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Lipid peroxidation and the reduction of ADP–Fe^{3+} chelate by NADH–ubiquinone reductase preparation from bovine heart mitochondria

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The NADH–ubiquinone reductase preparation (Complex I) of bovine heart mitochondria catalysed in the presence of reduced coenzymes and ADP–Fe^{3+} the lipid peroxidation of liposomes prepared from mitochondrial lipids. The apparent K_m values for the coenzymes and the optimal pH of the reactions agreed well with those of the lipid peroxidation of the submitochondrial particles treated with rotenone. On assay of the reduction of ADP–Fe^{3+} chelate by the reduction of cytochrome *c* in the presence of superoxide dismutase and antimycin A or by the oxidation of reduced coenzymes, the reactions were not affected by rotenone but were inhibited by thiol-group inhibitors. The properties of the ADP–Fe^{3+} reductase activity were highly consistent with those of the lipid-peroxidation reaction. These observations suggest that electrons from reduced coenzymes are transferred to ADP–Fe^{3+} chelate from a component between a mercurial-sensitive site and the rotenone-sensitive one of the NADH dehydrogenase and that the reduction of ADP–Fe^{3+} chelate by the NADH dehydrogenase is an essential step in the lipid peroxidation.

Lipid-peroxidation reactions catalysed by systems of isolated enzymes such as xanthine oxidase or NADPH–cytochrome *c* reductase have been studied for elucidating the mechanism of the enzymic lipid-peroxidation reactions, and the necessity for an artificial compound, EDTA, has been claimed (Pederson *et al.*, 1973; Svingen *et al.*, 1979). We have shown that bovine heart submitochondrial particles catalyse the peroxidation of endogenous lipids with reduced nicotinamide coenzymes as electron donors in the presence of ADP–Fe^{3+} chelate and proposed that the NADH dehydrogenase of the respiratory chain is responsible for the reaction [Takeshige & Minakami, 1975; the preceding paper (Takayanagi *et al.*, 1980)]. It seems necessary to simplify the mitochondrial lipid-peroxidation system so that we can show the role of the NADH dehydrogenase in the peroxidation reaction and elucidate the mechanism of the reaction.

We show in the present paper that the NADH–ubiquinone reductase preparation (Complex I) of bovine heart mitochondria catalyses the peroxidation of the lipids extracted from mitochondria and that the reduction of ADP–Fe^{3+} chelate through an electron carrier that is present between a mercurial-sensitive site and the rotenone-sensitive one of the respiratory chain is essential for the enzymic lipid peroxidation by mitochondria.

Experimental

Preparation of submitochondrial particles, NADH–ubiquinone reductase preparation and liposomes

Bovine heart submitochondrial particles were prepared as EDTA particles (Lee & Ernster, 1967), and the NADH–ubiquinone reductase preparation (Complex I) was prepared by the method of Hatefi & Rieske (1967). Mitochondrial lipids were extracted under N_2 by the method of Folch *et al.* (1957). Liposomes were prepared by sonicating the lipids in 50mM-Tris/acetate buffer, pH 7.4 at 4°C, under N_2 with a Tomy sonicator (UR-150P) at an output of 4A for 3min at 4°C. The amount of lipid was determined as lipid phosphorus by the method of Bartlett (1959).

Assays of lipid peroxidation

NADH- and NADPH-dependent lipid peroxidation of the submitochondrial particles and the malondialdehyde formation were determined as described in the preceding paper (Takayanagi *et al.*, 1980). The lipid peroxidation catalysed by Complex I was assayed by incubating liposomes (1μmol of

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lipid phosphorus) at 37°C for 10min with shaking in 1.0ml of a reaction mixture containing Complex I (5–7 μg), 2mM-ADP, 0.25mM-$FeCl_3$, 100mM-Tris/acetate buffer, pH7.4 at 37°C, and 0.1mM-NADH or 0.8mM-NADPH.

Assays of ADP–Fe^{3+} reductase activities

The rate of the reduction of ADP–Fe^{3+} by the NADH–ubiquinone reductase preparation was determined either by the oxidation of reduced coenzymes or the reduction of cytochrome *c* dependent on ADP–Fe^{3+}. The oxidation of reduced coenzymes was measured as follows: 1.0ml of a reaction mixture containing the NADH–ubiquinone reductase preparation (5–20 μg of protein), 2mM-ADP, 0.25mM-$FeCl_3$, 0.1mM-NADH or 0.8mM-NADPH and 100mM-Tris/acetate buffer, pH5.5–8.0 at 37°C, was incubated for 10min at 37°C with shaking. The oxidized coenzymes were extracted with $HClO_4$ and determined enzymatically (Estabrook *et al.*, 1967). The reduction of cytochrome *c* was measured at 37°C in dual-wavelength mode ($A_{550-540}$) with an Hitachi 556 spectrophotometer in a reaction mixture containing 100 μM native or acetylated cytochrome *c*, the reductase preparation (5–20 μg/ml), 2mM-ADP, 0.25mM-$FeCl_3$, 0.7 μM-antimycin A, 100mM-Tris/acetate buffer (pH5.5–8.0 at 37°C), 0.1mM-NADH or 0.8mM-NADPH and iron-superoxide dismutase (15 μg/ml), an absorption coefficient of $19.1 \times 10^3 M^{-1} \cdot cm^{-1}$ being used. Antimycin A was not necessary in the experiments with acetylated cytochrome *c*. The rate of the reduction of ADP–Fe^{3+} by the submitochondrial particles was determined by the oxidation of reduced coenzymes with a similar procedure as that described above, except that the submitochondrial particles (0.5mg/ml) replaced the reductase preparation and 2 μM-rotenone and 20 μM-2,6-di-t-butyl-*p*-cresol (butylhydroxytoluene) were added to the reaction mixture.

Other analytical procedures

$NAD(P)^+$ transhydrogenase and NADH–ubiquinone-1 reductase activities were measured by the method of Kaplan (1967) and Hatefi & Rieske (1967) respectively. NADH–ferricyanide reductase activity was measured by the decrease in A_{420}. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Reagents

Acetylated cytochrome *c* was prepared as described by Minakami *et al.* (1958). Iron-superoxide dismutase was purified from *Escherichia coli* by the method of Yost & Fridovich (1973) and the dismutase had activity of 5490units/mg of protein as determined by the method of McCord & Fridovich (1969).

Results

Lipid peroxidation by the NADH–ubiquinone reductase preparation

The NADH–ubiquinone reductase preparation (Complex I) catalysed malondialdehyde formation from the lipids isolated from mitochondria in the presence of ADP–Fe^{3+} chelate and NADH or NADPH. The rate of the formation increased linearly with the amount of the reductase preparation, and heat treatment of the preparation at 80°C for 10min completely destroyed the activity (Fig. 1*a*). The relationship between the rate of malondialdehyde formation and the amount of the lipids was as shown in Fig. 1(*b*). The NADH-dependent reaction was inhibited by NADH at high concentrations (higher than 0.1mM), which is similar to those observed for the NADH oxidase and NADH–ferricyanide reductase activities of mitochondria (Minakami *et al.*, 1962; Hatefi & Stempel, 1969), whereas the NADPH-dependent reaction was not inhibited by high concentrations of NADPH. Similar results were obtained by using NADH- and NADPH-generating systems (Fig. 1*c*), and the apparent K_m values for NAD^+ and $NADP^+$ were 0.88 ± 0.17 and 308 ± 13 μM (means ± S.E.M., $n = 3$) respectively. Both the NADH- and NADPH-dependent reactions had the optimal pH of 6.2 (Fig. 1*d*). These K_m values and optimal pH were highly consistent with those in the peroxidation reactions by rotenone-treated submitochondrial particles (0.9 μM for NAD^+ and 96 μM for $NADP^+$; Takayanagi *et al.*, 1980). The peroxidation by Complex I was inhibited by high ionic strength (50% inhibition at 0.3M-KCl) and not accelerated by the addition of EDTA–Fe^{3+} chelate, in contrast to the NADPH-dependent peroxidation catalysed by the NADPH–cytochrome *c* reductase preparation from rat liver microsomal fractions, which requires EDTA–Fe^{3+} and high salt concentrations for the maximal activity (Pederson *et al.*, 1973).

Reduction of ADP–Fe^{3+} chelate

Because we consider the reduction of ADP–Fe^{3+} to be a key step in the lipid peroxidation catalysed by Complex I, we determined the rate of the reduction of ADP–Fe^{3+}. One assay procedure is to measure the reduction of cytochrome c, which readily accepts electrons from the chelate. As shown in Fig. 2(*a*), acetylated cytochrome *c*, which does not interact with respiratory-chain components (Minakami *et al.*, 1958; Azzi *et al.*, 1975), was slowly reduced by Complex I, but the reduction was completely inhibited by superoxide dismutase, because this slow reduction is due to the O_2^- production supported by reduced coenzymes (Takeshige & Minakami, 1979). The addition of ADP–Fe^{3+} caused a rapid reduction of the acetylated

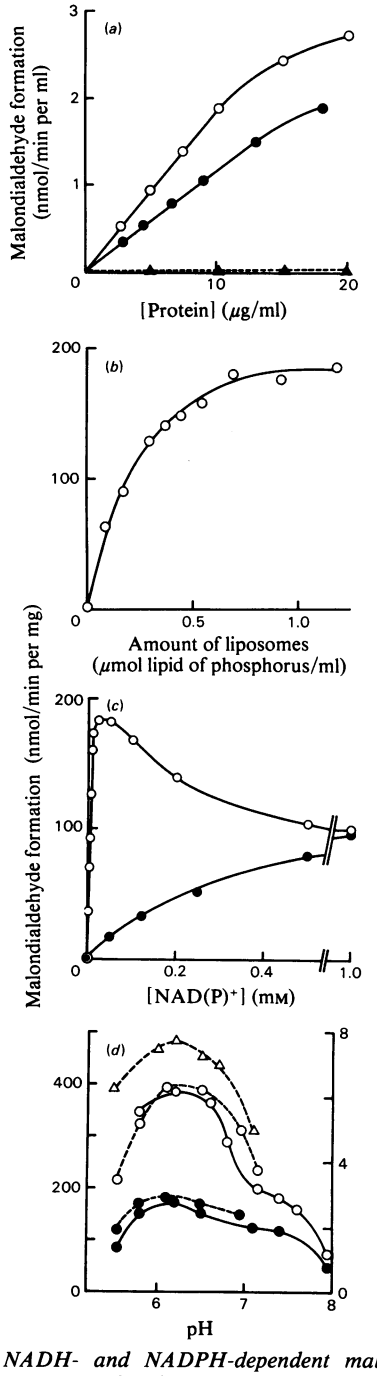

Fig. 1. *NADH- and NADPH-dependent malondialdehyde formation catalysed by Complex I: effects of (a) the amounts of Complex I preparation, (b) amounts of lipids, (c) coenzyme concentration and (d) pH*

The assay conditions were as described in the Experimental section except the parameters indicated on the abscissas. In the pH curves (*d*), the solid lines represent the values with 100mM-Tris/acetate buffer and the dotted lines represent those with 100mM-Tris/maleate buffer. Curves: O, Complex I preparation with NADH; ●, Complex I preparation with NADPH; ▲, the heated preparation (80°C, 10min) with NADH; △, the submitochondrial particles with NADH and 2μM-rotenone.

cytochrome *c*, which is supposed to be associated with the reduction of ADP–Fe^{3+}. Acetylated cytochrome *c* could be replaced by native cytochrome *c* if the determination was done in the presence of antimycin A, because of low activity of antimycin A-insensitive reduction of native cytochrome *c* in the NADH–ubiquinone reductase preparation (Fig. 2*b*).

The rate of the electron transfer to ADP–Fe^{3+} could also be estimated by the oxidation of NADH or NADPH in the presence of the chelate, in which NAD^+ or $NADP^+$ formed by the oxidation of the reduced coenzymes was measured. The results of the two procedures were essentially in good agreement with each other, and oxygen did not affect the assay procedures, as shown in Table 1, except that the addition of lipids to the assay systems caused increase in the NADH oxidation and decrease in the reduction of cytochrome *c*; these changes were overcome by the addition of butylhydroxytoluene. The observations indicate that the oxidation and reduction of the ADP–iron chelate is occurring during the lipid peroxidation and that the peroxidation competes with cytochrome *c* for electrons from ADP–Fe^{2+}. The oxidation of reduced coenzymes could be used for the assay of the ADP–Fe^{3+} reductase activity in the submitochondrial particles if butylhydroxytoluene and respiratory inhibitors such as rotenone, antimycin A and KCN were added in the assay system.

The apparent K_m values of the cytochrome *c* reduction via ADP–Fe^{2+} for NADH and NADPH were 3.3 and 660μM respectively, and the NADH-dependent reduction was inhibited by NADH at high concentrations, but the inhibition by excess NADPH was not observed. Both the NADH- and NADPH-linked ADP–Fe^{3+} reductions were maximal at about pH6.2. These properties of the reaction, to reduce ADP–Fe^{3+} chelate, are similar to those of NADH- and NADPH-dependent malondialdehyde formation by Complex I.

Effects of mercurials on the peroxidation and the reduction of ADP–Fe^{3+}

Rotenone did not affect the cytochrome *c* reduction via ADP–Fe^{2+} (not shown) but thiol-group inhibitors such as $HgCl_2$, *p*-hydroxymercuribenzoate and *N*-ethylmaleimide inhibited the reduction catalysed by the NADH–ubiquinone reductase preparation (Fig. 3*a*, curve B). The inhibition could be ascribed to the interaction of the

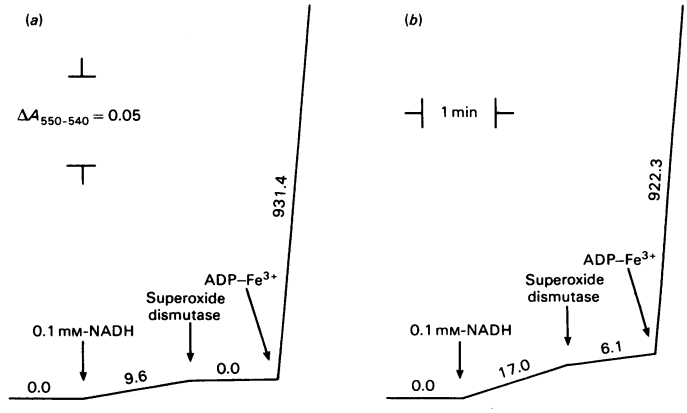

Fig. 2. *Reduction of cytochrome c via ADP–Fe^{2+} by the ubiquinone reductase preparation: (a) with acetylated cytochrome c, and (b) with native cytochrome c*

The assays were done in 100mM-Tris/acetate buffer, pH7.0 at 37°C, and with 15μg of the reductase preparation. Other conditions were as described in the Experimental section. Antimycin A was omitted in (*a*). Arrows indicate an addition of reagents. The rates of the reduction of cytochrome *c* are given on the traces as nmol/min per mg of protein. The concentrations of cytochrome *c* and $FeCl_3$ for the half-maximal reduction velocity were 30 and 46μM respectively.

Table 1. *NADH-dependent ADP–Fe^{3+} reductase activities of the ubiquinone reductase preparation*

NAD^+ formation and the reduction of native cytochrome *c* via ADP–Fe^{2+} were assayed in 100mM-Tris/acetate buffer, pH7.0 at 37°C, as described in the Experimental section and the reaction was initiated by the addition of 0.1mM-NADH. ADP–Fe^{3+} and EDTA–Fe^{3+} were prepared by mixing $FeCl_3$ and ADP or EDTA at 4°C in 50mM-Tris/maleate buffer, pH6.0, immediately before use, and the final concentrations of ADP, EDTA and $FeCl_3$ in a reaction mixture were made to 2, 0.26 and 0.25mM respectively. 'Control' in Expt. 1 represents the reaction mixture without ADP–Fe^{3+} and Thunberg tubes were used in Expt. 2. The values are given as means ± S.E.M. for four (NAD^+ formation) or three (cytochrome *c* reduction) experiments. The values with asterisks were means for two experiments.

	NAD^+ formation		Cytochrome *c*	
	(μmol/min per mg)	(%)	(μmol/min per mg)	(%)
Expt. 1				
Control	0.00*	0	0.01*	0
+ADP–Fe^{3+}	0.38 ± 0.01	100	0.91 ± 0.02	100
+ADP–Fe^{3+} (with heated preparation)	0.00*	0	0.00*	0
+EDTA–Fe^{3+}	0.07 ± 0.01	18	0.21 ± 0.01	23
+ADP–Fe^{3+} + EDTA–Fe^{3+}	0.44 ± 0.03	116	1.18 ± 0.02	130
+Lipid (1.3μmol/ml) + ADP–Fe^{3+}	0.74 ± 0.02	195	0.69 ± 0.06	76
+Butylhydroxytoluene (20μM) + ADP–Fe^{3+}	0.38 ± 0.01	100	0.90 ± 0.01	99
+Lipid (1.3μmol/ml) + ADP–Fe^{3+} + butylhydroxytoluene (20μM)	0.37 ± 0.01	97	0.89 ± 0.02	98
Expt. 2				
+ADP–Fe^{3+}, under atmosphere	0.44 ± 0.02	100	0.86 ± 0.02	100
+ADP–Fe^{3+}, under N_2	0.41 ± 0.03	93	0.90 ± 0.01	105

reagents with a component of the reductase preparation, because the preincubation of the preparation with the reagents was necessary for the inhibition, but the preincubation of ADP–Fe^{3+} or cytochrome *c* with the thiol-group inhibitors was ineffective. The ADP–Fe^{3+} reductase activity of the submitochondrial particles, as measured by the oxidation of NADH in the presence of the chelate, rotenone and butylhydroxytoluene, was also inhibited by *p*-hydroxymercuribenzoate (Fig. 3*b*, curve G). NADH-induced malondialdehyde formation by Complex I (Fig. 3*a*, curve C) and that by

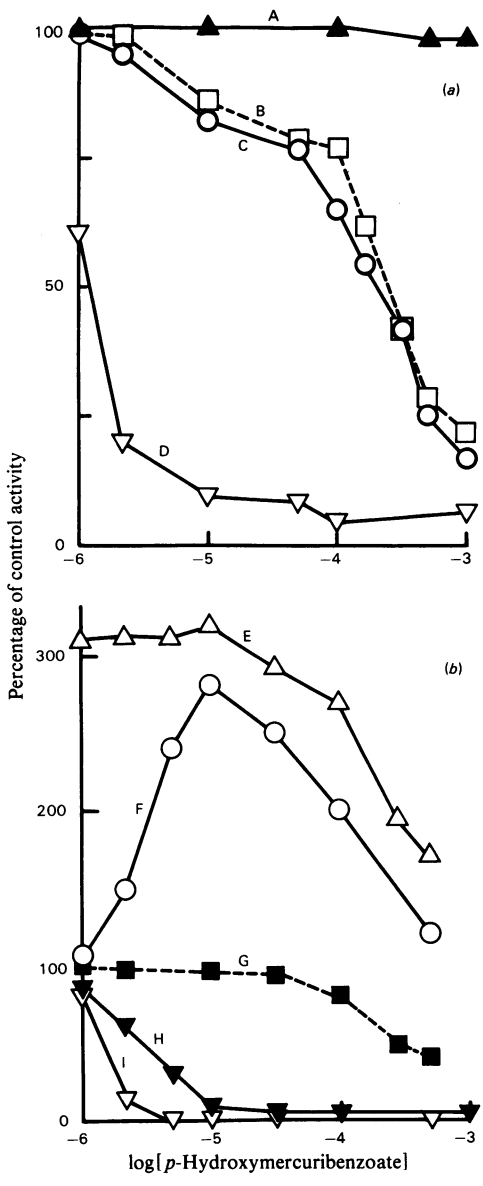

Fig. 3. *Effect of p-hydroxymercuribenzoate on the NADH-dependent malondialdehyde formation and the ADP-Fe^{3+} reductase activities of (a) the ubiquinone reductase preparation and (b) the submitochondrial particles*

The reductase preparation (15 μg/ml) or the particles (0.5 mg/ml) were incubated at 37°C for 20 min with the mercurial at the concentrations indicated on the abscissae in 1.0 ml of Tris/acetate buffer, pH 7.0 at 37°C, immediately before the assays. The assays were done at 37°C and pH 7.0. The values given below in parentheses are the control activities (nmol/min per mg of protein) without the mercurial. Curves: A (▲), ferricyanide reductase activity of the reductase preparation (80200); B (□), ADP-Fe^{3+} reductase activity of the reductase preparation measured by the reduction of cytochrome *c* (892); C (○) and F (○), the malondialdehyde formation of the reductase preparation (233) and the particles (1.86); D (▽) and I (▽), $NAD(P)^+$ transhydrogenase activities of the reductase preparation (461) and the particles (453); E (△), the malondialdehyde formation by the 2 μM-rotenone-treated particles (the activity without the rotenone treatment was used as the control activity; 1.86); G (■), ADP-Fe^{3+} reductase activity of the particles measured by the formation of NAD^+ in the presence of rotenone and butylhydroxytoluene (19.6); H (▼), ubiquinone reductase activity of the particles (420 with ubiquinone-1).

rotenone-treated submitochondrial particles (Fig. 3*b*, curve E) decreased with increasing concentration of the mercurial, and the inhibition of the peroxidation reactions appeared to be parallel with those of the ADP-Fe^{3+} reduction.

The sensitivities of the ADP-Fe^{3+} reduction and the malondialdehyde formation to the mercurial were much lower than those of the other activities catalysed by the NADH dehydrogenase, such as NADH–ubiquinone-1 reductase (Fig. 3*b*, curve H) or $NAD(P)^+$ transhydrogenase (Fig. 3*a*, curve D; Fig. 3*b*, curve I), except for the NADH–ferricyanide reductase activity, which is insensitive to the mercurial (Fig. 3*a*, curve A). Therefore two opposite effects of the mercurial were observed for malondialdehyde formation by the submitochondrial particles not treated with rotenone (Fig. 3*b*, curve F). The mercurial activated the peroxidation at low concentrations and inhibited it at high concentrations. The former effect can be ascribed to the inhibition of an antioxidant role of ubiquinol (Takayanagi *et al.*, 1980) and the latter effect to the inhibition of the ADP-Fe^{3+} reductase activity.

Discussion

We discussed in the preceding paper (Takayanagi *et al.*, 1980) the involvement of the NADH dehydrogenase of the respiratory chain in the lipid peroxidation of bovine heart submitochondrial particles supported by NADH or NADPH. The present paper gives more direct evidence for the contention that a reconstructed system consisting of the NADH–ubiquinone reductase preparation (Complex I) catalyses the peroxidation of lipids, and the peroxidation reactions by the ubiquinone reductase preparation and the submitochondrial particles were similar in respect to the K_m values for reduced coenzymes and the optimal pH. The NADH-dependent peroxidation by Complex I was not stimulated by rotenone, in contrast with the reaction by the submitochondrial particles, which is enhanced more than 2-fold by the rotenone treatment (Takayanagi *et al.*, 1980). The difference is explained by a low concentration of ubiquinone in the reconstructed system (about 8 μmol/mol of lipid

phosphorus) compared with that in the submitochondrial particles (about 1 mmol/mol of lipid phosphorus). The addition of 10 mmol of ubiquinone-1/mol of lipid phosphorus to the reconstructed system caused a complete inhibition of the peroxidation, and the inhibition was counteracted by the rotenone treatment (results not shown).

We also showed that the Complex I preparation as well as the submitochondrial particles could reduce ADP–Fe^{3+} chelate with NADH or NADPH and that the activity in Complex I could be assayed simply by the reduction of cytochrome *c* in the presence of superoxide dismutase. The NADH-linked ADP–Fe^{3+} reductase activity differs from the other activities catalysed by the NADH dehydrogenase of the respiratory chain in several respects: the reduction of ADP–Fe^{3+} was not inhibited by rotenone, but inhibited by a prolonged incubation with thiol-group inhibitors, and the pH optimum was 6.2. The ferricyanide reductase activity with a pH optimum at 8.0 is not affected by the modification of slowly reacting thiol group (Minakami *et al.*, 1964; Tyler *et al.*, 1965), the ubiquinone reductase activity is sensitive to rotenone and the transhydrogenase activity is more sensitive to thiol-group inhibitors than the ADP–Fe^{3+} reductase activity. These properties of the NADH-linked ADP–Fe^{3+} reductase activity were highly consistent with those of the NADH-dependent lipid peroxidation activity. The inhibition of the NADH-induced peroxidation by *p*-hydroxymercuribenzoate appeared to be parallel with that of the ADP–Fe^{3+} reduction, suggesting a possible participation of the ADP–Fe^{3+} reductase activity in the lipid peroxidation.

Although excess EDTA strongly inhibits lipid peroxidation (Gutteridge *et al.*, 1979; Pederson & Aust, 1972), Fe^{3+} chelated with a molar equivalent EDTA has been known to stimulate the lipid peroxidation catalysed by the NADPH–cytochrome *c* reductase of rat liver microsomal fractions (Pederson *et al.*, 1973). We, however, could not find the EDTA–iron chelate to be effective in our system, probably because of the sluggishness of EDTA–Fe^{3+} reduction by mitochondria (see Table 1).

We may conclude from the present and the preceding (Takayanagi *et al.*, 1980) papers that the NADH-dependent lipid peroxidation of bovine heart mitochondria is catalysed by the NADH dehydrogenase of the respiratory chain, which gives electrons to ADP–Fe^{3+} between a mercurial-sensitive site and the rotenone site, and the reduction of the chelate is an essential step for the lipid-peroxidation reaction.

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