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Effect of Succinate on Mitochondrial Lipid Peroxidation. 1. Comparative Studies on Ferrous Ion and ADP · Fe/NADPH-Induced Peroxidation

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Abstract

Lipid peroxidation in isolated rat liver mitochondria, mitoplast, and mitochondrial inner membrane fragments was induced either by ferrous ions, or in an NADPH-dependent process by complexing with adenine nucleotides (ADP or ATP) iron. The Fe^{2+}-induced lipid peroxidation is nonenzymic when inner membrane fragments are used, while the differences in the inhibitory effect of Mn^{2+} ions and the stimulatory effect of the ionophore A-23187 in mitochondria and inner membrane fragments suggest an enzymic mechanism for ferrous ion-induced lipid peroxidation in intact mitochondria. Contrary to this the ADP/Fe/NADPH-dependent lipid peroxidation is an enzymic process both in mitochondria and inner membrane preparations. We have shown that cytochrome P_{450} is involved in the ADP/Fe/NADPH-induced lipid peroxidation. Succinate, a known inhibitor of NADPH-dependent lipid peroxidation, inhibited the Fe^{2+}-induced process also, and there was no difference in this effect when inner membrane preparations, mitochondria, or mitoplasts were used.

Key Words: Mitochondria; submitochondrial particles; lipid peroxidation; cytochrome P_{450}; succinate (rat liver).

Introduction

Lipid peroxidation in mitochondria may have both physiological and pathological significance in the mammalian cell (Player *et al.*, 1977; Marshansky *et al.*, 1983; for review see Chance *et al.*, 1979; Vladimirov *et al.*, 1980). To study this process, lipid peroxide formation in isolated mitochondria was investigated using different agents (Schneider *et al.*, 1964; Bindoli *et al.*, 1982), especially ferrous ions (McKnight *et al.*, 1965; Marshansky *et al.*, 1983) or iron, complexed with ADP in an NADPH-dependent lipid

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peroxidation (Pfeifer and McCay, 1972; Player *et al.*, 1977). The latter process is inhibited by succinate in mitochondria from beef heart (Takayanagi *et al.*, 1980) or from rat liver (Mészáros *et al.*, 1982).

The mitochondrial membrane may be involved in lipid peroxidation reactions in intact cells as well. Increased lipid peroxidation was found in mitochondria a few days after the gamma irradation of rats (Kergonou *et al.*, 1981). Before the increase of lipid peroxidation could be detected, the inhibitory effect of succinate on lipid peroxidation declined as a consequence of ^{60}Co-gamma irradiation (Rónai *et al.*, 1984). This result may be explained by the physiologically important protective effect of succinate on peroxidation of membrane lipids.

These findings motivated us to compare the effect of succinate on Fe^2-induced and ADP/Fe/NADPH-dependent mitochondrial lipid peroxidation. This seemed to be even more interesting, since several mechanisms were proposed to explain the inhibitory effect of succinate on different types of lipid peroxidations (Cavallini *et al.*, 1984).

In the present paper the protective effect of succinate against lipid peroxidation promoted by different mechanisms in various mitochondrial fractions is demonstrated. In addition, our results suggest that contrary to earlier views (McKnight *et al.*, 1965; Pfeifer and McCay, 1972) the ferrous ion-induced lipid peroxidation in intact mitochondria may be regarded as an enzymic process.

Materials and Methods

Rat liver mitochondria were prepared by differential centrifugation as described earlier (Szabados *et al.*, 1979) with slight modifications. The mitochondrial pellets were washed twice with medium containing 0.15 M KCl and 20 mM Tris-HCl, pH 7.2, and were finally suspended in the same medium.

Mitoplasts were prepared by digitonin treatment of the mitochondria according to Schnaitman and Greenwalt (1968), using 0.15 mg digitonin/mg mitochondrial protein.

Mitochondrial inner membrane fraction was prepared from mitoplasts by desintegrating with an MSE 100 W Model sonic oscillator at 7 μm amplitudes for 5 periods of 30 sec at 0°C. The unbroken mitoplasts were removed by centrifugation at 10,000 $\times$ g for 15 min. The inner membrane fraction was sedimented by 105,000 $\times$ g in a Beckman L2-65 ultracentrifuge for 60 min and washed twice and resuspended in 0.15 M KCl and 20 mM Tris-HCl, pH 7.2, solution.

To characterize the subcellular particulate fractions marker enzyme activities were measured: glucose-6-phosphate dehydrogenase (Swanson, 1955)

for microsomal impurities and monoamine oxidase (Tabor *et al.*, 1955) for mitochondrial outer membrane contamination, respectively.

Mitochondria and submitochondrial particles were incubated at 37°C in a medium containing either 0.15 M KCl, 10 mM Tris-HCl, pH 7.2, 20 μM $FeCl_3$, 2.0 mM ADP, and 0.3 mM NADPH for measuring the rate of the ADP/Fe/NADPH-dependent lipid peroxidation or in a 0.15 M KCl, 10 mM Tris-HCl, pH 7.2, 20 μM $FeSO_4$-containing medium for detection of Fe^{2+}-induced lipid peroxidation, unless otherwise stated in the text. The protein concentrations of the reaction mixtures were 2 mg/ml for mitochondria and mitoplasts and 1 mg/ml for inner membrane preparations. Protein content of mitochondria and mitoplasts was determined by the biuret method (Gornall *et al.*, 1949) and by the modified Lowry method (Schacterle and Pollack, 1973) for inner membrane preparations using bovine serum albumin as standard.

The rate of lipid peroxidation was estimated by measuring the amount of malondialdehyde formed according to Buege and Aust (1978).

Glucose-6-phosphate dehydrogenase and NADPH were obtained from Boehringer, Mannheim. Inophore A-23187 was from Eli Lilly Lab., and SKF-525 A[2] from Calbiochem, respectively. All other reagents were of analytical grade and obtained from Reanal Fine Chemicals (Hungary).

Results

Lipid Peroxidation in Mitoplasts

Microsomal fractions exhibit substantially higher levels of lipid peroxidative activity (Player *et al.*, 1977; Wills, 1969) compared to mitochondrial preparations. Therefore it is always questionable whether mitochondria have intrinsic lipid peroxidative activity of enzymic nature (Player *et al.*, 1977; Marshansky *et al.*, 1983; Mészáros *et al.*, 1982). Our findings, presented in Fig. 1, show higher levels of malondialdehyde production in mitoplasts than in mitochondria although microsomal and mitochondrial outer membrane contamination of mitoplasts was negligible (data not shown).

The inhibitory effect of succinate on lipid peroxidation was detected in the mitoplasts also. At the same time the degree of inhibition was not so pronounced as in the case of mitochondria (Fig. 1).

In the absence of chelators, mitoplasts were prone to ferrous ion-induced lipid peroxidation (Fig. 2), similar to that found earlier in mitochondria (Schneider *et al.*, 1964; McKnight *et al.*, 1965). This process is succinate sensitive (Fig. 2), as has been described for the NADPH-dependent one (Mészáros *et al.*, 1982).

[2] Abbreviation: SKF-525 A, β-diethylaminoethyldiphenylpropyl acetate · HCl.

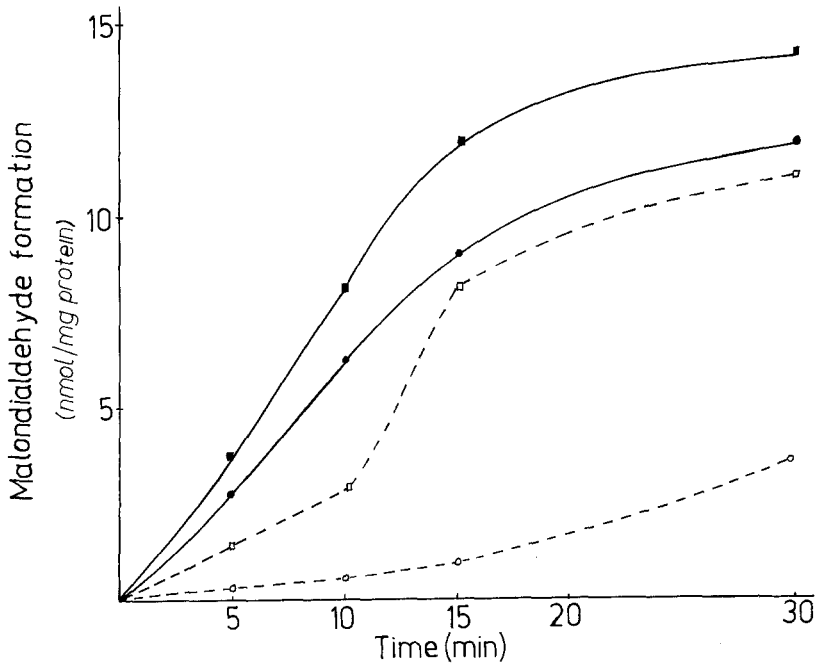

Fig. 1. Effect of succinate on ADP/Fe/NADPH-induced lipid peroxidation in mitochondria and mitoplasts. Experimental conditions are described in Materials and Methods. Malondialdehyde formed in the presence of mitochondria (●—●); mitoplast (■—■); mitochondria plus succinate (○—○); mitoplast plus succinate (□—□). The value of malondialdehyde formed (mean ± S.D., $n = 7$) after 30 min incubation of mitochondria was 11.7 ± 0.9 nmol/mg protein and 3.0 ± 1.9 nmol/mg protein in the absence and in the presence of succinate, respectively.

Mechanism of Fe^{2+}-Induced Lipid Peroxidation in Mitochondria

In the absence of NADPH, the adenine nucleotides or EDTA ferrous ion complexes exhibit only marginal lipid perioxidative activity as compared to lipid peroxidation driven by free Fe^{2+} ions (Table I). In the presence of reducing equivalents, lipid peroxidative activity of ADP · Fe^{2+} or EDTA · Fe^{2+} complexes was significantly higher than that of ferrous ions not complexed with chelator (Fig. 2, Table II).

It can be suggested that the chelated Fe^{2+} cannot be transported through the mitochondrial inner membrane, and its rapid oxidation abolishes lipid peroxidative capability, since Fe^{3+} does not promote peroxidation in mitochondria (Fig. 2). Investigating this possibility, we have found that the divalent cation ionophore A 23187 with a high affinity for Fe^{2+} (Young and Gomperts, 1977) increased malondialdehyde production in mitochondria in the presence of ATP/Fe^{2+}, while the Fe^{2+}-induced process remained unchanged (Table II). The proposed significance of Fe^{2+} transport in the

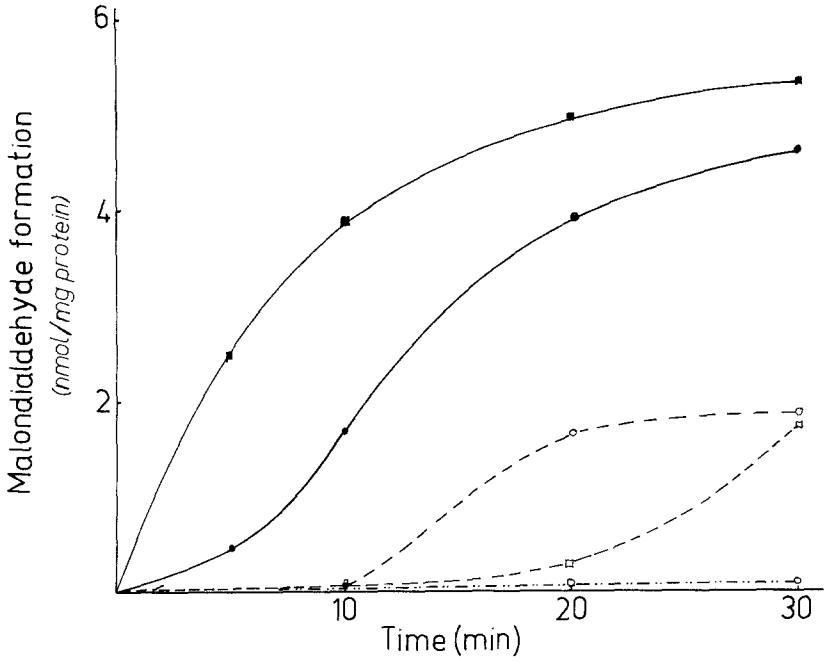

Fig. 2. Effect of succinate on Fe^{2+}-induced lipid peroxidation in mitochondria and mitoplasts. For experimental conditions, see Materials and Methods. Malondialdehyde production is expressed as nmol/mg protein in mitochondria with (○–○) and without (●–●) succinate and in mitoplasts without (■–■) and with (□–□) succinate respectively. The mean ± S.D., $n = 6$ after 30 min incubation of mitochondria was 5.01 ± 0.33 and 1.96 ± 0.29 in the absence and in the presence of succinate, respectively. Malondialdehyde formation in the presence of 20 μM Fe^{3+} ion (○–·–○).

Table I. Malondialdehyde Formation Induced by Different Iron Complexes[a]

Additions	Malondialdehyde formed nmol · mg protein^{-1}
Fe^{2+} 20 μM + ADP 2 mM	3.4 ± 0.7
Fe^{2+} 20 μM + ADP 2 mM + NADPH 0.3 mM	11.3 ± 1.5
Fe^{2+} 20 μM + EDTA 20 μM	1.35 ± 1.3
Fe^{2+} 20 μM + EDTA 20 μM + NADPH 0.3 mM	18.19 ± 4.9

[a]Incubation of mitochondria was performed for 30 min under conditions described in Materials and Methods. The values given are means ± S.D. of five experiments.

Table II. Effect of Ionophore A-23187 and Mn^{2+} Ions on Fe^{2+}-Induced Malondialdehyde Formation[a]

Additions	Malondialdehyde production nmol · mg protein^{-1}
None	5.01 ± 0.63
A-23187 0.4 μM	5.09 ± 0.57
$MnCl_2$ 20 μM	0.40 ± 0.20
ATP 2 mM	2.90 ± 0.80
ATP 2 mM + A-23187 0.4 μM	3.70 ± 0.85

[a]Mitochondria were incubated for 30 min in the presence of 20 μM Fe^{2+} as described under Materials and Methods. The mean ± S.D. of five preparations are given.

ferrous ion-promoted lipid peroxidation in intact mitochondria (Table II) is supported also by the strong inhibitory effect of Mn^{2+}, in concentration equimolar to Fe^{2+}, on ferrous ion-promoted lipid peroxidation (Table II), where Mn^{2+} efficiently competes with Fe^{2+} for the binding sites on membrane (Poyer and McCay, 1971).

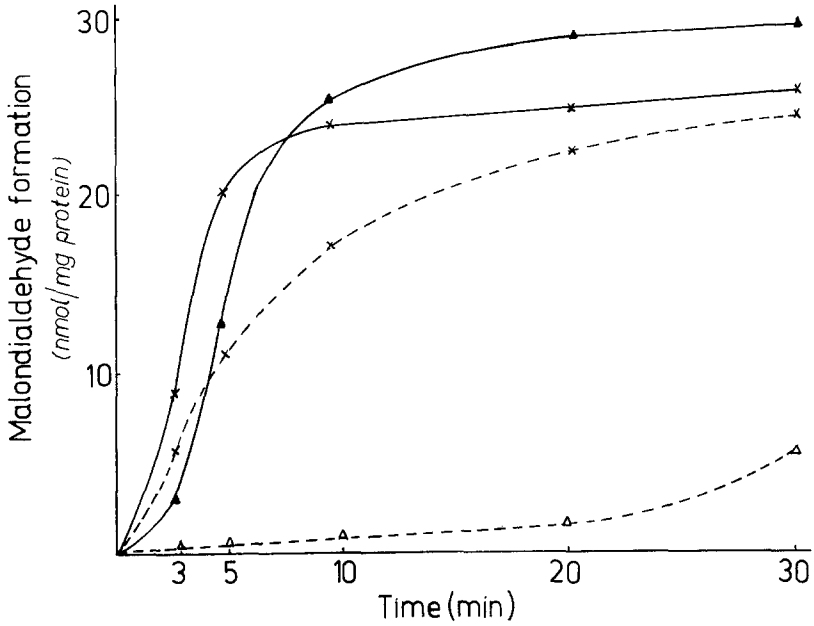

Fig. 3. Fe^{2+}-induced lipid peroxidation in mitochondrial inner membrane—effect of succinate. Heat treatment was performed by boiling the inner membranes for 5 min. Other experimental conditions are as described in Materials and Methods. Fe^{2+}-induced malondialdehyde production of inner membranes in the absence (▲–▲) and in the presence (△–△) of succinate and of heat-treated inner membranes in the absence (x–x) and in the presence of succinate (x– – –x).

Lipid Peroxidation in Inner Membrane Fractions

The rate of lipid peroxidation induced by ferrous ions in mitochondrial inner membrane preparations was substantially higher than that in mitochondria. The inhibitory effect of succinate was observed in both mitochondria and in the inner membranes (Fig. 3). When heat-treated preparations were used, the lipid peroxidative activity of inner membrane fraction induced by ferrous ions remained unaltered, but the succinate sensitivity was lost as a result of enzyme inactivation (Fig. 3).

Contrary to the NADPH-dependent lipid peroxidation of mitochondria and mitochondrial inner membranes, which was highly sensitive in a concentration-dependent manner to the known cytochrome P-450 inhibitor, SKF-525 A (Fig. 4), the Fe^{2+}-driven lipid peroxidation of inner membrane was not inhibited by SKF-525 A. This finding also supports the nonenzymic nature of ferrous ion-induced lipid peroxidation in mitochondrial inner membrane.

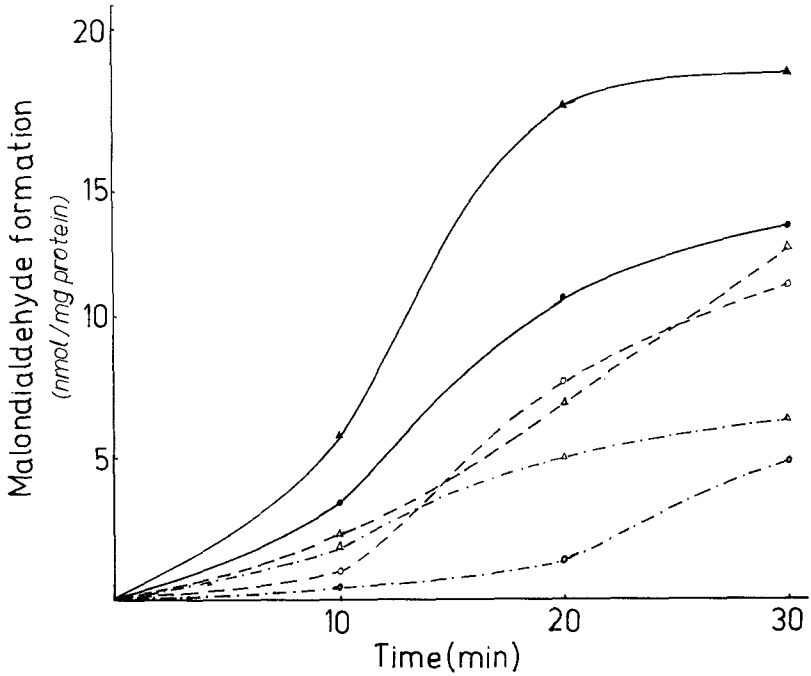

Fig. 4. Effect of SKF-525 A on the ADP/Fe/NADPH-induced lipid peroxidation. Mitochondrial malondialdehyde formation in the absence (●–●) and in the presence (○–○) of 250 μM or 500 μM (○–·–○) SKF-525 A. Malondialdehyde formation of inner membrane in the absence (▲–▲) and in the presence (△–△) of 250 μM or 500 μM SKF-525 A (△–·–△), respectively.

Discussion

Lipid peroxidation seems to have significance in the turnover of mitochondrial lipids (Pfeifer and McCay, 1972), in cation transport (Marshansky *et al.*, 1983), and in the disappearance of mitochondria during reticulocyte maturation (Rapoport *et al.*, 1979; Hartel *et al.*, 1982). However, because of the high lipid peroxidative activity of the microsomal fraction, there is a question whether the ADP/Fe/NADPH-promoted lipid peroxidative activity of isolated mitochondria is caused at least partially by microsomal contamination. Figure 1 demonstrates that mitoplasts sustained an ADP/Fe/NADPH-promoted peroxidative activity to the same, or even higher, extent than mitochondria; moreover, the inhibitory effect of succinate was shown both in mitochondria and in mitoplasts. These results together with results from the literature (Pfeifer and McCay, 1972; Takayanagi *et al.*, 1980; Mészáros *et al.*, 1982) strongly suggest that the ADP/Fe/NADPH-induced lipid peroxidation found in mitochondrial preparations was of mitochondrial origin, and cannot be ascribed to microsomal contamination.

The NADPH-dependent lipid peroxidation system of liver mitochondria seems to be closely associated with the inner membrane (Fig. 3). The transhydrogenase has been suggested to be responsible for lipid peroxidation, and the process to be mediated by free radicals produced by the components of the respiratory chain (Pfeifer and McCay, 1972; Boveris *et al.*, 1976; Takayanagi *et al.*, 1980; Turrens and Boveris, 1980).

The ADP/Fe/NADPH-dependent lipid peroxidation in mitochondria is sensitive to SKF-525A (Fig. 4), a known inhibitor of cytochrome P-450 in microsomes (Brodie *et al.*, 1958; Mukkerjee and Krishnamurty, 1980). This finding indicates that cytochrome P-450, a constituent of mitochondrial inner membrane (Sato *et al.*, 1977; Pedersen *et al.*, 1979), seems to be involved in the ADP/Fe/NADPH-driven lipid peroxidative sequences of mitochondria and mitochondrial inner membrane fraction.

Lipid peroxidation in mitochondria was shown to be protected by succinate (Bindoli *et al.*, 1982; Mészáros *et al.*, 1982; Cavallini *et al.*, 1984). The inhibitory effect of succinate was thought to be connected with the presence of reduced ubiquinones (Takayanagi *et al.*, 1980; Mészáros *et al.*, 1982; Cavallini *et al.*, 1984). This protective mechanism may also operate in mitochondrial inner membrane preparations.

According to earlier opinions (McKnight *et al.*, 1965; Pfeiffer and McCay, 1972) the ferrous ion-induced lipid peroxidation in intact mitochondria may be regarded as a nonenzymic process. Our findings that (1) complexed ferrous ions promote lipid peroxidation in intact mitochondria to a much smaller extent than Fe^{2+} ions do, (2) malondialdehyde formation in the presence of ADP/Fe is stimulated by ionophore A-23187 (Table II),

a potent Fe^{2+} carrier (Young and Gomperts, 1977), and (3) Mn^{2+} ions in equimolar concentration with Fe^{2+}, by competing for iron binding sites (Poyer and McCay, 1971), strongly inhibit the ferrous ion-promoted lipid peroxidation (Table II), firmly suggest that in intact mitochondria the transport of iron proceeds the Fe^{2+}-driven lipid peroxidation. These observations led us to suppose that in intact mitrochondria the Fe^{2+} ion-induced lipid peroxidation takes place on the matrix side of the inner membrane. There the Fe^{2+} ion could be complexed with intramitochondrial adenine nucleotides and the oxidized ferrous ions may be reduced by NADH or NADPH generated inside the mitochondrion. According to this opinion, the Fe^{2+}-induced lipid peroxidation in intact mitochondria might be considered an enzymic process, as was proved for the ADP/Fe/NADPH-dependent lipid peroxidation (Pfeifer and McCay, 1972; Takayanagi *et al.*, 1980).

Contrary to this, in the isolated inner membrane preparations, lacking matrix constituents, this mechanism of Fe^{2+}-induced lipid peroxidation cannot take place. In this case, malondialdehyde formation is induced by a nonenzymic process, since the heat inactivation did not inhibit it (Fig. 3), the ionophore A-23187 had no effect, and the inhibition of malondialdehyde formation by SKF-525 A was not observed. The ferrous ion-promoted lipid peroxidation was abolished in the inner membranes by succinate similar to that found in intact mitochondria, but its inhibitory effect was lost in heat-treated preparations having no oxidizing capacity (Fig. 3).

Induction of lipid peroxidation by ferrous ions was observed in heat-treated mitochondria also (McKnight *et al.*, 1965; Pfeifer and McCay, 1972). This finding is not inconsistent with the postulated enzymic mechanism of Fe^{2+}-promoted lipid peroxidation in intact mitochondria. It simply indicates that after heat inactivation of the enzymes ferrous ion can induce nonenzymic lipid peroxidation similarly to that observed in the inner membrane preparations lacking matrix constituents. The ADP/Fe/NADPH-dependent lipid peroxidation, not found in heat-treated mitochondria and inner membrane preparations (data not shown), represents enzymic process.

We conclude from our experiments that the Fe^{2+}-induced lipid peroxidation in intact mitochondria could be regarded as enzymic process contrary to that occurring in inner membrane preparations. However, the ADP/Fe/NADPH-dependent lipid peroxidation has enzymic character both in intact mitochondria and inner membrane fragments. The cytochrome P-450 seems to be involved in the ADP/Fe/NADPH-induced reaction sequences. Succinate inhibits both Fe^{2+}- and ADP/Fe/NADPH-induced lipid peroxidation in mitochondria and in inner membrane preparations, independently of the mechanism of induction.

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