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EFFECT OF FERROUS IONS ON MICROSOMAL PHOSPHOLIPID PEROXIDATION AND RELATED LIGHT EMISSION

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SUMMARY

Purified NADPH cytochrome *c* reductase catalyzes the oxidation of NADPH in the presence of Fe^{3+}, ADP and EDTA EDTA in this system appears to elevate the redox potential of ferric ion and of its iron complex thereby facilitating the transfer of one electron from NADPH to tri-valent iron (more rapidly than superoxide is formed) through a flavin moiety in the reductase, but it diminishes the concentration of free iron to be required for phospholipid peroxidation

The reduction of Fe^{3+} by the xanthine–xanthine oxidase system is different from that manifested by the NADPH–NADPH cytochrome *c* reductase system in the manner in which the former is carried out in the main by O_2^- generated by the substrate – O_2 – enzyme interaction

Reduced iron, which is free in the solution, plays an important role for the initiation and propagation of the phospholipid peroxidation, monitored by malondialdehyde assay and light emission

In the xanthine-induced lipoxygenation system, the OH radical, probably produced from hydrogen peroxide by the action of Fe^{2+}, is not involved in the initiation of the peroxidative cleavage of phospholipid in microsomal lipoprotein

INTRODUCTION

Microsomes isolated from rat liver have been reported to catalyze the NADPH-dependent peroxidation of endogenous lipid if ferric ions and a chelator such as ADP or pyrophosphate are present [1, 2] This reaction destroys polyunsaturated fatty acid moieties in microsomes and can be monitored by measurement of malondialdehyde or of lipid peroxides

It has also been shown that microsomes emit an ultra-weak luminescence during microsomal lipoxygenation, and postulated that luminescence other than that due to malondialdehyde is produced by lipid hydroperoxide interaction [3] Howes and Steele [4] emphasized that the chemiluminescence involves a singlet oxygen-mediated reaction However, attempts to demonstrate singlet oxygen ($^1O_2$) or its precursor (superoxide anion, O_2^-) as the peroxidative agents being produced by NADPH-

dependent microsomal electron transport have failed [2] If so, superoxide dismutase (scavenger of both $^1O_2$ and O_2^- may not affect the luminescence On the other hand, indirect evidence of superoxide anion generation by washed intact the microsome–NADPH system, i e the formation of compound III from added lactoperoxidase, has been offered [5] The studies described in this report indicate that, in the reconstituted microsomal lipoxygenation and xanthine–xanthine oxidase mediated lipoxygenation systems, the peroxidation of lipid in microsomal lipoprotein is initiated mainly by iron reduced by the flavoproteins directly or indirectly through O_2^- It is then propagated by a chain reaction involving possibly the lipid hydroperoxide radical Our studies also suggest that measurement of light intensity during lipoxygenation gives rapid and precise information with respect to the very early stage of peroxidative cleavage of phospholipid

MATERIALS AND METHODS

Reagents Horseradish peroxidase (type II) and ADP were obtained from Sigma Chemical Company The catalase and cytochrome *c* were produced by Calbiochem NADPH was purchased from Oriental Yeast Co , Ltd All other chemicals were of reagent grade

Enzyme preparation and assay Xanthine oxidase was prepared from buttermilk by trypsinization, heat treatment and ammonium sulfate fractionation by the method of Horecker and Heppel [6] It was purified by gel filtration on a Sephadex G-200 column Enzyme activity was determined by the rate of appearance of the reduced band of cytochrome *c* at 550 nm in the presence of oxidized cytochrome *c* and hypoxanthine [6] The enzyme (spec act , 16–20) could be stored in the frozen state for several weeks with little loss in activity but its activity decreased considerably after repeated freezing and thawing

Microsomal NADPH cytochrome *c* reductase (spec act , 10–16) and superoxide dismutase (specific activity 2 250–2 650) were prepared from rat liver microsomes [7] and bovine red blood cells [8], respectively, by established methods Their corresponding activities were determined by reduction of cytochrome *c* [7] and the ability to inhibit (50 %) the reduction of cytochrome *c* by milk xanthine oxidase [8], respectively When approximately 100–200 μg of protein (cytochrome *c* reductase or superoxide dismutase) was examined by disc electrophoresis, only one main band appeared on staining with Amido Black Heat inactivated superoxide dismutase was obtained by placing the enzyme solution in boiling water for 3 min

Preparation of microsomal lipoprotein particle This was prepared by the method of Nakano et al [9] and was free of a hemoprotein moiety, NADPH cytochrome *c* reductase [10] and trypsin activity

Conditions of incubation The standard reaction mixture for NADPH-dependent lipoxygenation consisted of lipoprotein, 1 10^{-4} M $Fe(NO_3)_3$, 1 67 mM ADP, 5 10^{-5} M EDTA, 0 16 mM NADPH, NADPH cytochrome *c* reductase, and 0 1 M Tris-HCl buffer (pH 6 8) in a total volume of 3 ml Xanthine oxidase and 3 3 10^{-4} M xanthine was used, instead of NADPH cytochrome *c* reductase and NADPH, for the xanthine induced lipoxygenation system In some cases, the components such as superoxide dismutase, catalase and radical scavengers (or trappers) were added to the standard incubation mixtures to examine their corresponding effect on malondi-

aldehyde formation When other assays were required, each of lipoprotein, Fe^{3+}-ADP and EDTA or a combination of two of them was omitted from the standard incubation media All components, except for NADPH (or xanthine oxidase) were preincubated for 3 minutes at 37 °C (in some cases, 25 °C) and the reaction was initiated by the addition of NADPH (or xanthine oxidase) The incubation was carried out for various time periods depending on the nature of the experiment

Assay of hydrogenperoxide H_2O_2 generation was indirectly determined by the formation of peroxidase compound II in the presence of horseradish peroxidase [5] and directly by ferrous thiocyanate method [11] with slight modification

Other methods NADPH-disappearance was monitored by fluorometry at 450 nm with an excitation wavelength at 365 nm in Hitachi Model 203 fluorescence spectrophotometer as described by Ernster and Nordenbland [12] Conversion of Fe^{3+} to Fe^{2+} was measured by the increase of absorbancy at 510 nm in the presence of added *o*-phenanthroline (3 6 μmoles/3 ml) Protein was determined by method of Lowry et al [13] Luminescence was measured by the single photoelectron counting method [14]

RESULTS

(1) *Consumption of NADPH by NADPH-dependent cytochrome c reductase*

A number of iron-containing compounds including Fe^{3+}–EDTA–ADP complex were examined as possible electron acceptors from $NADPH_2$ in the presence of NADPH-dependent cytochrome *c* reductase Their effects were evaluated by the decrease in the fluorescence of NADPH added to the reaction mixture with and without electron acceptors

TABLE I

$NADPH_2$ CONSUMPTION BY CYTOCHROME *c* REDUCTASE IN THE PRESENCE OF ELECTRON ACCEPTORS

The incubation medium consisted of NADPH cytochrome *c* reductase (0 48 units for Expt 1 and 1 5 units for Expt 2), 85 μM NADPH and 0 1 M Tris–HCl buffer (pH 6 8) in a total volume of 3 ml With the exception of cytochrome *c*, the assays were performed by fluorometry at 25 C as described under Methods $Fe(NO_3)_3$ in water was used as Fe^{3+}

Electron acceptors	$NADPH_2$ consumed (nmoles/ml per min)
Expt 1	
2 10^{-5} M Cytochrome *c*	31 30*
2 10^{-5} M Fe^{3+}+2 10^{-5} M EDTA+1 10^{-3} M ADP	3 30
5 10^{-6} M methemoglobin	0 65
2 10^{-5} M cytochrome b_5	0 0
Expt 2	
1 10^{-4} M Fe^{3+}	0 60
1 10^{-4} M Fe^{3+}+1 67 10^{-3} M ADP	0 70
1 10^{-4} M Fe^{3+}+1 2 10^{-3} M *o*-phenanthroline	2 60
1 10^{-4} M Fe^{3+}+1 67 10^{-3} M ADP+1 2 10^{-3} M *o*-phenanthroline	10 50
1 10^{-4} M Fe^{3+} + 1 67 10^{-3} M ADP+0 5 10^{-4} M EDTA	7 00

* Value was calculated from cytochrome *c* reductase activity (nmoles/min/ml), assuming that 2 moles of cytochrome *c* could be reduced by 1 mole of $NADPH_2$

As shown in Table I, the rate of NADPH consumption with the Fe^{3+}–EDTA–ADP equals about 10 % of that of $NADPH_2$ consumption during cytochrome *c* reduction Williams and Kamin [15] have reported that the ferric ion-reducing activity of NADPH-dependent cytochrome *c* reductase is only 3 % of that recorded with cytochrome *c* at pH 7 7 Very little $NADPH_2$ consumption was observed following the addition of methemoglobin, cytochrome b_5, Fe^{3+} or Fe^{3+}–ADP However, the addition of a strong iron-chelating agent (EDTA or *o*-phenanthroline) to Fe^{3+}–ADP system resulted in strong enhancement of $NADPH_2$ consumption

(2) *Effect of EDTA concentration on NADPH consumption and malondialdehyde formation*

When the initial rate of NADPH consumption by the NADPH-dependent cytochrome *c* reductase system was measured with a fixed concentration of Fe^{3+}–ADP (1 10^{-4} M as Fe^{3+}) and various concentrations of EDTA, it was found to increase greatly with increasing amounts of EDTA

The addition of a lipoprotein to the NADPH–NADPH cytochrome *c* reductase–Fe^{3+}–ADP–EDTA system enhanced NADPH consumption These results are

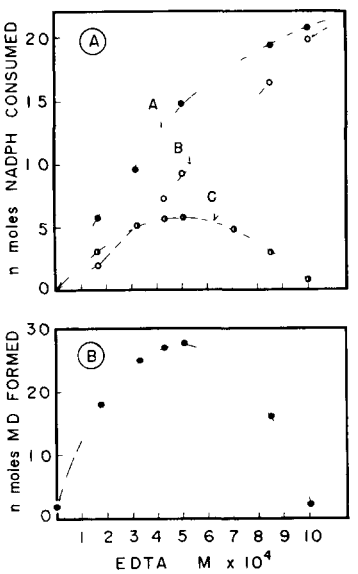

Fig 1 Effect of EDTA on NADPH consumption (A) and malondialdehyde formation (B) A, The incubation media consisted of 1 10^{-4} M Fe^{3+}, 1 67 mM ADP, 85 μM NADPH, EDTA in indicated concentrations, 1 6 units of NADPH cytochrome *c* reductase and 0 45 mg of lipoprotein (● — ●) or none (○ — ○), in a final volume of 3 ml NADPH consumption was measured fluorometrically at 25 °C and expressed as nmoles NADPH consumed per ml per min No or little NADPH consumption /min occurred without NADPH cytochrome *c* reductase The difference between NADPH consumption in the presence (curve A) and that in the absence of lipoprotein (curve B) is expressed as curve C (◑ — ◑) B, The incubation medium and conditions were identical to those listed in (A), save that units of the reductase (0 33 units) differed During incubation, a 0 5 ml aliquot was withdrawn from the medium for malondialdehyde measurement The non-enzymatic production of malondialdehyde was measured without NADPH and NADPH cytochrome *c* reductase and the value was subtracted from the value obtained with the complete system Malondialdehyde formation was expressed as nmoles MD/min/ml

shown in Fig 1A The hyperbolic curve, obtained by plotting the difference of initial velocity of NADPH consumption in the absence and in the presence of lipoprotein versus EDTA concentration, reached a peak at about 0 5 10^{-4} M EDTA

When the initial rate of malondialdehyde formation by NADPH-dependent lipoxygenation system was examined with a fixed concentration of Fe^{3+}–ADP as a function of EDTA, the concentration required for maximal activity was also 0 5 10^{-4} M (Fig 1B) This is in keeping with the results obtained during the peroxidation of liposomes in a similar system [16] The plot of the results closely resembles curve C in Fig 1A, indicating that the net consumption of NADPH in the presence of lipoprotein parallels the formation of malondialdehyde

To demonstrate the effect of EDTA on half-wave potential of ferric ion, EDTA was added to 1 mM $Fe(NO_3)_3$ in 0 5 M sodium citrate (pH, approximately 7 4) to obtain a certain ratio of EDTA/Fe^{3+} and the half-wave potentials of ferric ion and of iron-EDTA complex were measured by polarography at 23 °C The half-wave potential of ferric ion increased linearly from −0 079 V (ratio, $\frac{1}{4}$) to +0 08 V (ratio, $\frac{3}{4}$) with increasing the ratio of EDTA/Fe^{3+} Under the same condition, that of iron-EDTA complex was measured as follows, +0 20 V at ratio of $\frac{1}{4}$, + 0 29 V at ratio of $\frac{1}{2}$, +0 311 V at ratio of $\frac{3}{4}$, +0 325 V at ratio of 1, and + 0 34 V at ratio of 2

These results suggest that EDTA in NADPH–NADPH cytochrome *c* reductase system elevates the redox potential of ferric ion and iron–EDTA complex thereby facilitating the transfer of one electron from NADPH to tri-valent iron through flavoprotein but EDTA diminishes the concentration of free ferric ion to be required for the lipoxygenation

(3) *Effect of superoxide dismutase on iron reductase activity and on malondialdehyde formation*

When *o*-phenanthroline was added to NADPH–NADPH cytochrome *c* reductase system containing Fe^{3+}–ADP complex, absorbance at 510 nm involving Fe^{2+}–*o*-phenanthroline complex increased linearly with increasing time This rate however decreased linearly with increasing concentration of EDTA added This is in contrast to the findings that in the presence of Fe^{3+}–ADP complex EDTA accelerates NADPH oxidation by NADPH cytochrome *c* reductase, which may be parallel to iron reductase activity This discrepancy may be attributable to a competition of chelation between EDTA and *o*-phenanthroline for Fe^{2+} Therefore, absorbancy at 510 nm would not represent a true value for iron reduction, if the system contains other iron chelating agents However, this agent, under specified conditions, is very useful for determining the relative rate of iron reductase activity With NADPH cytochrome *c* reductase in various concentrations, absorbancy change of Fe^{2+}–*o*-phenanthroline per minute in the system containing 1 10^{-4} M Fe^{3+}, 1 67 mM ADP, 0 16 mM NADPH and 0 5 10^{-4} M EDTA had a linear relation with enzyme activity (0 01–0 1 units/ml of incubation mixture)

The reduction of Fe^{3+} induced by the NADPH–NADPH cytochrome *c* reductase system, as well as the formation of malondialdehyde by NADPH-dependent lipoxygenation, was not significantly affected either by intact or heat inactivated superoxide dismutase (0 1–10 μM)

As shown in Fig 2, both the reduction of iron by the xanthine–xanthine oxidase system and the malondialdehyde formation by xanthine induced lipoxygenation

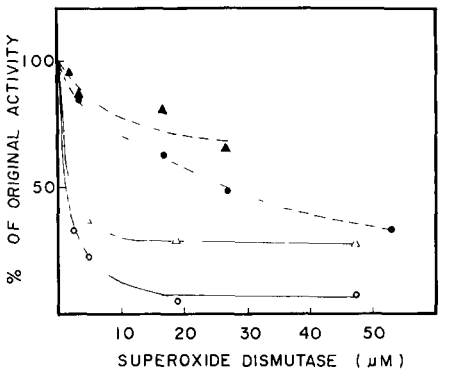

Fig 2 Effect of superoxide dismutase on Fe^{3+} reductase activity and malondialdehyde formation by xanthine–xanthine oxidase system and xanthine-induced lipoxygenation system, respectively The incubation medium consisted of xanthine oxidase (0 9 units for lipoxygenation system or 0 6 units for the iron reduction system), 1 10^{-4} M Fe^{3+}, 1 67 mM ADP, 0 5 10^{-4} M EDTA, 0 45 mg of lipoprotein or none, 0 1 M Tris–HCl buffer (pH 6 8), and intact (—) or heat inactivated superoxide dismutase (- - -) in various concentrations in a total volume of 3 ml Ortho-phenanthroline (3 6 μmoles/3 ml) was added to the lipoprotein-free system and the initial velocity (510 nm/min) of Fe^{3+} reduction (△, ▲) was assayed as described in text Malondialdehyde formed during 10 min incubation (○ ●) was determined by 2-thiobarbiturate method

system were considerably inhibited by superoxide dismutase in the concentration as low as 0 2 μM However, the heat inactivated superoxide dismutase also inhibited both iron reduction and lipoxygenation but to a lesser extent at less than 0 3 μM This may be due to the effect of metals (Cu^{2+} and Zn^{2+}) liberated from the enzyme during its inactivation on this system [17] Omission of Fe^{3+}–ADP–EDTA complex from the xanthine-induced system resulted in very little, but measurable, production of malondialdehyde These results indicate that the mechanism of iron reduction in the NADPH cytochrome *c* reductase system is quite different from that operative in the xanthine oxidase system in which generated O_2^- acts as a reductant of iron The data also suggest that both systems require iron in the reduced form for their maximal lipoxygenation

(4) *Possible formation of hydrogen peroxide and its effect on malondialdehyde formation*

Evidence of H_2O_2 generation was sought in a search for compound II (with specific absorption at 418 nm) in a system which contained horseradish peroxidase as well as by the ferrous thiocyanate method With these methods, no or little generation of H_2O_2 was detected during NADPH oxidation by NADPH cytochrome *c* reductase at pH ranging from 6 0–8 0

The addition of catalase to the NADPH-dependent lipoxygenation system (40 μg/3 ml of the reaction mixture) resulted in only slight enhancement of malondialdehyde formation At the same concentration of catalase, the xanthine-induced lipoxygenation system containing 0 63 units of xanthine oxidase and 0 45 mg of lipoprotein in 3 ml, however, greatly enhanced both the initial rate of malondialdehyde formation and the accumulation of this aldehyde (approximately 1 7 times those of the control without added catalase) Omission of either enzyme (xanthine oxidase or NADPH cytochrome *c* reductase) or the corresponding substrate (xanthine or

NADPH) and both components from each of the above systems including catalase resulted in no production of malondialdehyde, indicating that catalase can not act as a lipoxygenase or as xanthine oxidase

Agents known to be scavengers with a specificity for the OH radical [18], such as benzoate (3 mM) and mannitol (3 mM), had no effect on malondialdehyde formation by the above lipoxygenation systems This suggests that OH radicals produced from H_2O_2 were not involved in lipoxygenation

(5) *Luminescence during phospholipid peroxidation*

Under our experimental conditions, the two lipoxygenation systems emit the light which could be detected by single photoelectron counting method Fig 3 shows the typical results of the study of light emission from NADPH-dependent lipoxygenation system The addition of superoxide dismutase to the system (9 5 μM) did not significantly affect total light emission during a specified time interval (15 min) However, the time required for the development of maximal light intensity was prolonged Since a similar result was obtained with heat inactivated dismutase (9 5 μM), such a small observed effect may not be due to true dismutase action The luminescence, however, was strongly inhibited by the addition of an antioxidant, di-tert-butylhydroquinone, prior to and also during the incubation (data did not show) Under a similar condition, the other antioxidant (2,6-diiodo-4-aminophenol) and L-thyroxine, both at 5 10^{-6} M, produced 85–93 % and 40–55 % inhibition of both total light emission and malondialdehyde accumulation (both in 10 min), respectively Triethylendiamine, 0 22 M, which has been reported to be a scavenger of singlet oxygen [19], and which accelerates the NADPH cytochrome *c* reductase activity (1 5–1 7 fold) at the same concentration, stimulated slightly both total light emission and malondialdehyde accumulation (both in 10 min) The NADPH-dependent lipoxygenation system also required critical concentration of EDTA for

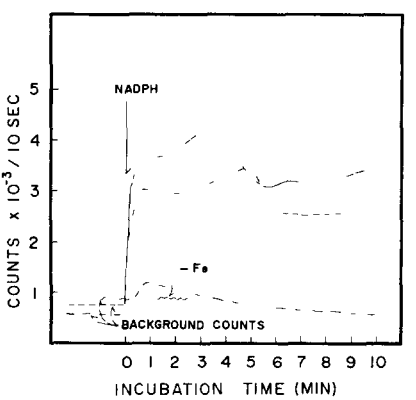

Fig 3 Effect of superoxide dismutase and di-tert-butylhydroquinone on luminescence by the NADPH-dependent lipoxygenation system The standard reaction mixture for NADPH-dependent lipoxygenation contained 0 4 unit of the reductase and 0 45 mg of lipoprotein without inhibitor (—) Superoxide dismutase (9 5 μM) (- - -) or di-tert-butylhydroquinone (5 10^{-6} M) (- - - -) was added to the media before the reaction was started Luminescence was measured at 37 °C in the dark The reaction was initiated by the addition of NADPH (at arrow) Light intensity was expressed as counts per 10 s No detectable luminescence was observed in the absence of added Fe^{3+}–ADP (–Fe)

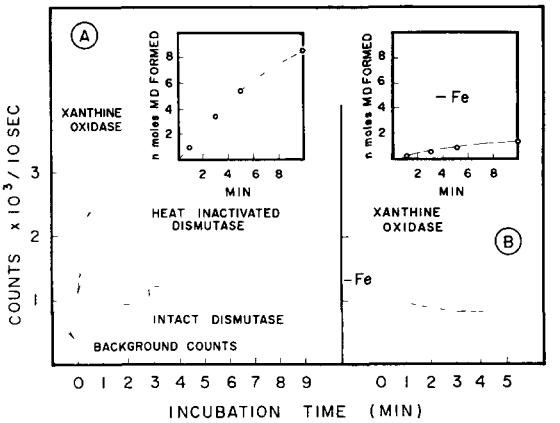

Fig 4 Effect of superoxide dismutase and di-tert -butylhydroquinone on luminescence by the xanthine-induced lipoxygenation system The standard reaction mixture for xanthine-induced lipoxygenation contained 0 9 units of xanthine oxidase 0 45 mg of lipoprotein, with or without inhibitor, in a total volume of 3 ml In some experiments Fe^{3+}–ADP (–Fe) was omitted from the standard reaction mixture (4B) Intact or heat inactivated superoxide dismutase (0 33 μM) (- - -) or di-tert -butylhydroquinone (5 10^{-6} M) (- - - -) was added to the standard reaction mixture prior to the initiation of the reaction by the addition of xanthine oxidase Malondialdehyde formation in the standard mixture (inset, 4A) and that in the system in which Fe^{3+}–ADP was omitted (inset, 4B) was measured by the method described in text

maximal light emission as well as maximal malondialdehyde formation

In contrast to the results of adding dismutase to NADPH-dependent lipoxygenation system, the dismutase, in the concentration as low as 0 33 μM, produced obvious inhibition (64 % in terms of total light emission in 10 min) of the light emission by xanthine-induced system With heat-inactivated dismutase in the same concentration and explanation, intact and heat-inactivated dismutase, both at 1 67 μM, inhibit the light emission to the extent of 84 4 and 37 7 %, respectively, in keeping with the effects of the enzyme on malondialdehyde accumulation by this system Di-tert-butylhydroquinone was also a potent inhibitor on the luminescence (Fig 4A) Even when the Fe^{3+}–ADP–EDTA was omitted from the xanthine-induced system, the system produced a weak but measurable light emission at the beginning of the reaction (Fig 4B), suggesting that singlet oxygen generated by xanthine–xanthine oxidase–O_2 interaction had attacked phospholipid In contrast to the results reported by Arneson [20], the reaction mixture containing xanthine and xanthine oxidase did not produce a detectable luminescence

DISCUSSION

The possibility that O_2^- or $^1O_2$ generated by a lipoxygenation system plays an important role in peroxidative cleavage of phospholipid has been proposed by several workers [4, 16, 17, 21] The evidence for this is indirect and based mainly on the inhibitory effect of superoxide dismutase or scavenger (or trapper) of singlet oxygen on malondialdehyde formation Nakamura and Yamazaki [22] have clearly demonstrated that NADPH cytochrome *c* reductase (enzyme constituting our reconstituted

system) produces superoxide anion during NADPH oxidation

The iron reduction by NADPH–NADPH cytochrome *c* reductase appears to proceed by the flux of one electron from NADPH to Fe^{3+} either via the semiquinone of flavin moiety (Reaction 1) flavin-O_2^- complex in the enzyme (reaction 2) or by

$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$ (Reaction 3)

As pointed out by Rapp et al [23], superoxide dismutase would react with O_2^- which is free in the solution Therefore, no inhibition by dismutase of the NADPH–Fe^{3+}–reductase may be explained if Fe^{3+} is reduced by the cytochrome *c* reductase through Reaction 1 or 2 more rapidly than superoxide is formed The iron reduction by xanthine and xanthine oxidase system, identical to the cytochrome *c* reduction by this system [24], should be carried out in the main by the free O_2^- generated by the substrate–O_2–enzyme interaction (Reaction 3) Even though the mechanism of Fe^{3+} reduction is different in the xanthine induced and NADPH-dependent system at pH 6 8, the initiation and propagation of lipid peroxidation are considered to be essentially the same in the two systems This is supported by the following findings (1) Omission of Fe^{3+} from the NADPH-dependent lipoxygenation system and xanthine-induced system result in less lipoxygenation, monitored by malondialdehyde value and total emission of luminescence (2) Antioxidants (radical acceptors or hydrogen donors), known to interfere with the propagation sequence of lipid peroxidation being mediated by peroxide radical [25], strongly inhibit the lipoxygenations by both systems

We have reported that Fe^{2+} alone and a combination of Fe^{2+} (or Fe^{3+}) and ascorbate catalyze the peroxidative cleavage of lipoprotein monitored by malondialdehyde formation [9, 10] However, no precise formulation of the mechanism of iron-catalyzed oxidation is yet possible It is likely that bivalent iron or an iron oxygen complex (Fe^{3+} O_2^-) plays an important role in the initial breakdown of the minimum concentration of lipid hydroperoxide presented in phospholipids of lipoprotein to form the alkoxyradicals essential for the chain reaction

Recently, Fong et al [18] have demonstrated that lysosomal lysis by purified NADPH-cytochrome *P*-450 reductase and xanthine oxidase acting with their corresponding substrates in the presence of Fe^{3+}-ADP complex is inhibited by the addition of catalase and by specific OH scavengers They postulated that the OH radical is a species responsible for the lipid peroxidation of lysosomal membranes However, the addition of catalase to our xanthine-induced lipoxygenation system caused obvious activation rather than inhibition of phospholipid peroxidation In addition OH radical scavengers did not affect phospholipid peroxidation in the NADPH-dependent and xanthine-induced systems, which may be due to the lipid source used for lipoxygenation and not an EDTA effect Such a significant effect of catalase on xanthine-induced system could involve the following mechanisms catalase catalyzed a rapid decomposition of H_2O_2 being generated by xanthine–O_2–xanthine oxidase interaction to supply O_2 for lipid peroxidation and also to protect from oxidation of Fe^{2+} by H_2O_2

In the presence of Fe^{3+}–ADP [26], the peroxidation of endogenous lipid in intact microsomes is mainly promoted by NADPH The enzymes which involve this lipoxygenation are considered to be NADPH cytochrome *c* reductase and NADPH cytochrome *P*-450 reductase [27] These enzymes are not yet fully characterized but

we do not believe that they are two distinct enzymes As suggested by others [2, 28], the role of ADP may be to prevent precipitation of ferric ion at near neutral pH or chelation of ferric ion by the other non-specific chelators in the reaction system

We feel that the reconstructed system which we have employed duplicates or closely resembles the main lipoxygenation pathway promoted in intact microsomes, save that EDTA is an artifact Even though the integrated light intensity emitted from the lipoxygenation system is in a good correlation with malondialdehyde accumulation in the experiments with several inhibitors of lipoxygenation, further study is needed to establish the relation between species of light and lipoxygenation.

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REFERENCES

1 Hochstein, P and Ernster, L (1963) Biochem Biophys Res Commun 12, 388–394
2 Poyer, J L and McCay, P B (1971) J Biol Chem 246, 263–269
3 Howes, R M and Steele, R H (1971) Res Comm Chem Pathol Pharmacol 2, 619–626
4 Howes, R M and Steele, R H (1972) Res Comm Chem Path Pharmacol 3, 349–357
5 Debey, P and Balny, C (1973) Biochimie 55, 329–332
6 Horecker, B L and Heppel, L A (1955) in Methods in Enzymology (Colowick S P and Kaplan N O eds) Vol 2 pp 482–485, Academic Press, New York
7 Omura, T and Takesue, S (1970) J Biochem (Tokyo) 67, 249–257
8 McCord, J M and Fridovich, I (1969) J Biol Chem 244, 6049–6055
9 Nakano, M , Tsutsumi, Y and Ushijima, Y (1971) Biochem Biophys Acta 252, 335–347
10 Ushijima, Y , Suwa, K and Nakano, M (1973) Biochim Biophys Acta 320, 284–294
11 Egerton, A C , Everett, A J , Minkoff, G J , Rudrakanchana, S and Salooja, K C (1954) Anal Chim Acta 10, 422–428
12 Ernster, L and Nordenbrand, K (1967) in Methods in Enzymology (Colowick, S P and Kaplan, N O , eds) Vol 10, pp 574–580, Academic Press, New York
13 Lowry, O H , Rosebrough, N J , Farr, A L and Randall R J (1951) J Biol Chem 193, 265–275
14 Shimizu, Y , Inaba H , Kumaki, K Mizuno K , Hata, S and Tomioka, S (1973) IEEE Trans Instrum Meas IM-22, 153–157
15 Williams, C H and Kamin, H (1962) J Biol Chem 237, 587–595
16 Pederson, T C and Aust, S D (1972) Biochem Biophys Res Commun 48, 789–795
17 Pederson, T C and Aust, S D (1973) Biochem Biophys Res Commun 3, 1071–1077
18 Fong, K L , McCay, P B and Poyer, J L (1973) J Biol Chem 248, 7792–7797
19 Quannes, C and Wilson, T (1968) J Am Chem Soc 90, 6527–6528
20 Arneson, R M (1970) Arch Biochem Biophys 136, 352–360
21 Goda, K , Chu, J W , Kimura, T and Schaap, A P (1973) Biochem Biophys Res Commun 52, 1300–1306
22 Nakamura, S and Yamazaki, I (1968) Biochim Biophys Acta 189, 29–37
23 Rapp, V , Adams, W C and Miller, R W (1973) Can J Biochem 51, 158–171
24 McCord, J M and Fridowich, I (1968) J Biol Chem 243, 5753–5750
25 Bolland, J L and Ten Have, P (1947) Trans Faraday, Soc 43, 201–210
26 Hochstein, P , Nordenbrand, K and Ernster, L (1964) Biochem Biophys Res Commun 14, 323–328
27 Pederson, T C , Buege, J A and Aust, S D (1973) J Biol Chem 248, 7134–7141
28 Tam, B K and McCay, P B (1970) J Biol Chem 245, 2295–2300