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Effect of Succinate on Mitochondrial Lipid Peroxidation. 2. The Protective Effect of Succinate Against Functional and Structural Changes Induced by Lipid Peroxidation

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Abstract

The damaging effects of ADP/Fe/NADPH-induced lipid peroxidation were studied on the enzymes and membranes of rat liver mitochondria. Succinate, an inhibitor of mitochondrial lipid peroxidation, prevented or delayed most of the damage caused by the peroxidation on different mitochondrial structures and functions. There were marked abnormalities on the electrophoretic pattern of mitochondrial proteins during the course of lipid peroxidation. The disappearance of particular polypeptide bands and the accumulation of high-molecular-weight aggregates could be observed. Succinate was found to delay these effects. As a consequence of lipid peroxidation the succinate oxidase activity of mitochondria was decreased. The succinate dehydrogenase enzyme and the component(s) of the respiratory chain were inactivated. Succinate prevented the inactivation of succinate dehydrogenase but did not protect the other components of terminal oxidation chain. From the matrix enzymes the glutamate dehydrogenase retained its full activity but the NADP-linked isocitrate dehydrogenase was inactivated. The mitochondrial membranes became permeable to large protein molecules. Succinate prevented the inactivation of isocitrate dehydrogenase and delayed the release of protein molecules from mitochondria.

Key Words: Lipid peroxidation; mitochondria; respiratory chain; effect of succinate; membrane permeability; isocitrate dehydrogenase; cross-linking (rat liver).

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Introduction

The peroxidation of membrane lipids has been widely investigated because of its suspected role in different pathological conditions (Harman, 1982; Plaa and Witschi, 1976; Sevanian and Hochstein, 1985). The mitochondrial lipids are targets prone to lipid peroxidation (Tappel and Zalkin, 1959). Various chemicals can induce the peroxidation of lipids in isolated mitochondria (Schneider *et al.*, 1964). Depending on the agents used for inducing lipid peroxidation, the kinetics and the proposed mechanisms of the processes differ significantly (Hunter *et al.*, 1963).

Some defense systems and materials, e.g., superoxide dismutase, catalase, glutathione peroxidase, vitamin E, flavonoids, and phenols were suggested to be involved in protecting mitochondria against lipid peroxidation (Nohl and Hegner, 1978; Forman and Boveris, 1982; Sies and Cadenas, 1983). Recently, it has been shown that succinate, a citric acid cycle intermediate, could play a role in the defense of mitochondria isolated from various sources (Bindoli *et al.*, 1982; Mészáros *et al.*, 1982; Takeshige and Minakami 1975). This phenomenon might have significance *in vivo* as well to protect organisms against ionizing irradiation (Rónai *et al.*, 1984).

The aim of the present investigations was to study the consequences of the ADP/Fe/NADPH-induced lipid peroxidation in rat liver mitochondria and the effect of succinate in preventing damages caused by the peroxidation.

In the present paper it is shown that the individual components of the mitochondrial respiratory chain are sensitive to the peroxidative attack to different extents. By measuring the release of the glutamate dehydrogenase activity, it was possible to draw conclusions concerning the membrane permeability changes. We report here that the succinate effect is not restricted to preventing or delaying malondialdehyde production but it also protects mitochondrial enzymes and structures against inactivation or destruction, respectively. On the electrophoretic pattern different abnormalities caused by the lipid peroxidation were observed. Succinate delayed the development of these changes.

Materials and Methods

Animals

Male Wistar albino rats (150–200 g) were used throughout the experiments. The animals were fed a standard chow diet and tap water *ad libitum* and fasted 15–20 h before killing.

Lipid Peroxidation

The liver mitochondria were prepared according to Szabados *et al.* (1979). To induce lipid peroxidation the mitochondria were incubated at 37°C in a medium containing 2 mg/ml mitochondrial protein, 2.4 mM ADP, 20 μM $FeCl_3$, 0.3 mM NADPH in 0.15 M KCl, and 20 mM Tris-HCl, pH 7.4 (+ LP medium). The effect of succinate on lipid peroxidation was studied in the same system containing succinate at 5 mM final concentration (LPS system). The control mitochondria were incubated in media identical with the lipid peroxidating one but NADPH and ADP/Fe^{3+} complex were omitted (− LP medium).

The peroxidation was stopped by addition of EGTA[3] at 2 mM final concentration. Samples were taken at fixed intervals and were used for further analyses.

Lipid peroxidation was followed by the thiobarbituric acid reaction as described (Buege and Aust, 1978). Proteins were determined by the biuret procedure (Gornall *et al.*, 1949) using bovine serum albumin as standard.

Respiratory Activities

Oxygen consumption was followed at 30°C with a Clarke type oxygen electrode (Estabrook, 1966) in a 3.0-ml thermostated cuvette at 0.5–1.2 mg/ml mitochondrial protein concentrations. The assay system contained 115 mM KCl, 20 mM Tris-HCl, and 2.5 mM potassium phosphate, pH 7.2 at 30°C.

Succinate oxidase activity was measured by following the oxygen uptake after successive addition of 10 mM succinate + 1.5 mM ADP and 2.5 μM oxidized cytochrome *c* in 1-min interval.

Cytochrome oxidase activity was assayed separately, after addition of 5 mM ascorbate + 6.0 mM TMPD and 2.5 μM cytochrome *c* in 1-min intervals (the concentrations above indicate the final concentrations in the cuvette).

Succinate Dehydrogenase

The succinate dehydrogenase activity was followed by the reduction of iodonitrotetrazolium chloride (Bernath and Singer, 1962). The absorbance changes were recorded at 490 nm. The assay system contained 0.2 mg/ml mitochondrial protein, 2 mM $CaCl_2$, 1 mM KCN, 20 mM succinate, and 0.1% iodonitrotetrazolium chloride in 50 mM potassium phosphate buffer, pH 7.6 at 37°C. The reaction was stopped by addition of acetone to the test tubes.

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[3] Abbreviations: SDS, sodium dodecyl sulfate; TMPD, *N,N,N,N*-tetramethyl-*p*-phenylenediamine; EGTA, ethyleneglycol bis(2-aminoethylether)-*N,N*-tetraacetic acid; MDA, malondialdehyde.

Glutamate Dehydrogenase, Permeability Changes

Glutamate dehydrogenase was measured as described (Schmidt, 1974). After incubation for lipid peroxidation, mitochondrium samples were treated with Triton X-100 in 0.66% final concentration in order to disrupt permeability barriers and obtain maximal rates. Cuvettes containing 40 μg protein were assembled with 100 mM ammonium acetate, 0.15 mM NADH, 0.5 mM ADP, 0.5 mM rotenone, 2 mM EGTA, 2 mM oxoglutarate in 250 mM sucrose, and 10 mM Tris-HCl buffer, pH 7.2. The changes of absorbance were followed at 340 nm.

To check the permeability changes due to lipid peroxidation, the mitochondria from control (− LP), lipid peroxidating (+ LP), and succinate-containing (LPS) media were sedimented at 10,000 × g for 15 min without preceding Triton X-100 treatment, and the glutamate dehydrogenase activity of the supernatant fractions was tested.

Isocitrate Dehydrogenase

The enzyme activity was assayed as described (Plaut, 1962). After terminating lipid peroxidation by addition of EGTA to the media described above, the mitochondria were solubilized by addition of 0.66% Triton X-100. The reaction mixture contained 0.2 mg/ml protein, 1.25 mM $MnCl_2$, 0.5 mM rotenone, and 0.5 mM NADP in 33 mM Tris-HCl, pH 7.2. The reaction was started by the addition of isocitrate at 2 mM final concentration.

Ultrasonic Treatment

In some experiments mitochondria were sonicated prior to incubation for lipid peroxidation. The mitochondria were suspended (2.5 mg/ml protein) in 150 mM KCl and 20 mM Tris-HCl buffer, pH 7.4. Sonication was performed in an MSE type sonicator at 0°C with 25 sec bursts at full power. After sonication, intact mitochondria were sedimented at 10,000 × g for 15 min and the protein concentration of the supernatant was adjusted to 2.0 mg/ml. Lipid peroxidation was performed as described for intact mitochondria.

Electrophoresis

For SDS/polyacrylamide gel electrophoresis, 30-μl samples were taken from the control, lipid-peroxidated, and succinate-pretreated mitochondria, respectively. Samples were lysed with 5 μl of 20% SDS mixed with 15 μl solution containing 30% w/v sucrose, 2 mM EDTA, 150 mM Tris-HCl buffer, pH 6.8, 1% mercaptoethanol, and 0.02% bromphenol blue. The samples were applied to a 10–15% gradient slab gel prepared essentially as described (Douglas *et al.*, 1979). The gels were stained with 0.1% Coomassie Brilliant Blue R250.

Results

Electrophoresis

The electrophoresis shows that the protein pattern of control mitochondria is not changed even after 30-min incubation (Fig. 1A, B). As the lipid peroxidation advances, the almost complete disappearance of two protein bands can be observed (Fig. 1A). In the stacking gel there is a densely stained area which does not show any bandlike structure. This seems to indicate the formation of high-molecular-weight protein aggregates (Fig. 1B). If mitochondria are incubated in the presence of succinate (LPS medium) the changes on the electrophoretogram are less pronounced compared to the control run and are postponed to later time points (Fig. 1A, B).

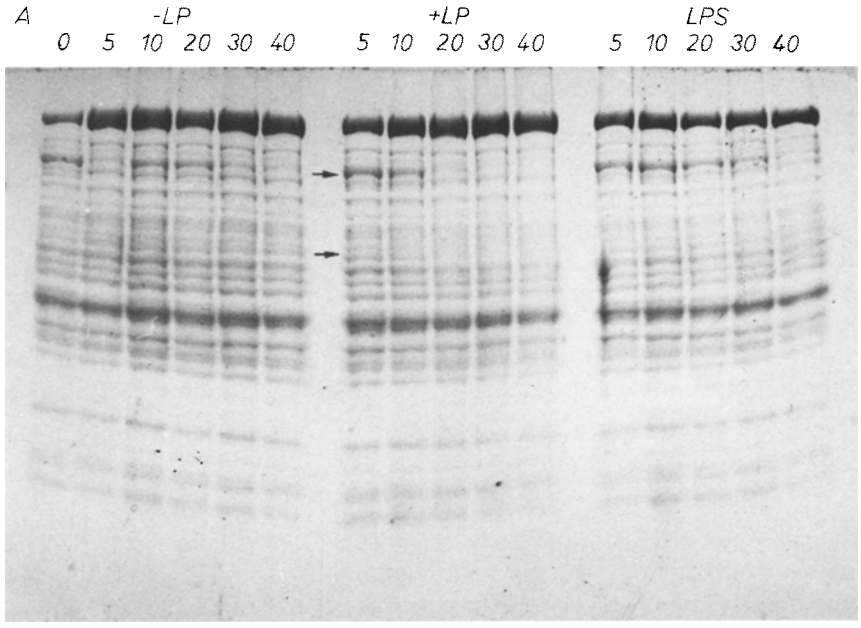
Succinate Oxidation

The experimental data are listed in Table Ia. Without incubation the oxygen consumptions of mitochondria derived from −LP, +LP, and LPS media are essentially the same in the presence of ADP + succinate. Addition of cytochrome *c* does not enhance oxygen uptake, there being no differences between the three systems. After 30-min incubation, the respirations of peroxidated (+LP) and succinate-treated (LPS) mitochondria are much slower than that of the control (−LP). Exogenous cytochrome *c* restores the activity of control mitochondria to the maximal level, but fails to restore the succinate oxidase activity of mitochondria of the +LP and LPS systems. The succinate does not have significant protective effect.

Cytochrome Oxidase

Without incubation for lipid peroxidation, the oxygen consumption data are in the same range when ascorbate and TMPD are present. Again no further stimulation of the rate is observed on the addition of cytochrome *c*. After 30-min incubation, the rate of oxidation decreases markedly in each preparation. The decrease of cytochrome *c* content (washing out) could be responsible for the diminished oxygen uptake, because the added exogenous cytochrome *c* stimulates the respiration to the maximal level. The cytochrome oxidase is not affected by the lipid peroxidation (Table IB).

In Fig. 2, the correlation between the intensity of malondialdehyde production and the decrease of succinate oxidase activity (%) in lipid peroxidating preparations is shown. The straight line is drawn by the least-squares method. There is a correlation between the malondialdehyde accumulation and the succinate oxidase inactivation. For this type of experiment,


A
-LP
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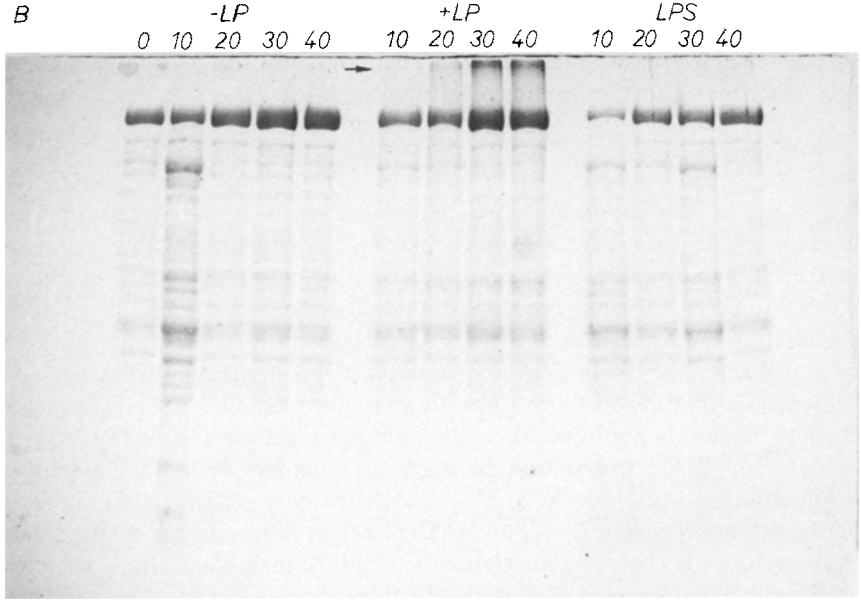
B
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Table I. The Effect of Lipid Peroxidation and Its Inhibition by Succinate on the Oxygen Consumption of Mitochondria[a]

(a) Succinate oxidase activity

Additions	Time of incubation at 37°C					
	0 min			30 min		
	Incubating media					
	−LP	+LP	LPS	−LP	+LP	LPS
	ng atom O_2 consumption · min^{-1} · mg $protein^{-1}$					
Succinate + ADP	110 ± 22	110 ± 32	127 ± 48	47 ± 18	33 ± 8	42 ± 14
Cytochrome *c*	127 ± 27	129 ± 32	156 ± 30	121 ± 21	69 ± 13	77 ± 9

(b) Cytochrome oxidase activity

Additions	Time of incubation at 37°C					
	0 min			30 min		
	Incubating media					
	−LP	+LP	LPS	−LP	+LP	LPS
	ng atom O_2 consumption · min^{-1} · mg $protein^{-1}$					
TMPD + ascorbate	161 ± 33	163 ± 25	217 ± 33	69 ± 25	64 ± 14	74 ± 28
Cytochrome *c*	187 ± 27	195 ± 45	218 ± 40	227 ± 29	267 ± 57	220 ± 39

[a] The details of the reaction conditions are described in the Materials and Methods section. −LP medium contained KCl, Tris-HCl, mitochondrial protein. +LP medium is the same as −LP, NADPH, ADP/Fe^{3+} added. LPS medium is the same as +LP, succinate added. Results are expressed as the mean value ± S.D., $n = 6$.

animals without overnight fasting were used in order to obtain a broad range of malondialdehyde production data.

Succinate Dehydrogenase

The data are shown in Fig. 3. After 30 min of lipid peroxidation, 60% of the succinate dehydrogenase activity is lost. In the presence of succinate only 16% of succinate dehydrogenase is inactivated.

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the mitochondria. Electrophoretic procedures are described in the Materials and Methods section. The abbreviations −LP, +LP, and LPS represent mitochondrial proteins derived from control, lipid peroxidated, and succinate-treated lipid peroxidating mitochondria. Numbers above the lanes indicate the different incubation times in minutes at 37°C. Arrows indicate the disappearance of polypeptide bands after lipid peroxidation (A). Densely stained areas in the high-molecular-weight region can be seen after lipid peroxidation (B).

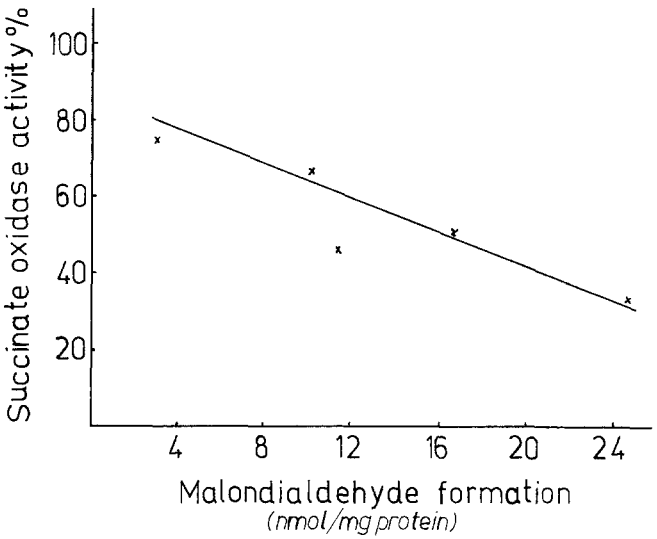

Fig. 2. Correlation between the inactivation of succinate oxidase and the malondialdehyde accumulation. The mitochondria were incubated at 37°C for 30 min, and the oxygen consumption was monitored after addition of ADP + succinate + cytochrome *c* as described in Materials and Methods. Straight line was fitted through the experimental points by the method of least squares. Slope = −4.81%, intercept on *y* axis at 87%; Pearson's correlation coefficient $r = 0.92$.

Glutamate Dehydrogenase and Membrane Permeability

In order to study the possible alterations of matrix proteins, the inactivation of glutamate dehydrogenase and isocitrate dehydrogenase is measured. Glutamate dehydrogenase activity is assayed in Triton X-100-treated mitochondria to obtain maximal activities. After 30 min incubation there is no significant differences between mitochondria incubated in −LP, +LP, and LPS media (Table IIa).

The resistance of glutamate dehydrogenase activity to peroxidative destruction made it possible to study the permeability of mitochondrial membranes toward large molecules after induction of lipid peroxidation. Without incubation at 37°C about 2.5% of total activity is released into the postmitochondrial supernatant. In the supernatant of control mitochondria (incubated at −LP medium) not more than 6% of glutamate dehydrogenase activity can be detected even after 30-min incubation. After 10, 20, and 30 min of incubation in +LP medium, the recoveries of total enzyme activities are 23, 52, and 118% in the postmitochondrial supernatant, respectively. The protective effect of succinate runs parallel with its inhibitory effect on lipid peroxidation, i.e., it delays very effectively the increase of permeability in the first 20 min. After 10, 20, and 30 min incubations in LPS medium, 7, 11, and

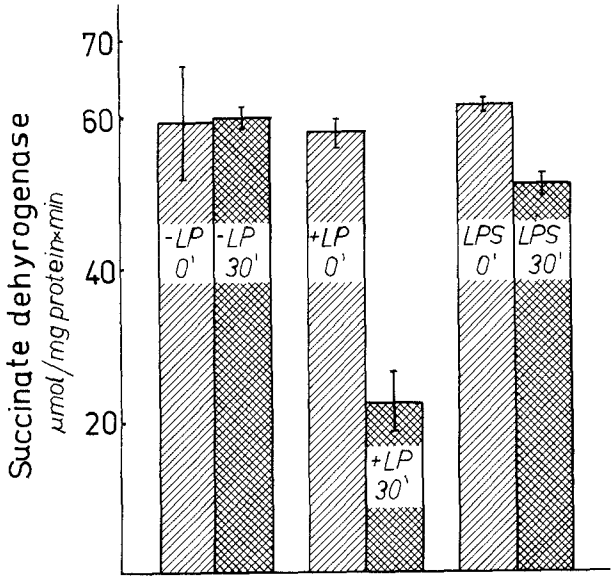

Fig. 3. Succinate dehydrogenase activity. The mitochondria were incubated for 0 and 30 min. The media −LP, +LP, and LPS, represent control, lipid peroxidated, and succinate-treated lipid peroxidated mitochondria, respectively. After addition of EGTA, the succinate dehydrogenase activity was determined as described in Materials and Methods. The bars represent the mean ± S.D., $n = 5$.

69% of total enzyme activity appears in the supernatant. In Table IIc, the dynamics of malondialdehyde production is shown. The lipid peroxidation increases very rapidly in the first 20 min, then the accumulation is slower and reaches a plateau after 30 min (data not shown). Succinate is an effective inhibitor of malondialadehyde production in the first 20 min.

Isocitrate Dehydrogenase

The isocitrate dehydrogenase activity of mitochondria shows marked decrease during lipid peroxidation. The extent of inactivation compared to the control mitochondria is 53, 58, and 58% after 10, 20, and 30 min incubations, respectively. Under these circumstances, 91% of inactivation has occurred in the first 10 min and further increase of malondialdehyde production did not enhance the extent of enzyme inactivation (Table IIc for malondialdehyde production and Table IIIa for isocitrate dehydrogenase). There is no positive correlation between the amount of malondialdehyde formed and the decrease of enzyme activity. The protective effect of succinate against inactivation is statistically significant. In order to clarify whether the tight organization of matrix enzymes inside the mitochondria (D'Souza and

Table II. Membrane Permeability Changes as a Consequence of Lipid Peroxidation[a]

(a) The glutamate dehydrogenase activity of mitochondria

Media	Time of incubation	
	0 min	30 min
	Glutamate dehydrogenase (mU · mg protein^{-1})	
−LP	911 ± 109	780 ± 185
+LP	873 ± 130	815 ± 75
LPS	844 ± 107	717 ± 108

(b) The recovery of glutamate dehydrogenase activity from postmitochondrial supernatant fraction

Media	Time of incubation			
	0 min	10 min	20 min	30 min
	Glutamate dehydrogenase activity (mU)			
−LP	25 ± 14.4	30 ± 17	46 ± 42	46 ± 38
+LP	32 ± 16	187 ± 102	425 ± 208	968 ± 226
LPS	33 ± 20	47 ± 18	78 ± 62	492 ± 216

(c) The dynamics of malondialdehyde formation

Media	Time of incubation			
	0 min	10 min	20 min	30 min
	Malondialdehyde (nmol · mg protein^{-1})			
+LP	0	7.6 ± 2.4	12.4 ± 1.9	16 ± 1.2
LPS	0	2.7 ± 2.6	7.0 ± 4.0	9.5 ± 3.0

[a] In Part (a), the mitochondria were solubilized by Triton X-100 in order to obtain maximal rates. In Part (b), after lipid peroxidation the samples were treated with EGTA, then intact mitochondria were sedimented by 10,000 × *g* for 15 min. The details of the reaction conditions are described in the Materials and Methods section. −LP medium contained KCl, Tris-HCl, mitochondrial protein. +LP medium is the same as −LP, NADPH, ADP/Fe^{3+} added. LPS medium is the same as +LP, succinate added. Results are expressed as the mean value ± S.D., $n = 5$.

Srere, 1983) is a necessary prerequisite of their inactivation caused by free radicals or other reactive substances, the mitochondria were sonicated prior to induction of lipid peroxidation. It can be seen that the inactivation of isocitrate dehydrogenase in the sonically treated samples takes place rather gradually and reaches a slightly higher maximum at 30 min (70%) than in the nonsonicated preparations (Table IIIb), but the differences were statistically insignificant.

Table III. The Inactivation of Mitochondrial NADP-Dependent Isocitrate Dehydrogenase during Lipid Peroxidation[a]

(a) Intact mitochondria

Media	Time of incubation			
	0 min	10 min	20 min	30 min
	Isocitrate dehydrogenase activity (mU · mg protein^{-1})			
−LP	51.2 ± 5.9	50.7 ± 10.0	42.3 ± 10.0	37.8 ± 6.5
+LP	51.2 ± 6.4	23.0 ± 6.2	18.0 ± 5.2	16.0 ± 6.0
LPS	50.0 ± 6.0	36.0 ± 7.9	28.3 ± 5.0	24.7 ± 5.8

(b) Sonically treated mitochondria

Media	Time of incubation			
	0 min	10 min	20 min	30 min
	Isocitrate dehydrogenase activity (mU · mg protein^{-1})			
−LP	50.1 ± 5.5	43.0 ± 2.2	36.3 ± 2.3	36.0 ± 4.5
+LP	49.0 ± 5.2	23.4 ± 5.6	15.5 ± 3.4	11.0 ± 7.8

[a]The details of the reaction conditions are described in the Materials and Methods section. Ultrasonic treatment was performed at 0°C with 25-sec bursts. Mitochondrial protein concentration was 2.5 mg/ml. −LP medium contained KCl, Tris-HCl, mitochondrial protein. +LP medium is the same as −LP, NADPH, ADP/Fe^{3+} added. LPS medium is the same as +LP, succinate added. Results are expressed as the mean value ± S.D., $n = 5$.

Discussion

In the present study we investigated the targets of lipid peroxidation in mitochondria and the effect of succinate in preventing the damages caused by the peroxidation. On the electrophoretogram (Fig. 1A, B) basically two changes could be observed. On one hand, densely stained areas were shown at the stacking gel (Fig. 1B), and on the other hand, certain protein bands were lost in the lipid-peroxidated preparations (Fig. 1A). Similar changes were described on beef heart submitochondrial particles (Narabayashi *et al.*, 1982). The simplest explanation for the first observation might be the development of cross-links between polypeptide chains. Malondialdehyde seems to be a good candidate for bridge formation betwen lysin residues of proteins (Chio and Tappel, 1969). Succinate was shown to delay the appearance of densely stained areas.

The disappearance of polypeptide bands could be explained either by the development of cross-links between some proteins showing high sensitivity to lipid peroxidation or by increased proteolytic activity. There was no sign of peptide fragmentation due to lipid peroxidation. As far as we know, there

was no protein degradation described in mitochondria during the course of lipid peroxidation. Succinate prevented to a certain extent the disappearance of bands as well.

The changes in the activity of the respiratory chain were also examined. It was known that the substrate-oxidizing capacity of mitochondria isolated from different sources decreased significantly as a consequence of lipid peroxidation (Pfeifer and McCay, 1972). Since the cytochrome oxidase activity in our experiments was unchanged, the peroxidation had to damage the respiratory chain at a more proximal segment. There should have been a considerable loss of cytochrome *c* content during the incubation, but this was not specific for lipid peroxidation (Table Ib).

The activity of succinate dehydrogenase (described previously on a different lipid-peroxidating system; McKnight and Hunter, 1966) decreased markedly. The inactivation of succinate dehydrogenase to a certain extent could be responsible for the decreased substrate oxidation, but the loss of this activity could be efficiently abolished by adding succinate to the peroxidating media (LPS), whereas the succinate failed to restore the inactivation of succinate oxidase.

From these data it is concluded that the most vulnerable area of terminal oxidation (investigated by the oxidation of succinate) is situated between succinate dehydrogenase and cytochrome *c*. These results are in good agreement with an earlier report on beef heart submitochondrial particles (Narabayashi *et al.*, 1982), i.e., that during lipid peroxidation the ubiquinone and cytochrome *b* contents of the membrane were decreased.

We also investigated the sensitivity of two matrix enzymes toward the inactivation due to ADP/Fe/NADPH-induced lipid peroxidation. The results were qualitatively in line with data of others on ferrous ion-induced lipid peroxidation (McKnight and Hunter, 1966). The NADP-dependent isocitrate dehydrogenase was inactivated but the glutamate dehydrogenase was not susceptible to peroxidative damage. A possible explanation might be provided on the basis of a close spatial association between the free-radical-generating enzyme system and the isocitrate dehydrogenase which might not exist in the case of glutamate dehydrogenase. To challenge this hypothesis, the lipid peroxidation was induced in sonically treated mitochondria, but the extent of inactivation was basically the same as was in the nonsonicated preparations.

Another possibility might be that the structure of a particular enzyme itself may determine its own susceptibility to inactivation by free radicals. Synthetic hydroperoxides can inactivate the isocitrate dehydrogenase (Green *et al.*, 1971). Both the isocitrate dehydrogenase and succinate dehydrogenase are known to have essential sulfhydryl groups, and therefore it can be presumed that in mitochondria the lipid hydroperoxides and/or oxygen free

radicals inactivate these enzymes by blocking their reactive thiol groups. Succinate had a significant protective effect on these enzyme activities.

The retained activity of glutamate dehydrogenase gave us the chance to follow the sequences of mitochondrial membrane damage caused by the process. On liposomes during the lipid peroxidation, induced by ferrous ions, the release of high-molecular-weight bipolymers was described (Kunimoto *et al.*, 1981). It is shown here that in mitochondria the enhancement of permeability ran parallel with the progression of malondialdehyde production and that succinate could prevent the permeability changes of mitochondria, proportionally to the inhibition of formation of thiobarbituric acid-reactive material.

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