INTERACTIONS OF ORGANIC HYDROPEROXIDES WITH HEME PROTEINS

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SUMMARY

The decomposition of *p*-methylbenzyl hydroperoxide by cytochrome *c* and other selected heme systems in the absence of reducing agents was investigated. *p*-Methylbenzaldehyde was identified as the major product. A mechanism for this reaction has been suggested. H_2O_2 and tertiary cumyl hydroperoxide do not react under these conditions.

The ability of organic hydroperoxides to act as oxygen donors in the cytochrome-mediated 7-ethoxyresorufin-O-deethylation was studied. Cumyl and tert.butyl hydroperoxides are able to substitute oxygen in the absence of NADPH while *p*-methylbenzyl hydroperoxide is not.

KEY WORDS

hydroperoxide (4-methylbenzyl) - decomposition, hydroperoxide (cumyl), monooxygenase (cytochrome P-450 - dependent), 7-ethoxyresorufin deethylation, hemoproteins.

INTRODUCTION

Volatile monoaromatic hydrocarbons (toluene, xylenes) are widely used (and misused) in human practice. Hydroperoxidic derivatives of these hydrocarbons (easily formed by photocatalyzed autooxidation processes) can enter the organism either directly or as intermediates of metabolic pathways /1/. The aim of our work was to examine the degradation of *p*-methylbenzyl hydroperoxide (XyHP) by heme proteins and to compare in this respect XyHP with other hydroperoxides, especially the cumyl hydroperoxide (CuHP). One of the important questions is the mechanism of degradation of hydroperoxides and their involvement in the biotransformation of other xenobiotics. We paid some attention also to adverse effects of hydroperoxides on heme proteins and cellular lipids.

MATERIALS AND METHODS

Materials

XyHP was prepared by photo-catalyzed oxidation of *p*-xylene by dioxygen /2/ and separated by thin layer chromatography (TLC) on commercial starch-containing silica plates (Silufol; Kavalier, Czechoslovakia) from the liquid portion of the bulk reaction mixture partly solidified by crystallization (upon cooling) of the unreacted xylene; TLC on alumina layer was also used for obtaining larger amounts. *p*-Methylbenzaldehyde was prepared as described by Law and Perkin /3/. Other biochemicals and their sources: NAD^+ (Imuna, Czechoslovakia), $NADPH.Na_4$, horseradish peroxidase and horse liver alcohol dehydrogenase (Boehringer), CuHP (Merck, 80% solution in cumene), tert.butyl hydroperoxide (tBHP, Institute of Macromolecular Research, Czechoslovakia), *p*-xylene (Reachim, USSR), 7-ethoxyresorufin (Pierce), resorufin and 3-methylcholanthrene (Aldrich), 3,3',4,4'-tetrachlorobiphenyl (Riedl-de Haen), cytochrome *c* (BDH Chemicals) and oxyhemoglobin (Institute of Hematology, Charles University, Czechoslovakia).

Abbreviations: XyHP – *p*-methylbenzyl hydroperoxide; CuHP – cumyl hydroperoxide; tBHP – tert.butyl hydroperoxide; EROD – 7-ethoxyresorufin-O-deethylase.

Methemoglobin was prepared by ferricyanide oxidation of oxyhemoglobin and purified by dialysis; reduced cytochrome *c* from cytochrome *c* (Fe^{3+}) by reduction with $Na_2S_2O_4$ and separated on Sephadex G25 fine (Pharmacia) in the dark. Hemin was prepared from oxyhemoglobin /4/. Other analytical grade chemicals were purchased from Lachema (Czechoslovakia).

Fertile eggs from hisex brown layer hens (Přemyslovice, Czechoslovakia) were incubated at approximately 38°C and 70% humidity and turned every 6 h. 3 μg of 3,3'4,4'-tetrachlorobiphenyl in 100 μl hexane were injected into allantoic sac on incubation day 13. 24 h after the administration, the livers were homogenized in the IKA homogenizer (Janke & Kunkel, Germany), microsomes were obtained by differential centrifugation and the 105,000 *g* sediment was resuspended in 50 mM phosphate buffer, pH 7.3, containing 20% glycerol and 0.5 mM EDTA and frozen at -80°C until used. All procedures were carried out at 4°C. Microsomes from 35-day-old chicks after pretreatment with 3-methylcholanthrene (3 days, 20 mg/kg/day) were isolated as described by Machala *et al.* /5/.

Equipment

Spectrophotometers Shimadzu UV-3000 and Varian DMS-100, luminescence spectrometer Perkin-Elmer LS-50, IR spectrophotometer Perkin-Elmer 783, NMR spectrometer Tesla BS 567 (100.035 MHz), oxygen analyzer (based on Clark electrode) SOPS 32/ ADLC2 (Laboratorní přístroje, Czechoslovakia), ultracentrifuge Beckman L-8.

Methods

Hydroperoxides were assayed by the ferrimetric method /6/ calibrated by the standard $FeCl_3$ solution and (for XyHP and H_2O_2) verified by the iodometric method /7/. The concentration of CuHP was calculated from Eq. 1, that of other hydroperoxides from Eq. 2 /6/:

$$c_{CuHP}\,[mM] = 285 \,.\, A_{512.5} / \text{sample volume}\,[\mu l] \qquad [1];$$

$$c\,[mM] = 321 \,.\, A_{512.5} / \text{sample volume}\,[\mu l] \qquad [2].$$

For very low XyHP concentrations, its ability to inhibit very strongly the activity of horse liver alcohol dehydrogenase /8/ was used. Enzyme activity depression was plotted against the concentration of XyHP (less than one nanomole) added to 1 ml of the standard assay mixture /9/ to obtain the concentration curve.

7-Ethoxyresorufin-O-deethylase (EROD) activity was measured by fluorometric determination of resorufin as described by Prough *et al.* /10/; an excitation wavelength of 530 nm and an emission wavelength of 585 nm were used. Concentrations of heme proteins were determined spectrophotometrically /11-13/ and expressed in μmoles heme Fe; hemin concentration was measured as described by Hrkal and Klementová /14/.

RESULTS AND DISCUSSION

Degradation of hydroperoxides by heme proteins

We first determined the rates of hydroperoxide degradation by selected heme proteins. After 1 hour incubation in mixtures of 9 μM cytochrome *c* (in 0.01 M phosphate, pH 7.1, at 25°C) with 7.5 mM CuHP and 6.0 mM XyHP, respectively, the concentration of CuHP did not change while XyHP disappeared completely. Fig. 1 shows the changes in the UV spectrum during the reaction of XyHP with cytochrome *c*. The rise of the absorption peak at 260 nm is very pronounced. No absorption change was observed in similar mixtures with 90 μM CuHP or 130 μM H_2O_2.

The absorption at 260 nm was used also for monitoring the time dependence of the XyHP degradation by other heme proteins, i.e., methemoglobin, horse radish peroxidase and liver microsomes from a 35-day-old chicken, which contained cytochromes P-450 and b_5 and NADPH-cytochrome P-450-reductase (Fig. 2).

Characterization of XyHP degradation products

In a subsequent experiment, we searched for the compound responsible for the 260 nm absorption in mixtures of XyHP and cytochrome *c*. The total A_{260} increase is directly proportional to XyHP concentration (Fig. 3a) while the cytochrome *c* concentration influences only the initial rate and not the total amount of the reaction

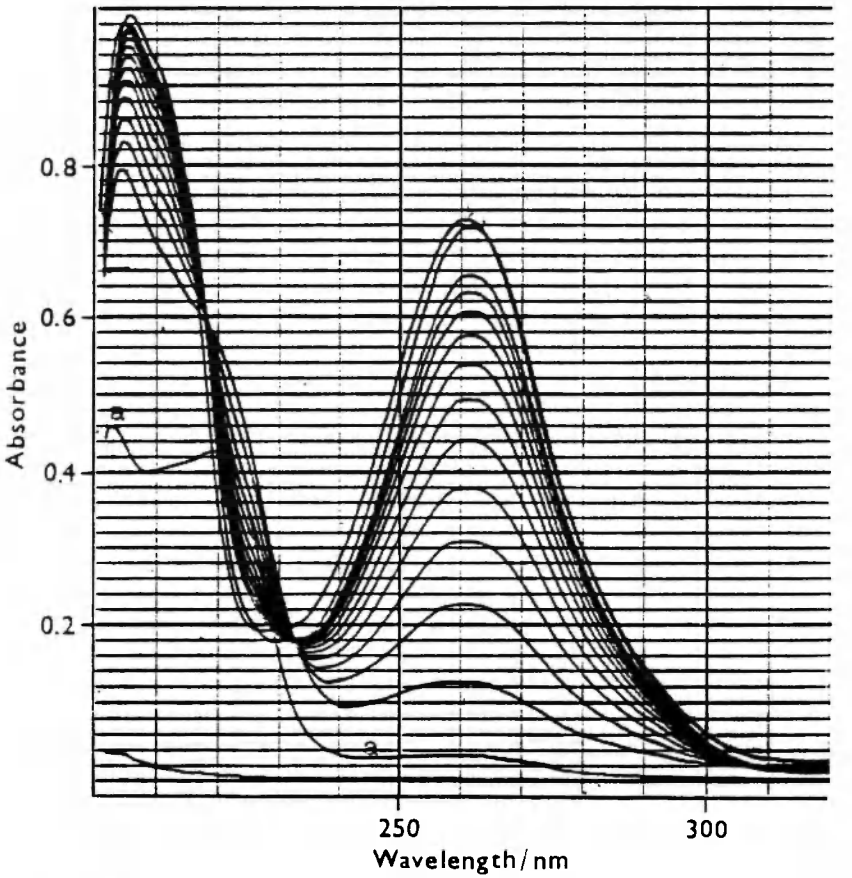

Fig. 1: Spectral changes of the reaction of XyHP with cytochrome *c*. The curve (a) represents the UV-spectrum of 45 μM XyHP in 0.1 M phosphate buffer, pH 7.1 at 25°C. Other curves represent difference spectra of the mixture of 45 μM XyHP with 1.05 μM cytochrome *c* against 1.05 μM cytocrome c in the same buffer. Spectra were scanned at time intervals of 2 min, the last two were scanned after 30 min and 2 hours, respectively. The scan speed was 100 nm/min.

product (Fig. 3b). The final value of A_{260} does not increase after the addition of cytochrome *c* or H_2O_2 but increases again after the addition of XyHP (data not shown).

The decrease of XyHP concentration in the reaction mixture with cytochrome *c* correlates with the A_{260} increase (Fig. 4). XyHP and

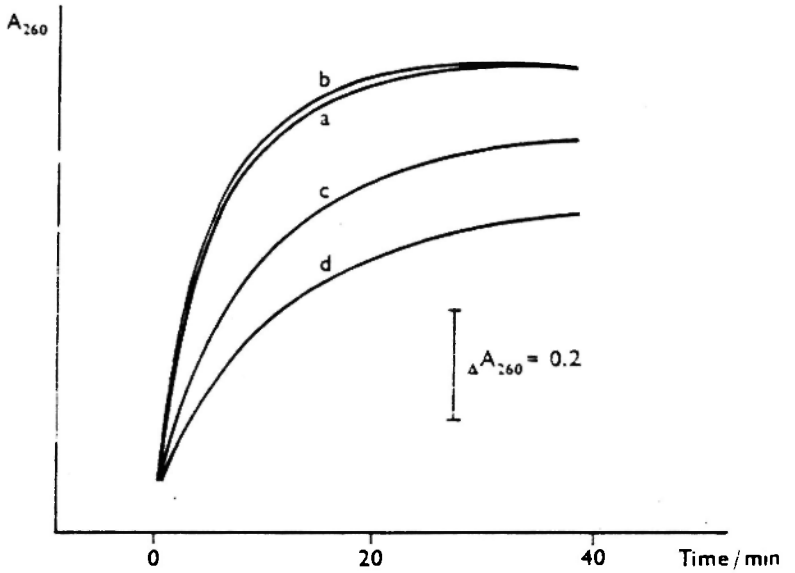

Fig. 2: Time course of the A_{260} increase. The following mixtures were used: 45 μM XyHP + 1.05 μM cytochrome *c* (curve a), 50 μM XyHP + 2.2 μM methemoglobin (curve b), 70 μM XyHP + 0.7 μM horseradish peroxidase (curve c), and 50 μM XyHP + microsomal fraction containing 0.10 μM cytochrome P-450 + 0.05 μM cytochrome b_5 (curve d). Reaction conditions see Fig. 1.

benzyl hydroperoxide are strong selective inhibitors of horse liver alcohol dehydrogenase /8/; actual concentration of XyHP in the reaction mixture with cytochrome *c* was measured by its inhibitory power towards the alcohol dehydrogenase reaction.

We attempted to isolate and to characterize the major XyHP degradation product resulting from the heme protein-dependent reaction by several methods. An obvious alternative of the XyHP transformation is the reduction to the corresponding alcohol. In our case the absorption peak at 260 nm could not be associated with *p*-methylbenzyl alcohol which has a very different absorption spectrum /15/. On the contrary, another possible product, *p*-methylbenzaldehyde, has an absorption maximum at 260 nm /16/ and it was this product which we were able to identify by both NMR and IR spectra and also by its chromatographic behavior on TLC when compared with the respective authentic sample.

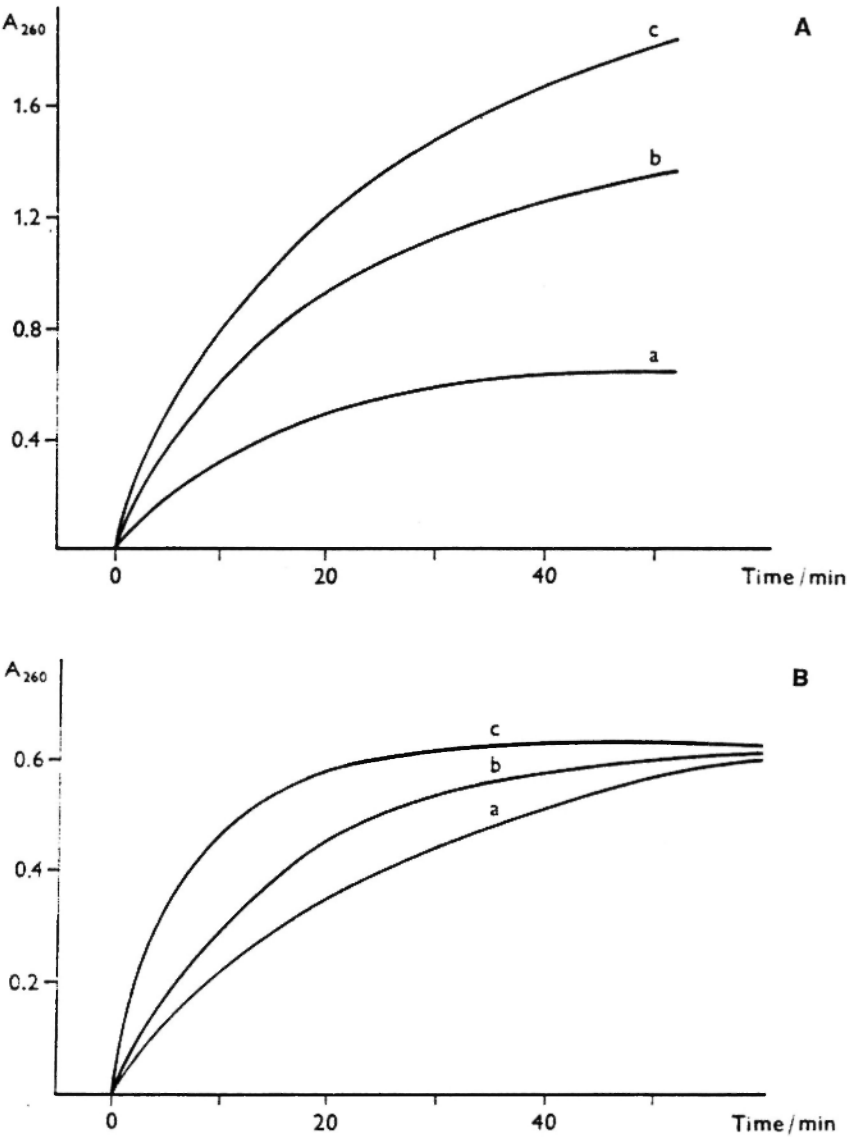

Fig. 3: Dependence of A_{260} increase rates on various concentrations of XyHP (A) and cytochrome c (B). **A:** Concentration of cytochrome *c* was 2.4 μM; concentrations of XyHP were 50 μM (a), 100 μM (b), and 150 μM (c). **B:** Concentration of XyHP was 65 μM; concentrations of cytochrome *c* were 0.67 μM (a), 1.7 μM (b), and 3.3 μM (c). Reaction conditions see Fig. 1.

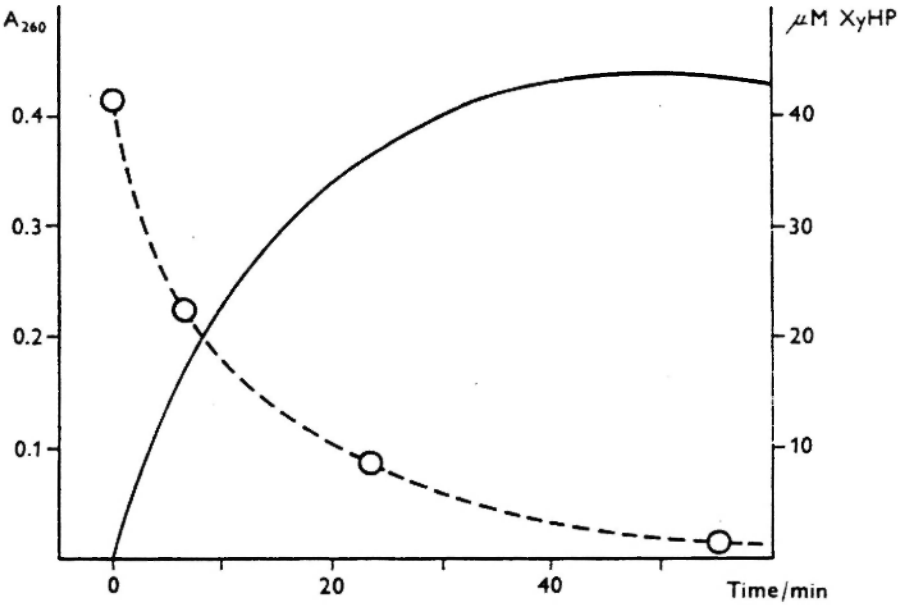

Fig. 4: Correlation between increasing absorbance at 260 nm (solid line) and decreasing concentration of XyHP (dashed line) measured as a potency to inhibit horse liver alcohol dehydrogenase /8/. Samples of the reaction mixture (40 μM XyHP + 0.75 μM cytochrome c) were added to the mixture containing 2 μg/ml horse liver alcohol dehydrogenase, 0.5 mM NAD^+, 10 mM ethanol, and 0.1 M glycine buffer, pH 10.0 placed in the 1-cm quartz cuvette, and the decrease of the alcohol dehydrogenase reaction rate (dA_{340}/dt) was measured. The reaction of XyHP with cytochrome c was performed under the same conditions as those in Fig. 1.

After the completion of the reaction, the mixture of XyHP and cytochrome *c* was extracted by an organic solvent (CCl_4). The 260 nm-absorbing compound was found in the organic, not in the aqueous phase. The NMR spectrum in deuterochloroform (after evaporation of CCl_4) revealed one aldehydic hydrogen (9.95 ppm), eight hydrogen atoms of two nonidentical *p*-substituted benzene rings (within the range of 6-7 ppm), two hydrogens of the methylene group (4.64 ppm), six hydrogens of two different methyl groups (~2.4 ppm) and one hydroxyl hydrogen (1.78 ppm). The spectrum was interpreted as that of a mixture of *p*-methylbenzaldehyde and *p*-methylbenzyl alcohol.

The IR spectrum also corresponded to the values tabulated for these two compounds /17/. In TLC on Silufol® with chloroform-benzene 3:1 (v/v) as the mobile phase, the isolated product and the authentic sample had identical R_f of 0.7 and showed a yellow stain after spraying with 2,4-dinitrophenylhydrazine. The yield of the reaction of XyHP with heme proteins, expressed as a concentration percentage of the *p*-methylbenzaldehyde (calculated on the 260 nm absorption coefficient $\varepsilon = 15.4\ mM^{-1}.cm^{-1}$ /16/), related to the starting XyHP concentration, was higher than 90% (Table 1). This leads us to the conclusion that *p*-methylbenzyl alcohol, found in the reaction mixtures, was rather an unremovable contaminant of the XyHP solutions used than a real XyHP conversion product.

TABLE 1

Yields of *p*-methylbenzaldehyde in the reaction of XyHP with various heme proteins. Extinction coefficient of 15.4 $mM^{-1}.cm^{-1}$ /15/ was used for the calculation of the concentration of *p*-methylbenzaldehyde. Hydroperoxide was determined by the ferrimetric method (see Materials & Methods). The reaction was performed in 0.1 M phosphate buffer, pH 7.1 at 25°C and monitored as an A_{260} increase.

Concentration of hydroperoxide /μM	Heme protein in reaction	Concentration of heme iron /μM	% yield of aldehyde
45	cytochrome *c*	1.1	102
65	cytochrome *c*	0.7	92
50	methemoglobin	2.2	104
40	microsomes	*	95
150	horseradish peroxidase	2.3	91

*9.5 nM cytochrome P-450, 4.5 nM cytochrome b_5.

Comparison of the kinetics of XyHP degradation by different heme systems

Since the determination of the K_m and V_{max} values of the XyHP degradation by heme proteins proved to be problematic due to an oxidative damage of heme proteins at higher XyHP concentrations, we decided to compare the effects of heme proteins on XyHP by using the k constant of the initial reaction rate (Eq. 3) which proved

to be valid up to 0.2 mM XyHP in the reaction mixture (data not shown):

$$dc_{RCHO} / dt_o = k \cdot c_{XyHP} \cdot c_{hem} \quad [3]$$

c means molar concentration, RCHO is *p*-methylbenzaldehyde. The values obtained are summarized in Table 2.

TABLE 2

Values of k from Eq. 3 for various heme systems. Initial rates were measured as described in Table 1. The values represent means of series of results obtained with various concentrations of XyHP (40 to 150 μM) and heme proteins (0.5 to 2.5 μM, data not shown, standard deviations did not exceed 10%).

Heme system	$k/mM^{-1}.s^{-1}$
cytochrome *c* (Fe^{3+})	1.3
cytochrome *c* (Fe^{2+})	1.1
methemoglobin	4.0
horseradish peroxidase	1.4
hemin	1.2
protoporphyrin IX + $FeCl_3$	0.01
$FeCl_3$	0

Interactions of hydroperoxides with chick embryo liver microsomal cytochromes P-450

Liver microsomal cytochromes P-450 (the key enzymes of the monooxygenase system) exhibit a peroxidase-like activity with organic hydroperoxides in absence of NADPH and utilize them in various monooxygenase reactions /18, 19/. 7-Ethoxyresorufin-O-deethylase (EROD) activity, which is the specific reaction of the methylcholanthrene-inducible forms of cytochrome P-450, can also be supported by CuHP /20/. We measured the hydroperoxide-dependent and the NADPH-dependent EROD activities of the liver microsomes from 14-day-old embryos after pretreatment with 3,3', 4,4'-tetrachlorobiphenyl, a methylcholanthrene-type inducer. Fig. 5 shows the curves of the reaction started by tBHP, CuHP or XyHP.

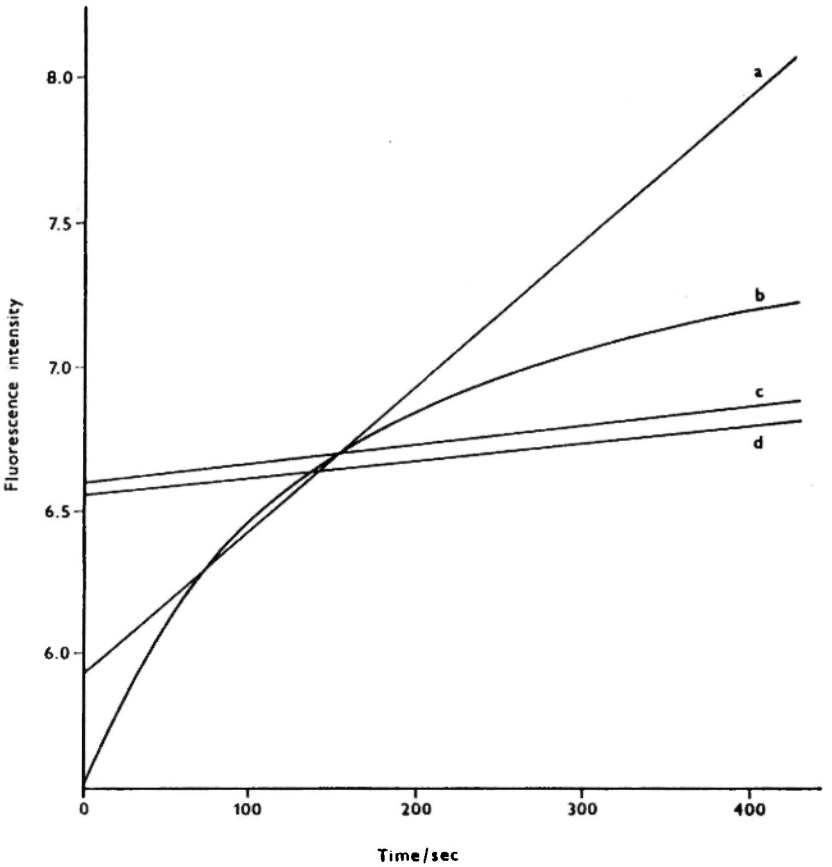

Fig. 5: Hydroperoxide-dependent EROD reaction. Reaction mixtures contained 1.85 ml phosphate buffer (0.01M, pH 7.3 with 0.15 M KCl and 1mM ethylenedianminetetraacetic acid), 0.1 ml of 7-ethoxyresorufin solution (5 mg per 1 l of the same buffer with 1% ethanol v/v), and 0.5 ml of chick embryo liver microsomal fraction (see Materials and Methods). The reactions were started by addition (to the final concentrations) of 1.35 mM tBHP (a), 1.35 mM CuHP (b), 1.35 mM XyHP (c), and 13.5 mM XyHP (d) and monitored fluorometrically (excitation wavelength 530 nm, emission wavelength 585 nm) at 25°C.

While both the hydroperoxides bearing their functional group on a tertiary carbon supported the EROD activity, XyHP did not, even at a higher concentration.

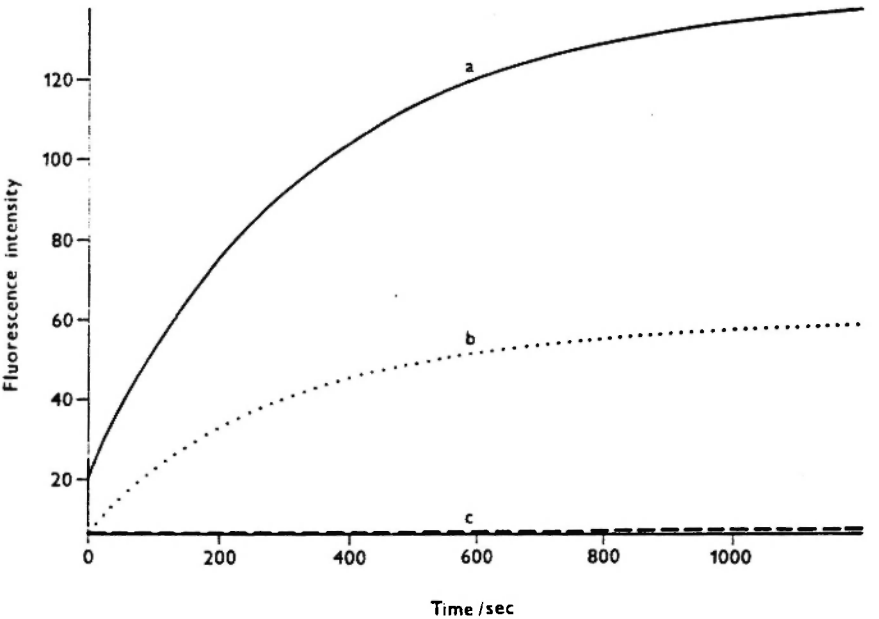

Fig. 6: Comparison of NADPH- and hydroperoxide-dependent reactions. The reactions were started by additions (to the final concentration) of 0.5 mM NADPH (a), 13.5 mM tBHP (b), and 13.5 mM XyHP (c). Reaction conditions were the same as those in Fig. 5.

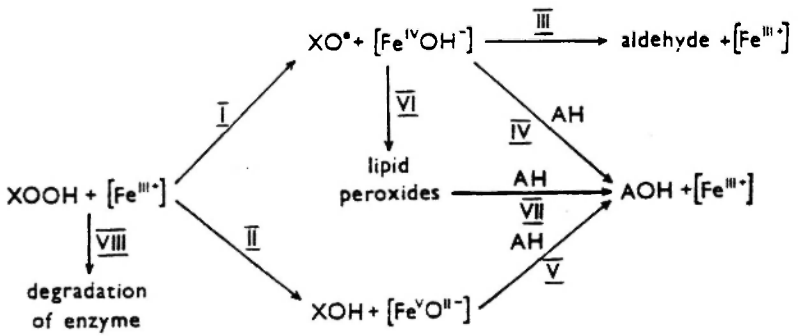

Fig. 7: Schematic overview of possible reactions of hydroperoxide with cytochrome P-450. Square brackets symbolize the porphyrin ring, AH means a substrate of the hydroxylation reations. The pathways I-VIII are described in the text.

Fig. 6 shows a comparison of the NADPH-dependent and the hydroperoxide-dependent EROD activities which unequivocally demonstrates different kinetic parameters of the reaction.

Proposed mechanism of the XyHP-heme proteins interactions

Based on data in the literature which deal primarily with interactions of microsomal cytochromes P-450 and hydroperoxides, and taking into account the above experimental data, we present here a schematic overview of possible reactions of hydroperoxides with heme proteins (Fig. 7). This scheme involves certain extrapolations from interactions of hydroperoxides with cytochrome *c*.

Generally, it is well known that both homolytic (type I in our scheme) and heterolytic cleavages (type II) of the hydroperoxide O-O bond can occur in liver microsomes. As suggested by Marnett *et al.* /21/, the microsomal cytochrome P-450 reduces hydroperoxides preferentially by the one-electron mechanism producing the corresponding alkyloxyl radical and a ferryl-hydroxo complex (I). The same authors demonstrated a possibility of the oxidation of alkyloxyl to aldehyde under the concomitant heme regeneration (reaction III); simultaneously, also the monooxygenation of the substrate AH can occur (reaction IV). Another pathway is the degradation of the cytochrome by the hydroperoxides (VIII), especially in absence of a suitable substrate for the monooxygenation reaction /19/. Reaction VIII was observed as a significant decrease of the absorbance of heme proteins in the Soret band during the interactions with XyHP (Zídek and Skurský, unpublished data).

The homolytic reaction I prevails /22/; under certain conditions also the $[Fe\text{-}O]^{3+}$ complex formation (reaction II) might be preferred. The ferryl complex is supposed to be effective as a hydroxylating or epoxidating agent of the substrate AH (reaction V).

CuHP is an intensively studied hydroperoxide. Most authors suggest the homolytic mechanism of CuHP degradation followed by monooxygenase reaction IV. An alternative pathway, involving the initiation of lipoperoxidation by cumene oxyl radical has also been considered; the peroxyl radicals of unsaturated lipids are then thought to be the ultimate monooxygenating agents (reaction VI and VII), e.g., /21/. Our own experiments have shown that the reactions I and III take place in the XyHP-heme protein interaction most

probably and that the aldehyde formation can be interpreted as a result of the homolytic scission of XyHP.

Although we performed detailed experiments with cytochrome *c* only, we believe that the homolytic cleavage mechanism can be assumed also in interactions of XyHP with other heme proteins, since the characteristic increase of A_{260} was observed with all heme compounds studied. On the other hand, in contrast to the behavior of CuHP, XyHP did not support the hydroperoxide-dependent monooxygenase reaction IV in the chick embryo liver microsomal fraction (Fig. 5). However, XyHP is, like CuHP, able to interact with rabbit liver cytochrome $P\text{-}450_{LM2}$ to form the so-called "C-complex" described by Blake and Coon /23/.

Alternative mechanisms of the XyHP degradation were also considered. The dismutation of two peroxyl radicals into aldehyde and alcohol plus one molecule of dioxygen /24/ seems to be excluded. The high yields of the aldehydic product contradict it and our attempts to register any O_2 concentration increase (by the Clark oxygen electrode) failed under experimental conditions. The possibility that *p*-methylbenzyl alcohol as a product of the peroxidase-supported decomposition of XyHP could also be effective as a reducing agent in the peroxidase reaction cycle seems unlikely because no increase of absorbance around 250 nm (typical for benzaldehyde /15/) was observed in mixtures of benzyl alcohol with CuHP and H_2O_2 in the presence of cytochrome *c*.

CONCLUSIONS

Different types of interactions of heme proteins with CuHP (or tBHP) and XyHP were investigated. Our results demonstrate the degradation of XyHP to *p*-methylbenzaldehyde which is catalyzed by the heme prosthetic group in absence of reducing compounds. In contrast to the common peroxidase reactions, the influence of the apoprotein type upon the reaction rate is very small. The radical mechanism involving the homolytic cleavage of the hydroperoxidic O-O bond seems to be most probable. As the degradation of XyHP was observed also in the microsomal fraction, we can suppose the same mechanism of interactions between benzyl hydroperoxides (with the functional group on a primary carbon atom) and cytochrome *c* or microsomal cytochromes. This conclusion is also sup-

ported by the results of our experiments studying the role of hydroperoxides as active oxygen donors in the EROD reaction.

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