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THE ACTION OF HYDROGEN PEROXIDE ON THE FORMATION OF THIOBARBITURIC ACID-REACTIVE MATERIAL FROM MICROSOMES, LIPOSOMES OR FROM DNA DAMAGED BY BLEOMYCIN OR PHENANTHROLINE. ARTEFACTS IN THE THIOBARBITURIC ACID TEST

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Incubation of rat-liver microsomes, previously azide-treated to inhibit catalase, with H_2O_2 caused a loss of cytochrome P-450 but not of cytochrome b_5. This loss of P-450 was not prevented by scavengers of hydroxyl radical, chain-breaking antioxidants or metal ion-chelating agents. Application of the thiobarbituric acid (TBA) assay to the reaction mixture suggested that H_2O_2 induces lipid peroxidation, but this was found to be due largely or completely to an effect of H_2O_2 on the TBA assay. By contrast, addition of ascorbic acid and Fe(III) to the microsomes led to lipid peroxidation and P-450 degradation: both processes were inhibited by chelating agents and chain-breaking antioxidants, but not by hydroxyl radical scavengers. H_2O_2 inhibited ascorbate/Fe (III)-induced microsomal lipid peroxidation, but part of this effect was due to an action of H_2O_2 in the TBA test itself. H_2O_2 also decreased the colour measured after carrying out the TBA test upon authentic malondialdehyde, tetraethoxypropane, a DNA-Cu^{2+}/*o*-phenanthroline system in the presence of a reducing agent, ox-brain phospholipid liposomes in the presence of Fe(III) and ascorbate, or a bleomycin-iron ion/DNA/ascorbate system. Caution must be used in interpreting the results of TBA tests upon systems containing H_2O_2.

KEY WORDS: Microsomes, iron, lipid peroxidation, ascorbate, cytochrome P-450, hydrogen peroxide, bleomycin, DNA, copper-phenanthroline.

INTRODUCTION

Microsomes are a heterogeneous collection of vesicles formed upon homogenization of tissues; they can be pelleted by high-speed centrifugation[1]. Many (although by no mean all) of the vesicles in the pellet arise from the endoplasmic reticulum of the cell, and so microsomes are enriched in cytochrome P-450 and NADPH-cytochrome P-450 reductase.[1] Despite their heterogeneity, microsomes are popular substrates for studies of lipid peroxidation and tests of the efficacy of chain-breaking antioxidants.[2,3] Microsomal lipid peroxidation can be induced by adding Fe^{2+} ions, Fe^{2+}/Fe^{3+} mixtures, Fe^{3+}/ascorbate or Fe^{3+}-chelates and NADPH[2-4]. Despite the extensive studies that have been carried out on microsomal systems, the mechanism of iron-stimulated microsomal lipid peroxidation is uncertain.[4,5] For example, hydroxyl

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radicals (·OH) are formed in peroxidizing microsomal systems and their production is inhibited by catalase. However, neither catalase nor scavengers of ·OH usually inhibit iron ion-dependent peroxidation in microsomal or liposomal systems.[4,6–8] During microsomal lipid peroxidation induced by NADPH and Fe^{3+}-chelates[9–11], or by addition of organic hydroperoxides[12,13], cytochrome P-450 is destroyed.

Although hydrogen peroxide is formed in peroxidizing liposomal or microsomal systems[4,6–8,12,14], it is not essential for peroxidation to proceed. Indeed, addition of H_2O_2 has been reported not to stimulate[15], or even to inhibit[8,15], microsomal lipid peroxidation. In these experiments, it is necessary to add azide to inhibit catalase, which contaminates microsomal fractions.[1] Azide itself has no effect on the peroxidation process.[14] H_2O_2 at high concentrations has been shown to cause haem degradation and iron ion release from several haem proteins, including haemoglobin,[16,17] leghaemoglobin,[18] myoglobin[19] and purified cytochrome P-450.[20] However, Mannering *et al.*[21] were unable to detect loss of P-450 in microsomes exposed to O_2^- and H_2O_2.

In the present paper, we show that addition of H_2O_2 to rat-liver microsomes causes degradation of cytochrome P-450 and leads to an apparent lipid peroxidation, which turns out to be an artefact of the thiobarbituric acid (TBA) assay. The properties of this system are compared with those of the lipid peroxidation induced by adding $FeCl_3$/ascorbate to microsomes, or to liposomes made from ox-brain phospholipids.[26] The effect of H_2O_2 on the reaction of TBA with malondialdehyde, tetraethoxypropane and the products produced by breakdown of DNA in the presence of Cu^{2+}-phenanthroline and reducing agents[22] or bleomycin-Fe^{3+} and ascorbate[23] is also reported.

MATERIALS AND METHODS

Reagents

Desferrioxamine was a kind gift from CIBA-Geigy. All other reagents were of the highest quality available from Sigma Chemical Corp. or from BDH Chemicals Ltd. Catalase was Sigma type C-40; 1 unit decomposes 1 μmol of H_2O_2 per minute under the reaction conditions given in the Sigma catalogue.

Microsomes were prepared from the livers of adult male rats, fasted overnight before sacrifice, by differential pelleting.[1,24] Rats were killed by cervical dislocation and the livers perfused *in situ* with 10 ml of cold KCl (1.15% [w/v]). Livers were removed, discarding connective tissue and bile ducts and placed in ice-cold 1.15% (w/v) KCl. They were minced with scissors, washed well to remove blood and then gently homogenized in a Potter homogenizer at 4°C using a Teflon pestle. The homogenate was centrifuged at 500 g for 10 min, the supernatant at 10,000 g for 15 min and the supernatant from that at 100,000 g for 1 h. All centrifugations were carried out at 4°C. The microsomal pellet was washed three times in 1.15% (w/v) KCl and finally resuspended in 20 mM KH_2PO_4-KOH buffer, pH 7.4, using two strokes of the Potter homogenizer. If not used immediately, the microsomal suspension was aliquoted and stored at −20°C for a period not exceeding 3 weeks.[24] Protein content was measured by a modification[25] of the Lowry method.

Ox-brain phospholipid liposomes were prepared as in.[26]

Peroxidation of microsomes and liposomes

Reaction mixtures contained, in a final volume of 1.0 ml, the following reagents at the final concentrations stated: KH_2PO_4-KOH buffer pH 7.4 (10 mM), microsomes (0.5–1.0 mg protein) or liposomes (0.2 ml of 5 mg/ml in phosphate-buffered saline)[26] and, where indicated, either $FeCl_3$ and ascorbic acid (each 100 μM) or H_2O_2 (usually 4 mM). Reaction mixtures were incubated at 37°C, usually for 25 min. When H_2O_2 was used, the microsomes were pre-incubated with sodium azide (final concentration 3 mM) to inhibit any traces of contaminating catalase, at 37°C for 30 min. Azide had no effect on peroxidation in any system. After incubation, peroxidation was measured by the TBA test. To each reaction mixture, 1 ml of 1.0% (w/v) TBA in 50 mM NaOH was added, together with 1 ml of 2.8% (w/v) trichloracetic acid. Tubes were heated for 30 min at 80°C in a water bath or at 100°C for 15 min on a heating block. Sometimes 0.1 ml of a 0.2% (w/v) solution of BHT in ethanol was included with the TBA reagents. Solutions of H_2O_2, $FeCl_3$ and ascorbic acid were made up fresh just before use.

Cytochromes P-450 and b_5 in microsomes were measured as in.[27] Reaction mixtures were as described for lipid peroxidation. Molar extinction coefficients of 91 cm^{-1} mM^{-1} for cytochrome P-450 and 171 cm^{-1} mM^{-1} for cytochrome b_5 were used.

DNA degradation by Cu-phenanthroline and Fe-bleomycin in the presence of ascorbic acid were measured as in.[22,23]

"Malondialdehyde" preparation. 50 μl of 1, 1, 3, 3-tetraethoxypropane was diluted to 25 ml with 40% (v/v) ethanol. Then 0.5 ml of the resulting solution was further diluted to 25 ml with 40% (v/v) ethanol, to give a stock solution.

RESULTS

Destruction of microsomal cytochrome P-450 by H_2O_2

Rat liver microsomes, treated with azide to inhibit catalase, were incubated with H_2O_2 at millimolar concentrations. There was a rapid loss of cytochrome P-450 (Figure 1 and 2) but no significant change in the cytochrome b_5 content of the microsomes (data not shown).

Table I summarizes a representative experiment showing the effects of various oxidant scavengers on this system. The hydroxyl radical scavengers sodium formate or mannitol had no significant effect on P-450 destruction, nor did the chain-breaking antioxidants gossypol[28] or butylated hydroxytoluene (BHT). Addition of excess catalase inhibited, as would be expected (data not shown).

Apparent microsomal lipid peroxidation stimulated by H_2O_2

In view of previous reports that H_2O_2 has no effect on[15] or inhibits[8,15] microsomal lipid peroxidation, we were surprised to find an apparent peroxidation in our reaction system, as measured by the thiobarbituric acid (TBA) test (Figure 1 and Table I). This peroxidation was inhibited by including chain-breaking antioxidants in the reaction mixture (gossypol and BHT, Table I) but not by scavengers of $^{\cdot}OH$. Desferrioxamine,

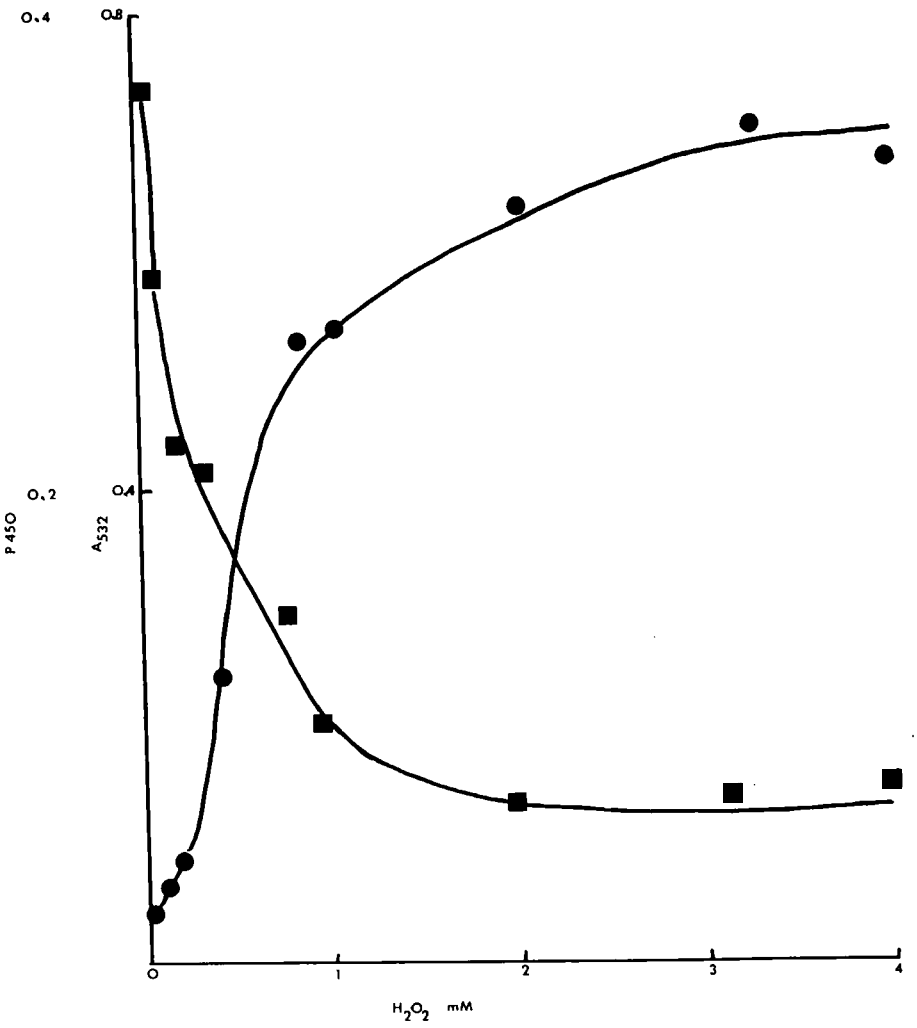

FIGURE 1 Cytochrome P-450 destruction and apparent lipid peroxidation in microsomes incubated with H_2O_2. Microsomes were incubated with H_2O_2 at the final concentration indicated. Lipid peroxidation was measured as thiobarbituric acid reactivity (A_{532}) and cytochrome P-450 measured by differential spectral analysis at 450–490 nm between the reduced form and its carbon monoxide complex. ●Thiobarbituric acid reactivity (TBA) A_{532} ■Cytochrome P-450 concentration, nmol/mg protein.

a chelating agent known to inhibit iron ion-dependent lipid peroxidation[2] and ·OH generation,[29] inhibited the peroxidation (Table II) but had little effect on P-450 degradation. By contrast, ferrioxamine (Fe^{3+}-desferrioxamine) did not inhibit (data not shown). EDTA also strongly inhibited the apparent peroxidation (Table II) but not the P-450 degradation. By contrast, the chelator *o*-phenanthroline *increased* the apparent rate of lipid peroxidation (Table II) but not that of P-450 degradation. Transferrins are known to inhibit iron-ion dependent free radical reactions (discussed

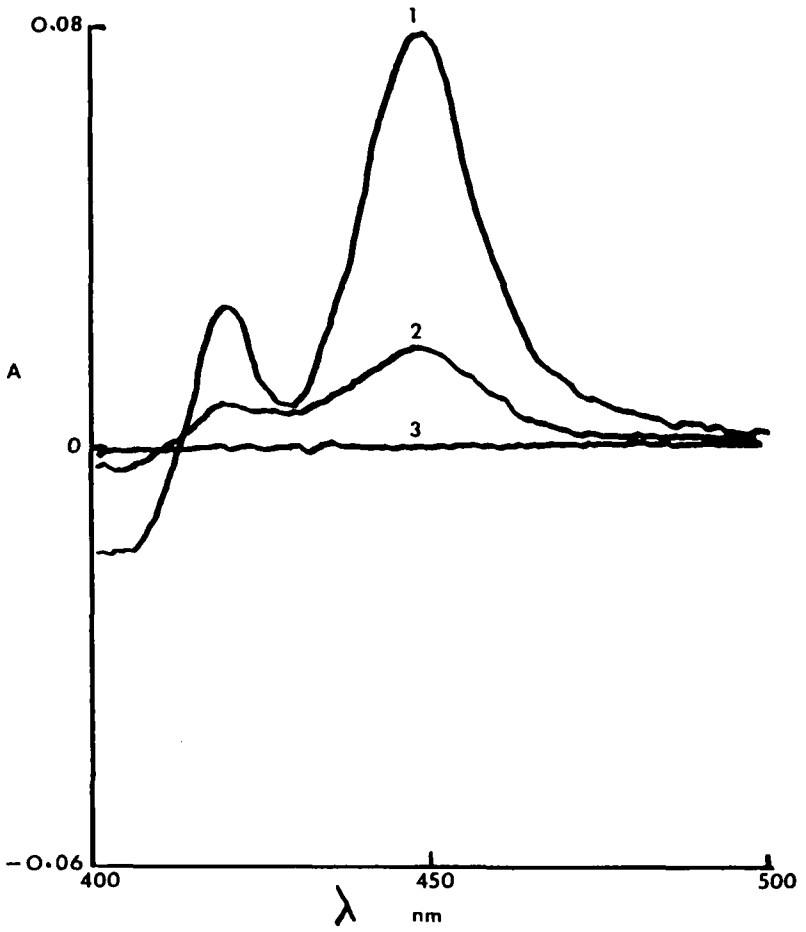

FIGURE 2 Effect of H_2O_2 on microsomal cytochrome P-450. Reaction mixtures were as described in the legend to Figure 1. Spectra were run after 30 min incubation. 1 — control, without H_2O_2 2 — with 4 mM H_2O_2. The loss of cytochrome P-450 (measured at 450 nm after reaction with CO) can be clearly seen. 3-Baseline.

TABLE 1

Effect of antioxidants on cytochrome P-450 destruction and apparent lipid peroxidation induced by H_2O_2 in rat-liver microsomes. H_2O_2 was present at 4 mM final concentration. Gossypol and butylated hydroxytoluene were added dissolved in ethanol, which itself had no effect on the peroxidation process.

Antioxidant added to reaction mixture	Final concentration	TBA reactivity measured A_{532}	Cytochrome P-450 nmol/mg protein
None (omit H_2O_2)	-	0.000	0.71
None	-	0.556	0.22
Mannitol	20 mM	0.551	0.28
Sodium formate	20 mM	0.555	0.26
Gossypol	10 μM	0.000	0.31
Phenobarbital	10 μM	0.523	0.20
Butylated hydroxytoluene	10 μM	0.089	0.32

in [30]), but ovotransferrin (conalbumin) had no inhibitory effect on H_2O_2-induced lipid peroxidation or P-450 degradation; indeed, it reproducibly *stimulated* peroxidation (Table II).

The TBA test is a very complex assay system, prone to many artefacts.[31-36] Control experiments showed that the apparent stimulation of microsomal lipid peroxidation by H_2O_2 is also largely an artefact of the TBA test, in that it was still seen when H_2O_2 was added immediately after the TBA reagents (before heating) instead of being included in the incubation mixture (Tables II and III). The effects of the metal ion chelating agents on H_2O_2-dependent peroxidation could also be reproduced when they were added during the assay (Table II, second column). The spectrum of the chromogen produced (Figure 3) was the same in all cases and is similar to that of a $(TBA)_2$-MDA adduct.[31] Incubation of H_2O_2 with the TBA reagents in the absence of

TABLE II

Effects of chelating agents on apparent microsomal lipid peroxidation stimulated by H_2O_2. H_2O_2 (4 mM final concentration) was included either during the incubation with microsomes (column A) or added immediately after the TBA reagents but before heating (column B). A_{532} values are the means of duplicate determinations that differed by < 10%. A representative experiment is shown. Results have been corrected for the background peroxidation in reaction mixtures to which H_2O_2 was not added.

Chelator added	Concentration	Extent of peroxidation (A_{532})	
		A	B
None	-	0.294	0.303
Desferrioxamine	1 mM	0.000	0.000
	100 μM	0.000	0.000
o-Phenanthroline	0.8 mM	0.418	0.480
	0.4 mM	0.451	0.503
	100 μM	0.467	0.436
EDTA	100 μM	0.000	0.000
	50 μM	0.000	0.000
	20 μM	0.000	0.000
	10 μM	0.000	0.070
Conalbumin	100 μM	0.494	0.610

TABLE III

Effects of H_2O_2 on microsomal lipid peroxidation: concentration dependence. H_2O_2 at the final concentrations stated was included either during the incubation with microsomes (column A) or added immediately after the TBA reagents (column B). A_{532} values are the means of duplicate determinations that differed by < 8%. A representative experiment is shown. Results have been corrected for the background rate of peroxidation in reaction mixtures to which H_2O_2 was not added.

$[H_2O_2]$ mM	Extent of peroxidation A_{532}	
	A	B
0.10	0.051	0.017
0.25	0.149	0.028
0.50	0.285	0.108
1.00	0.450	0.205
4.00	0.603	0.320
10.00	0.649	0.321
15.00	0.629	0.248
20.00	0.493	0.066

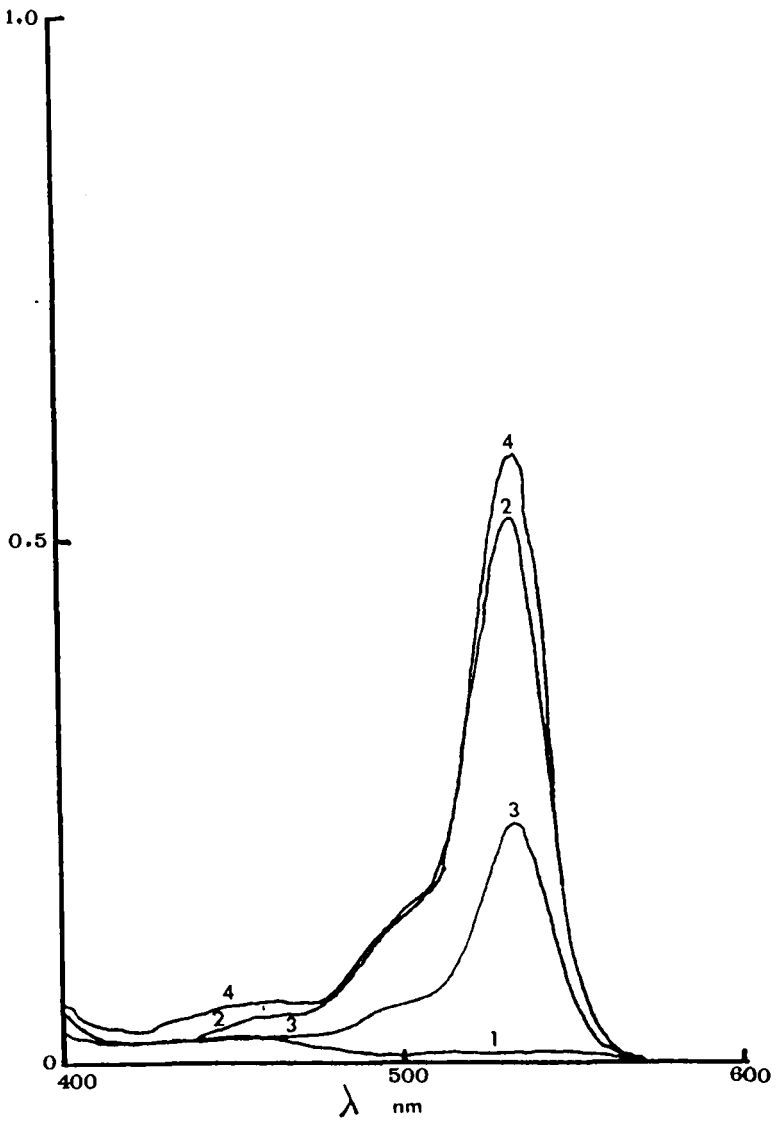

FIGURE 3 Spectrum of the chromogen generated in the TBA reaction Line 1. H_2O_2 (4 mM) incubated with TBA reagents. 2. Microsomes incubated with H_2O_2, then TBA test performed. 3. Microsomes incubated, then TBA reagents and H_2O_2 added and TBA test performed. 4. Microsomes incubated with $FeCl_3$ and ascorbic acid, then TBA test performed.

microsomes produced no chromogen (Figure 3). In some experiments an identical amount of peroxidation was seen whether H_2O_2 was included in the incubation mixture or added with the TBA reagents (e.g. Table II) but in others the peroxidation observed when H_2O_2 was added with the TBA reagents, was somewhat less (e.g. Table III). However, if peroxidation during the TBA test was blocked by adding BHT[33] with the TBA reagents, no stimulation of peroxidation by H_2O_2 was observed (Table IV).

TABLE IV

Effect of BHT on H_2O_2 – stimulated apparent microsomal lipid peroxidation. Microsomes were incubated with H_2O_2 at the concentrations stated. Reaction mixtures were then aliquoted. On one aliquot, the TBA test was performed as described in the Materials and Methods section (A), in the second it was performed with addition of BHT just before the TBA reagents (B).

$[H_2O_2]$ mM	Other reagents added	Extent of peroxidation (A_{532})	
		A	B
0		0.091	0.059
5		0.699	0.062
10		0.730	0.065
15		0.720	0.065
20		0.584	0.066
20	Desferrioxamine (1 mM)	0.098	0.053
20	Conalbumin (100 μM)	0.873	0.045
20	Gossypol (10 μM)	0.095	0.037
20	o-Phenanthroline (800 μM)	0.976	0.097
20	EDTA (100 μM)	0.135	0.053

Action of H_2O_2 on other systems generating TBA-reactive material

Authentic malondialdehyde was generated in the TBA test by heating tetraethoxypropane with the TBA reagents. Alternatively, the tetraethoxypropane was heated with the acid component of the reagent to hydrolyse it and then the TBA added, followed by further heating. In both cases, inclusion of H_2O_2 in the TBA assay mixture *decreased* the amount of chromogen detected as expected from previous work.[26] Table 5 shows a representative result. If H_2O_2 was added after the chromogen had been developed, some of the pink colour was destroyed (Table V, column B).

Microsomes contain ribosomal RNA in addition to lipid, and DNA may also be present if the pellet is contaminated with nuclei. However, inclusion of DNA or RNA (tested up to 1 mg/ml) in our reaction mixtures produced no significant TBA reactivity. When microsomes were replaced by ox-brain phospholipid liposomes as a substrate for peroxidation, stimulation by H_2O_2 was variable and much less marked than with microsomes. Table VI shows some representative results. Similar variable results to those with liposomes were obtained using arachidonic acid as a substrate (data not shown).

TABLE V

Effect of hydrogen peroxide on the reactivity of malondialdehyde, derived from tetraethoxypropane (TEP), in the TBA test. 5 μM TEP was included in the TBA reagents together with H_2O_2 at the final concentration stated. Tubes were heated at 100°C for 15 min (column A). In column B, the H_2O_2 was added after the chromogen had been developed by 15 min heating, and heating was continued for a further 15 min.

$[H_2O_2]$ added mM	$(TBA)_2$-Chromogen formed A_{532}	
	A	B
0	1.01	1.04
1.12	0.99	0.97
2.24	0.90	0.82
3.36	0.84	0.53
4.48	0.75	0.50
4.00 (no TEP)	0.00	–

TABLE VI

Action of H_2O_2 on liposomal lipid peroxidation. The peroxidation of ox-brain phospholipid liposomes (final concentration 1 mg/ml) was measured by the TBA test. H_2O_2 was included in the reaction mixture at the final concentrations stated. Note the fairly-small stimulation of peroxidation. Gutteridge[26] previously reported that H_2O_2 did not significantly stimulate peroxidation in liposomes.

$[H_2O_2]$ mM	Amount of peroxidation A_{532}
0	0.100
0.5	0.128
1.0	0.142
2.0	0.180
3.0	0.179
4.0	0.178

When DNA is exposed to certain transition metal ion complexes in the presence of O_2 and reducing agents, DNA degradation takes place to form compounds that decompose in the TBA assay to give malondialdehyde and thereby produce the $(TBA)_2$-MDA adduct. One such metal ion complex is copper-phenanthroline.[22] When H_2O_2 was added with the TBA reagents at the end of the copper-phenanthroline assay, it again decreased the amount of chromogen detected (Table VII). A bleomycin-iron ion complex also degrades DNA to TBA-reactive material[23] and H_2O_2 added after the end of the reaction again decreased the amount of chromogen measured (Table VIII, column B). In addition, H_2O_2 inhibited the actual formation of TBA-reactive material from DNA by the bleomycin system (Table VIII, column A).

Peroxidation of microsomes and liposomes stimulated by ascorbate/$FeCl_3$

It was of interest to compare the apparent H_2O_2-induced microsomal peroxidation with that known[2] to be stimulated by incubation of microsomes with ascorbate and $FeCl_3$. Ascorbate/iron ion-dependent peroxidation was, as expected, almost completely inhibited by ovotransferrin (Table IX). The spectrum of the chromogen produce in the TBA test was indentical to that of the expected $(TBA)_2$-MDA adduct (Figure 3). This peroxidation also resulted in cytochrome P-450 degradation, as expected[9,10] (Table IX). No peroxidation was seen if either Fe^{3+} or ascorbate was

TABLE VII

Action of H_2O_2 on the measurement of TBA-reactive material generated from DNA by a copper ion-phenanthroline complex in the presence of a reducing agent. Assays were carried out essentially as described in [22] using either mercaptoethanol (column A) or ascorbate (column B) as the reducing agent. H_2O_2 was added to give the concentrations stated in the reaction mixture immediately before adding TBA and HCl to develop the chromogen.[22]

$[H_2O_2]$ added mM	Amount of chromogen measured A_{532}	
	A	B
0	0.47	0.61
2.8	0.41	0.48
4.8	0.41	0.43
5.6	0.35	0.40
11.2	0.33	0.31
22.4	0.22	0.22

TABLE VIII

Action of H_2O_2 on the measurement of TBA-reactive material generated from DNA by the bleomycin-iron ion/ascorbate system. DNA, Fe^{3+}, bleomycin, ascorbate, $MgCl_2$ and sufficient HCl to adjust the pH to 7.4 were incubated at 37°C as described in [23]. H_2O_2 at the final concentration stated was added either at the beginning of the assay (column A) or immediately after 0.1 M EDTA had been added to stop the reaction and just before colour was developed (column B). The H_2O_2 both decreased colour formation in the TBA test (column B) and inhibited the actual generation of TBA-reactive material from DNA by the bleomycin-iron ion complex (column A).

$[H_2O_2]$ mM	DNA degradation to TBA-reactive material A_{532}	
	A	B
0	2.02	2.02
0.05	1.78	1.83
0.10	1.45	1.82
0.50	0.76	1.82
1.00	0.80	1.78
2.00	0.72	1.70
3.00	0.41	1.28
4.00	0.27	0.92

TABLE IX

Effect of scavengers and chelating agents on microsomal peroxidation induced by $FeCl_3$/ascorbate. Gossypol and butylated hydroxytoluene were dissolved in ethanol, which itself had no effect on the peroxidation process. Results of a representative experiment are presented. Absorbance values are the average of duplicate determinations that differed by < 11%.

Addition to reaction mixture	Final concentration	Lipid peroxidation A_{532}	Cytochrome P-450 nmol/mg protein
None (omit $FeCl_3$ or ascorbate)		0.000	0.79
None		2.452	0.19
Mannitol	20 mM	2.390	0.34
Sodium formate	20 mM	2.300	0.21
Gossypol	10 μM	0.106	0.69
Butylated hydroxytoluene	10 μM	0.090	0.81
EDTA	10 μM	0.700	0.40
	100 μM	0.142	0.79
o-Phenanthroline	100 μM	0.156	0.80
	800 μM	0.149	0.83
Conalbumin	100 μM	0.128	0.78
Desferrioxamine	100 μM	0.121	0.90
H_2O_2	4 mM	1.355	0.20

omitted from the reaction mixture, suggesting that the iron contamination of the microsomes was insignificant (otherwise addition of ascorbate would have led to peroxidation[2]). Peroxidation was unaffected by ·OH scavengers (mannitol, sodium formate) or by catalase, but it was inhibited by chain-breaking antioxidants (BHT, gossypol) and by chelating agents (desferrioxamine, EDTA, *o*-phenanthroline). All these agents also prevented P-450 degradation. Thus, in the ascorbate/$FeCl_3$ system, inhibition of peroxidation is associated with prevention of P-450 degradation (Table IX). Control experiments showed that the peroxidation measured in the $FeCl_3$/ascor-

bate system did not occur only during the TBA assay, in that little peroxidation was observed when microsomes were incubated alone and ascorbate and $FeCl_3$ were then added with the TBA reagents, provided that fairly fresh microsomal preparations (with a low background rate of peroxidation) were used.

H_2O_2 *inhibited* ascorbate/$FeCl_3$-dependent microsomal peroxidation (Table IX, last line; Table X). It also inhibited peroxidation of ox-brain phospholipid liposomes induced by $FeCl_3$ and ascorbate. In both cases, part of this inhibition was due to an effect of H_2O_2 on the TBA assay, but much of the action of H_2O_2 was exerted in the reaction mixture itself. This was shown both by adding H_2O_2 with the TBA reagents, and also by performing the TBA test in the presence of BHT to block peroxidation during the test (Table X).

DISCUSSION

Incubation of microsomes with $FeCl_3$ and ascorbate leads to rapid lipid peroxidation[2,4-7,13] and destruction of cytochrome P-450. Blocking the lipid peroxidation by adding iron ion chelators or chain-breaking antioxidants inhibits the peroxidation. It seems likely that P-450 is destroyed by a reaction with lipid peroxides.[9-12] Hydroxyl radicals were not required for lipid peroxidation or for P-450 degradation, since neither process was significantly inhibited by catalase or by ·OH scavengers. Addition of ovotransferrin to bind iron ions (Table 6) inhibited the peroxidation. H_2O_2 at high concentrations also inhibited the peroxidation partially, as was also observed with liposomes (Table X).

Incubation of microsomes with H_2O_2 led to loss of cytochrome P-450, but not of cytochrome b_5. The loss of P-450 was unrelated to lipid peroxidation: it was not prevented by chain-breaking antioxidants (BHT, gossypol) or iron chelating agents. Since haem proteins are readily degraded by exposure to excess H_2O_2,[16-19] we suggest

TABLE X

Effect of H_2O_2 on microsomal and liposomal peroxidation stimulated by $FeCl_3$ and ascorbate. *A* H_2O_2 added during the incubation. *B* H_2O_2 added with the TBA reagents. *C* H_2O_2 added during the incubation, BHT added with the TBA reagents. *D* Both H_2O_2 and BHT added with the TBA reagents. The H_2O_2 concentrations quoted below are the final concentrations in the reaction mixtures. Note the much more striking inhibitory effect of H_2O_2 when BHT is added with the TBA reagents to block peroxidation during the assay.

$[H_2O_2]$ mM	Extent of peroxidation A_{532}			
	A	B	C	D
Microsomes				
0	2.19	2.19	1.51	1.51
1.0	2.00	2.10	0.42	1.20
2.0	1.80	2.08	0.24	1.06
3.0	1.68	1.94	0.20	0.93
4.0	1.57	1.83	0.18	0.87
Liposomes				
0	1.77	1.77	1.31	1.31
1.0	1.70	1.74	0.83	0.89
2.0	1.57	1.68	0.70	0.81
3.0	1.54	1.63	0.58	0.75
4.0	1.47	1.61	0.52	0.72

that the H_2O_2 penetrates into the microsomal lipid bilayers and directly destroys the cytochrome P-450. The inability to detect such a reaction in the studies of Mannering *et al.*[21] may have been due to the use of too low a concentration of H_2O_2.

Our control experiments showed that the apparent lipid peroxidation observed on incubating microsomes with H_2O_2 was largely or completely an artefact of the TBA test. Addition of H_2O_2 with the TBA reagents produced almost as much stimulation of peroxidation, whereas inclusion of BHT in the TBA reagents to prevent peroxidation during the assay abolished the stimulation by H_2O_2. It was especially interesting to note that this stimulation of peroxidation was not seen when the TBA test was performed on reaction mixtures containing $FeCl_3$ and ascorbic acid (Table X), which is presumably why it has not been observed previously. Instead, H_2O_2 inhibited somewhat. It also inhibited peroxidation of liposomes by $FeCl_3$ and ascorbate, as well as measurement of the TBA reactive material released from DNA by iron-bleomycin and copper-phenanthroline complexes. The fact that it also inhibits[26] chromogen detection from hydrolysed tetraethoxypropane suggest that H_2O_2 may inhibit by oxidizing MDA (perhaps to malonic acid) and/or by destroying the $(TBA)_2$-MDA adduct after it has been formed (Table V, column B). However, we have no explanation of why H_2O_2 stimulates formation of TBA-reactive material during the assay of microsomes whereas much less stimulation is evident when other lipid substrates (liposomes,[26] arachidonic acid) are used.

The TBA test is known to be chemically very complex and prone to artefacts,[31-36] yet the controls necessary to check for such artefacts are rarely performed (discussed in[31,32,37]). Our results show that caution should be employed in applying the TBA test to any system in which H_2O_2 is being generated, such as studies of the ability of O_2^--generating systems to induce lipid peroxidation. Interference by H_2O_2 can be minimized by adding BHT with the TBA reagents or by adding arsenite.[26,38]

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