried out by utilizing a Beckman liquid scintillation counter (LS-100) with an off-mode coincidence circuit. The background luminescence with a scintillation vial containing the buffer solution showed a value below 2000 cpm. In the presence of mitochondria, the value of 9000 cpm was obtained without the addition of any Fe^{2+}. The reaction mixtures used for chemiluminescence measurements are described in the figures. The reaction was carried out at 22 °C.

RESULTS

Effects of aging of adrenal cortex mitochondrial preparation on the content of cytochrome P-450 and malondialdehyde

Fig. 1 shows the relationship between cytochrome *P*-450 and the malondialdehyde content as a function of mitochondrial age, these being prepared without the addition of EDTA. In the fresh mitochondrial preparation, there was a negligible amount of malondialdehyde. As this preparation was aged at 0 °C, the malondialdehyde content increased with a concomitant loss of cytochrome *P*-450 content, strongly suggesting a close correlation between these two events. Both of these reactions are strongly inhibited by the addition of 1 mM EDTA and stimulated by the addition of Fe^{2+}, suggestive of their mediation by Fe^{2+}. We observed that addition of ADP to the reaction mixture stimulates the Fe^{2+} - mediated lipid peroxidation and cytochrome *P*-450 degradation. The stimulation observed by complexing anions is in good agreement with that observed in liver microsomes by earlier workers [16].

Effects of Fe^{2+} on the loss of cytochrome P-450 and malondialdehyde formation

Fig. 2 shows the effects of Fe^{2+} on the formation of malondialdehyde, an index of lipid peroxidation and the extent of both heme and cytochrome *P*-450 degradation. As the concentration of iron increased in the reaction mixture, the lipid

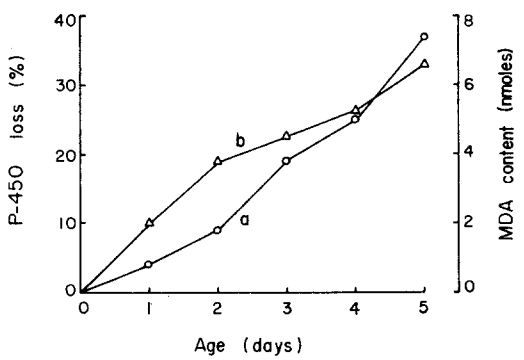

Fig. 1. Relationship between cytochrome *P*-450 and malondialdehyde content during aging of mitochondria. 36 mg protein of bovine adrenal cortex mitochondria containing 0.8 nmol of cytochrome *P*-450/mg protein were incubated at 0° using a 10 mM phosphate buffer (pH 7.4) in a total volume of 36 ml. At the times indicated, a 2-ml aliquot of the sample was removed for the cytochrome *P*-450 determination (curve a), and a 1-ml aliquot was taken for the determination of malondialdehyde (curve b). MDA stands for malondialdehyde.

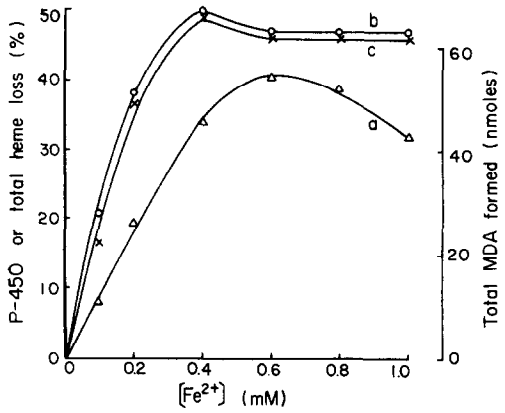

Fig. 2. Effects of Fe^{2+} on lipid peroxidation, cytochrome *P*-450 degradation and heme degradation. 2.2 mg mitochondrial protein containing 1.9 nmol of cytochrome *P*-450 and 2.3 nmol of total heme were incubated at 37 °C in a total volume of 2.5 ml using a 10 mM phosphate buffer (pH 7.4). Various concentrations of Fe^{2+} were added to the reaction mixture as indicated in the figure. Malondialdehyde (curve a), cytochrome *P*-450 (curve b) and total heme (curve c) were determined 60 min after these additions.

peroxidation activity and the extent of cytochrome *P*-450 and heme degradation all increased in a parallel manner. The optimum concentrations of iron were 0.4 and 0.6 mM for the heme degradation and the malondialdehyde formation, respectively. At iron concentrations higher than 0.6 mM, the amount of malondialdehyde formed tends to decrease. This was found to be due to further oxidation of malondialdehyde to malonic acid by Fe^{2+} under aerobic conditions.

From the measurements of the spectral changes in cytochrome *P*-450 during the degradative reaction it was observed that no significant amount of cytochrome *P*-420 was formed. Levin et al. [6] reported that the loss of cytochrome *P*-450 is the result of the degradation of the heme moiety of the cytochrome *P*-450 molecules. Our results show a parallel relationship between the loss of cytochrome *P*-450 and total heme (Fig. 2, curves b and c) supporting their conclusion.

Direct effects of malondialdehyde on cytochrome P-450

Since malondialdehyde is a cross-linking agent between proteins resulting in the formation of a Schiff base of the aldehyde with an amine [2], we have examined whether or not the malondialdehyde formation is a prerequisite for the cytochrome *P*-450 degradation. When the Fe^{2+} - mediated reaction gave 14.6 nmol of malondialdehyde, the percentage of the cytochrome *P*-450 loss was 36 %. When the corresponding amount (17.5 nmol) of malondialdehyde was added to the mitochondrial preparation, the cytochrome *P*-450 loss was only 2.8 %. Even at the high concentration of malondialdehyde (70 nmol) the loss was also found to be 2.8 %. From these results, it is evident that malondialdehyde, as a product of lipid peroxidation reaction, is not directly responsible for the degradation of cytochrome *P*-450.

Chemiluminescence associated with lipid peroxidation reaction

In order to examine whether or not lipid peroxidation emits light, chemi-

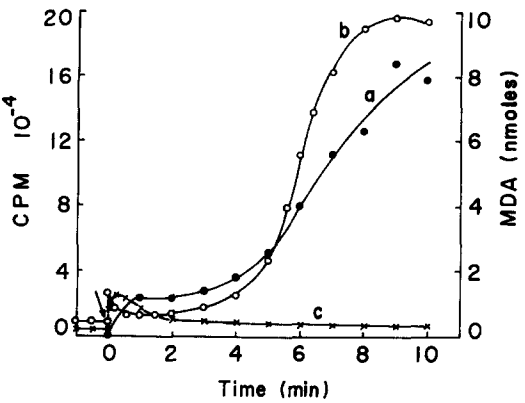

Fig. 3. Relationship between lipid peroxidation and chemiluminescence in the Fe^{2+} and O_2 system. For determining malondialdehyde formation (curve a), the reaction mixture contained 1 mg of mitochondrial protein, 10 mM phosphate (pH 7.4) and 0.2 mM Fe^{2+} in a total volume of 1 ml. For the chemiluminescence measurements, the reaction mixtures contained 10 mM phosphate (pH 7.4) and 0.2 mM Fe^{2+}, along with mitochondria (1 mg/ml) (curve b) or without mitochondria (curve c) in a total volume of 15 ml. The reactions were started by addition of Fe^{2+} as indicated by the arrow and were carried out at 22 °C.

luminescence was monitored by the use of a liquid scintillation counter. Fig. 3 shows that the $Fe^{2+}+O_2$ system produces a chemiluminescence in the presence of mitochondria. The chemiluminescence reaction showed two phases for its light emission. A small emission sharply increased after the addition of Fe^{2+} and was then quickly quenched. This reaction was insensitive to superoxide dismutase (140 units), 1,4-diazabicyclo-(2,2,2)-octane (1 mM) (singlet oxygen quencher) [17] and ethanol (30 mM) (OH· scavenger) [18, 19]. After 4–5 min, a large gradual emission was observed. The later phase, which was absent without the addition of mitochondria, was also not inhibited by either superoxide dismutase (140 units), 1,4-diazabicyclo-(2,2,2)-octane (1 mM) or ethanol (30 mM); but it was completely inhibited by diphenylamine (8 μM). When the time course of malondialdehyde formation is viewed along with the light emission intensities, the slowly increasing portion of the chemiluminescence appears related to the formation of malondialdehyde (Fig. 3).

We then examined the effects of superoxide dimutase, catalase and 1,4-diazabicyclo-(2,2,2)-octane on the loss of cytochrome *P*-450 and the formation of malondialdehyde. Neither superoxide dismutase (140 units) nor catalase (100 μg) had any significant inhibitory effects on the formation of malondialdehyde, as well as on the degradation of cytochrome *P*-450. Similarly, 1,4-diazabicyclo-(2,2,2)-octane (1 mM) and OH· scavengers (0.1 M *t*-butanol, 0.5 M formate and 0.1 M mannitol) [20–22] had no effect on both cytochrome *P*-450 degradation and the malondialdehyde formation reactions. Diphenylamine (10 μM), a free radical scavenger, showed again an inhibitory effect on both reactions. Therefore, in our system oxygen species such as $O_2^{\cdot-}$, H_2O_2, OH· and $^1O_2$ are not likely reagents to attack cytochrome *P*-450 or unsaturated fatty acids. The inhibitory effect of diphenylamine suggests the involvement of radical(s) other than $O_2^{\cdot-}$ and OH· in these reactions. Unlike the Fe^{2+} - mediated reaction, the reduced glutathione-induced lipid peroxidation was inhibited by superoxide dismutase [23].

Cumene hydroperoxide-mediated cytochrome P-450 degradation and malondialdehyde formation

The possibility of the involvement of a hydroperoxide in the cytochrome *P*-450 degradation and malondialdehyde formation reactions was then examined. As shown in Fig. 4, the amount of cytochrome *P*-450 lost and the amount of malondialdehyde formed are increasing in a parallel fashion as the amount of cumene hydroperoxide is increased in the reaction mixture. Under anaerobic conditions, both of these reactions are largely diminished indicating the requirement of O_2. The addition of 1 mM EDTA had no effect on both reactions, showing that these cumene hydroperoxide reactions are not mediated by contaminating metal ions.

The cumene hydroperoxide-mediated chemiluminescence pattern (Fig. 5) was very similar to that of the Fe^{2+} - mediated reaction. This pattern showed both the

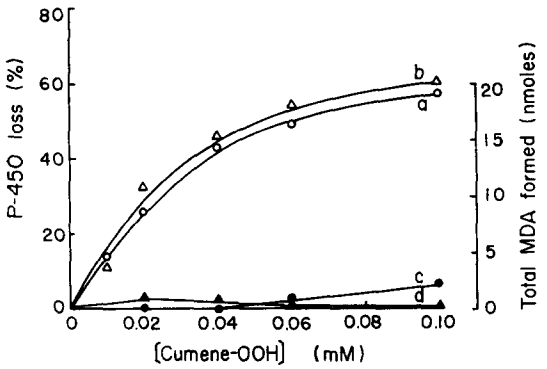

Fig. 4. Effects of cumene hydroperoxide on cytochrome *P*-450 degradation and lipid peroxidation. 2.5 mg mitochondrial protein containing 2 nmol cytochrome *P*-450 were incubated at 37 °C in a total volume of 2.5 ml of 10 mM phosphate (pH 7.4) under aerobic (curves a and b) or anaerobic (curves c and d) conditions. Various concentrations of cumene hydroperoxide were added to the reaction mixtures as indicated in the figure. Cytochrome *P*-450 (curves a and c) and malondialdehyde (curves b and d) were determined 10 min after these additions. Cumene-OOH stands for cumene hydroperoxide.

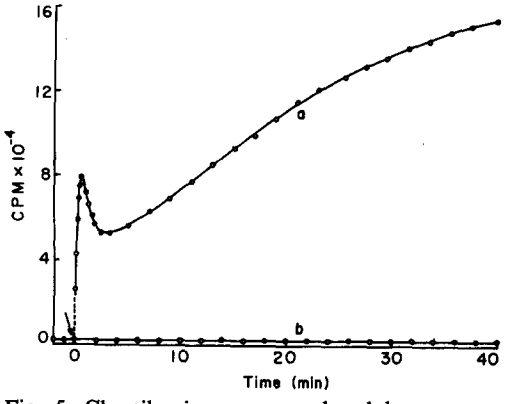

Fig. 5. Chemiluminescence produced by cumene hydroperoxide and O_2. The reaction mixture contained 10 mM phosphate (pH 7.4) and 0.1 mM cumene hydroperoxide, with mitochondria (1 mg/ml) (curve a) or without mitochondria (curve b) in a total volume of 15 ml. The reactions were carried out at 22 °C. The addition of cumene hydroperoxide is indicated by the arrow.

early rapid emission and the later gradually increasing reactions. This chemiluminescence was not inhibited by either superoxide dismutase (140 units) or 1,4-diazabicyclo-(2,2,2)-octane (1 mM). The later chemiluminescence appears to be associated with the formation of malondialdehyde.

*Effects of Fe^{2+} - pretreatment of adrenal mitochondria on steroid 11β-*hydroxylase *activity*

The mitochondria were pretreated with Fe^{2+} for 60 min at 37 °C, this reaction being terminated by the addition of 1 mM EDTA. The mitochondria were reisolated by centrifugation. The steroid 11 β-hydroxylation activity of these treated mitochondria was measured by the addition of adrenal ferredoxin and its reductase. After this limited treatment, the 11 β-hydroxylase (EC 1. 14. 1.6) activity of these mitochondria decreases by 30 % after one-third of the cytochrome *P*-450 decomposes. The amount of malondialdehyde is increased 4–5 times by this pretreatment. These results indicate that when free Fe^{2+} and O_2 are present, the degradation of cytochrome *P*-450 and the concomitant formation of malondialdehyde will occur; this resulting in a decrease in the steroid hydroxylation activity.

DISCUSSION

Our present results show that adrenal cortex mitochondrial cytochrome *P*-450 degradates with a concomitant formation of malondialdehyde. These phenomena are similar to those observed in liver microsomes [6–8]; both the degradation reaction of cytochrome *P*-450 and the formation of malondialdehyde being mediated by the presence of Fe^{2+}. Adrenal cortex mitochondria contain large amounts of cytochrome *P*-450 and an iron-sulfur protein called adrenal ferredoxin: they are present in approx. 2 nmol per mg of mitochondrial protein, which is about a 10 molar excess over the level of cytochrome oxidase. Upon the decomposition of the iron-sulfur chromophore, as in the case initiated by its binding to a cytochrome *c* molecule [24]; the iron ions would then be supplied enough quantity in order to induce the degradation of cytochrome *P*-450 and formation of malondialdehyde. In fact, Tappel [2] reported that in testis, which has an identical electron transport system to that of adrenal cortex mitochondria, a lipofuscin-like Schiff base compound was accumulated as the age of the rats increased. Therefore, mitochondria with a high content of ferredoxin, which are the cases of adrenal, testis and ovary, would be subject to this type of physiologically significant iron-mediated lipid peroxidation reaction.

The chemical reaction which leads to the degradation of cytochrome *P*-450 and the production of malondialdehyde is not fully understood through the present study. In this relation, Cheremisina et al. [25] studied on the mechanism of chemiluminescence coupled with Fe^{2+} - mediated lipid peroxidation reaction. Nakano and his colleagues [26] directly observed the emission spectra of $^1O_2$ during the NADPH- and molecular oxygen-dependent lipid peroxidation reaction by liver microsomes in the presence of Fe^{2+}. They claimed that based upon this result and on the inhibition experiments by dimethylfuran (a singlet oxygen trapper), $^1O_2$ is generated in the self-reaction of peroxy radicals: at a dimethylfuran concentration of 6.4 mM, both a 35 % inhibition of light emission and a 10 % inhibition of lipid peroxidation were observed. From our experiments, both the malondialdehyde formation and the chemilumines-

cence were not inhibited by a singlet oxygen quencher. Therefore, $^1O_2$ appears not to be responsible for the lipid peroxidation and the light emission reactions. In a recent work by Pederson and Aust [27], they concluded that $O_2^{\cdot-}$, $OH\cdot$ and $^1O_2$ are not involved in liver microsomal NADPH-dependent lipid peroxidation reaction. Consequently, all three laboratories agree as to the non-participation of $^1O_2$ in the Fe^{2+}-mediated lipid peroxidation reaction, although the source of light emission remains to be of debate.

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REFERENCES

1 Nakano, M., Tsutsumi, Y. and Uschijima, Y. (1971) Biochim. Biophys. Acta 252, 335–347
2 Tappel, A. L. (1973) Fed. Proc. 32, 1870–1874
3 Neubert, D., Wojtczak, A. B. and Lehninger, A. L. (1962) Proc. Natl. Acad. Sci. U.S. 48, 1651–1658
4 Hunter, Jr., F. E., Scott, A., Hoffstein, P. E., Guerra, F., Weinstein, J., Schneider, A., Schutz, B., Fink, J., Ford, L. and Smith, E. (1964) J. Biol. Chem. 239, 604–613
5 Hunter, Jr., F. E., Scott, A., Hoffstein, P. E., Gebicki, J. M., Weinstein, J. and Schneider, A. (1964) J. Biol. Chem. 239, 614–621
6 Levin, W., Lu, A. Y. H., Jacobson, M., Kuntzman, R., Boyer, J. L. and McCay, P. B. (1973) Arch. Biochem. Biophys. 158, 842–852
7 Jacobson, M., Levin, W., Lu, A. Y. H., Conney, A. H. and Kuntzman, R. (1973) Drug Metab. Disposition 1, 766–774
8 Vatsis, K. P., Kowalchyk, J. A. and Schulman, M. P. (1974) Biochem. Biophys. Res. Commun. 61, 258–264
9 Kamataki, T. and Kitagawa, H. (1973) Biochem. Pharmacol. 22, 3199–3207
10 Hunter, Jr., F. E., Gebriski, J. M., Hoffstein, P. E. and Weinstein, J. (1963) J. Biol. Chem. 238, 828–835
11 Greim, H. (1970) Naunyn-Schniedeberg's Arch. Pharmakol. 266, 261–275
12 Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2379–2385
13 Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370–2378
14 Kimura, T. and Suzuki, K. (1967) J. Biol. Chem. 242, 4085–4091
15 Gornall, A. G., Baradwill, C. S. and David, M. M. (1949) J. Biol. Chem. 177, 751–766
16 Hochstein, P., Nordenbrand, K. and Ernster, L. (1964) Biochem. Biophys. Res. Commun. 14, 323–334
17 Goda, K., Chu, J., Kimura, T. and Schaap, A. P. (1973) Biochem. Biophys. Res. Commun. 52, 1300–1306
18 Beauchamp, C. and Fridovich, I. (1970) J. Biol. Chem. 245, 4641–4646
19 Cohen, G., Heikkila, R. E. and MacNamee, D. (1973) J. Biol. Chem. 249, 2447–2452
20 Anbar, M. and Pecht, I. (1964) J. Phys. Chem. 68, 352–355
21 Pecht, I. and Faragii, M. (1972) Proc. Natl. Acad. Sci. U.S. 69, 902–906
22 Fridovich, I. (1972) Acc. Chem. Res. 5, 321–326
23 Zimmermann, R., Flohe, L., Weser, U. and Hartmann, H. (1973) FEBS Lett. 29, 117–120
24 Manabe, T. and Kimura, T. (1974) FEBS Lett. 47, 113–116
25 Cheremisina, Z. P., Olenev, V. I. and Vladimirov, Y. A. (1972) Biofizika 17, 605–610
26 Nakano, M., Noguchi, T., Sugioka, K., Fukutama, H., Sato, M., Shimizu, T., Tsuji, Y. and Inaba, H. (1975) J. Biol. Chem. 250, 2404–2406
27 Pederson, T. C. and Aust, S. (1975) Biochim. Biophys. Acta 385, 232–241