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Abstract

Effect of inorganic phosphate on ferrous ion- and ascorbate-induced lipid. peroxidations of isolated rat liver mitochondria was investigated. As a result it has been shown that phosphate accelerates the ferrous ion.induced lipid peroxidation; namely, phos. phate shortens the induction lag period of the lipid peroxidation reaction but the malondialdehyde after onset of its production is yielded at the same rate in various concentrations of phosphate. On the other hand, phosphate inhibits ascorbate.induced lipid peroxidation. There are stoichiometric interactions between the concentration of phos. phate and the induction period. Oxygen uptake by mitochondria was observed in the presence of both ferrous ion and phosphate at initial step of the reaction without being accompanied by malondialdehyde production, and afterwards there occurred malondialdehyde production with rapid rate of the oxygen uptake. Possible mechanisms and interactions among ferrous ion, ascorbate and phosphate were discussed.

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STUDIES ON FERROUS ION-INDUCED LIPID PEROXIDATION OF RAT LIVER MITOCHONDRIA. I. EFFECT OF INORGANIC PHOSPHATE

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Abstract: Effect of inorganic phosphate on ferrous ion- and ascorbate-induced lipid peroxidations of isolated rat liver mitochondria was investigated. As a result it has been shown that phosphate accelerates the ferrous ion-induced lipid peroxidation; namely, phosphate shortens the induction lag period of the lipid peroxidation reaction but the malondialdehyde after onset of its production is yielded at the same rate in various concentrations of phosphate. On the other hand, phosphate inhibits ascorbate-induced lipid peroxidation. There are stoichiometric interactions between the concentration of phosphate and the induction period. Oxygen uptake by mitochondria was observed in the presence of both ferrous ion and phosphate at initial step of the reaction without being accompanied by malondialdehyde production, and afterwards there occurred malondialdehyde production with rapid rate of the oxygen uptake. Possible mechanisms and interactions among ferrous ion, ascorbate and phosphate were discussed.

The presence of unsaturated fatty acids in the phospholipid of mitochondrial membrane appeared to be essential for energy transfer reactions (1). It is of importance to consider the possibility that some intermediates of oxidative phosphorylation are associated with the polyunsaturated fatty acids which lead to lipid peroxide formation or accumulation under certain conditions (2). It has been reported that lipid peroxides may be formed during normal activity of electron transport chain in vitamine E-deficient rat liver mitochondria (3). Phosphate induces mitochondrial swelling which is dependent on electron transport and generation of high energy intermediates in the process of oxidative phosphorylation (4, 5). It is of interest to know the interaction of phosphate with lipid peroxidation in mitochondria.

Mitochondrial lipid peroxidation has been demonstrated correlatively with mitochondrial membrane alterations (swelling, lysis and disintegration) (2, 6, 7). Ferrous ion seems to be specific to the initiation of lipid peroxidation in mitochondria (8-11). However, the mechanism of mitochondrial lipid peroxidation

is not understood quite well. Ferrous ion-induced lipid peroxidation of mitochondria has a lag of induction period (8, 12). The lag is observed far longer in high concentration of Fe^{++} (8), and is shortened by addition of Fe^{+++}, suggesting that oxidation-reduction rate in the incubation system is an important factor (8). HUNTER *et al.*(2) reported that phosphate, which induces no lipid peroxidation, inhibits the lipid peroxidation of mitochondria induced by the mixture of oxidized and reduced glutathion (GSSG+GSH) and by ascorbate. But many workers (9, 11, 13–16), who have observed lipid peroxide formation in mitochondria under various conditions, have overlooked the effect of phosphate used in their study. To clarify the interaction of phosphate in mitochondrial lipid peroxidation, effect of inorganic phosphate on lipid peroxidation of isolated rat liver mitochondria induced by Fe^{++} or ascorbate was studied in particular on the lag of induction period. Our report describes that inorganic phosphate shortens the lag of the induction period of Fe^{++}-induced lipid peroxidation, and lengthens that of ascorbate-induced one.

MATERIALS AND METHODS

Preparation of mitochondria: Albino rats (200 to 300 g) derived from the Donryu strain were maintained on mouse-rat diet, MF type of Oriental Co.

Rat was killed by decapitation and the liver was immediately immersed in a chilled STE-solution (0.25 M sucrose, 0.003 M Tris-HCl and 0.0002 M EDTA, pH 7.4). All procedures of preparation were carried out at 0–4°C by a modified method of HOGEBOOM (17). The liver was cut with scissors to small pieces, washed in the STE solution, homogenized roughly by a glass homogenizer in ten volumes of the STE-solution, and gently homogenized by a teflon homogenizer. The homogenate was centrifuged at 100×g for 10 min. Nuclei and cell debris were removed and supernatant was layered on an equal volume of 0.34 M sucrose containing 0.003 M Tris-HCl, pH 7.4, and centrifuged at 700×g for 10 min. The upper layers were gathered and centrifuged at 5,000×g for 10 min to reduce contamination of microsomal materials. After cautious removal of fluffy layers, the precipitate was suspended in the STE-solution, centrifuged, and then washing was repeated in an ST-solution (0.25 M sucrose and 0.003 M Tris-HCl, pH 7.4). Final mitochondrial precipitate was resuspended in the ST-solution and maintained in a concentrated suspension in an ice-bath. Mitochondria were washed in a large volume of KT-medium (0.15 M KCl and 0.01 M Tris-HCl, pH 7.4), centrifuged, and suspended in the KT-medium by a teflon homogenizer before use.

Incubation system and reagents: Incubation system was composed of about 1 mg of mitochondrial protein per ml in KT-medium and/or in KP-medium (0.15 M KCl and 0.01 M Na-K-phosphate buffer). The medium was preincubated for 10 min and the reaction was started by adding 0.25 mM Fe^{++} and/or 1 mM ascorbate in final concentration. Incubation at 4°C was carried out in an ice-water bath at 0–4°C. Fe^{++} used was ammonium ferrous sulfate pur-

chased from Katayama Kagaku Co., and other reagents were of special grade. Distilled and deionized water was used. Protein content was determined by Buiret method (18) with bovine serum albumin (Armour Laboratories, Fraction V) as a standard.

TBA color reaction: As a diagnostic test for lipid peroxidation, malondialdehyde (MA) produced was detected. The detection of MA by thiobarbituric acid (TBA) was carried out by a modified method of HUNTER *et al.* (8). After incubating for various lapses of time, 2ml of medium was mixed with the solution composed of 0.5ml of 40% trichloroacetic acid, 0.25ml of 5N HCl and 0.5ml of 2% TBA to stop the reaction, and then heated in a boiling-water bath for 10 min. The mixtures were chilled and centrifuged. The absorbance of clear supernatant was measured by spectrophotometer at 532nm and 600nm. TBA value was represented by $OD_{532-600}$/mg of protein. Peroxide is expressed in terms of MA, using the molar extinction coefficient, $E_{532}=1.56\times10^5 cm^2$ $mmole^{-1}$ (19).

Oxymetric method: Oxygen consumption was measured with a Bioxygraph (Kusui-Kagaku Co.) (20) with a 2ml-chamber kept at 25°C. Other details are described in the legend of figure.

RESULTS

Effect of phosphate on MA formation: The characteristic curve for MA production in Fe^{++}-induced lipid peroxidation of mitochondria in KT-medium shows a lag of induction period followed by a rapid increase. Fe^{++} at a high concentration had a far longer lag but produced a larger amount of MA after

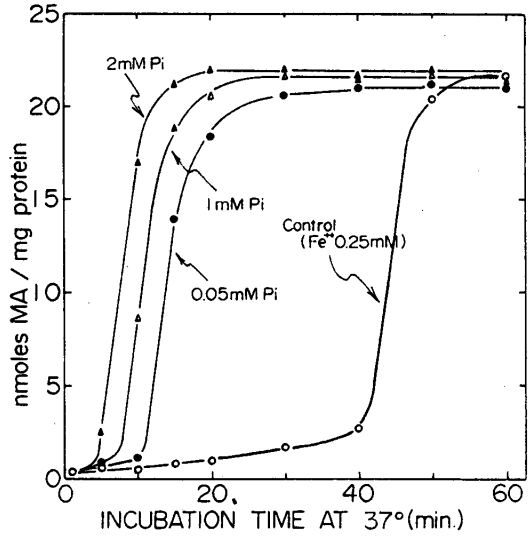

Fig. 1. Effect of inorganic phosphate (Pi) on the lipid peroxidation of rat liver mitochondria induced by Fe^{++} in KT (0.15M KCl, 0.01M, Tris-Cl, pH 7.4)-medium at 37°. Pi concentration in the medium is given in figure and reaction was started by addition of Fe^{++} (0.25mM).

the onset of the reaction than at a lower concentration of Fe^{++}. As shown in Fig. 1, in the presence of phosphate, Fe^{++}-induced lipid peroxidation at 37°C reflected the shortening of the lag period of induction. Increment of phosphate concentration further shortened the lag, but the velocity of MA production and the amount of MA produced were almost identical regardless of difference in the lag period. The changes of the induction period induced by phosphate in various concentrations of Fe^{++} were proportional to those in the control system without phosphate, indicating a linear correlation between the lags of induction and concentrations of Fe^{++} (Fig. 2).

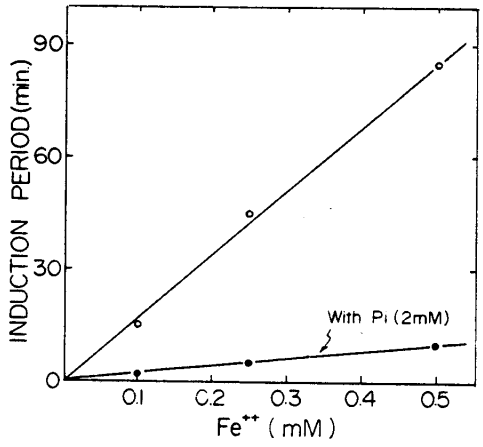

Fig. 2. Relation between the concentration of Fe^{++} and the induction period of mitochondrial lipid peroxidation induced by Fe^{++}. Incubation was carried out at 37° in KT-medium with or without 2 mM Pi. Induction period was calculated from the intervals before the onset of rapid MA formation.

On the contrary, phosphate prevented the appearance of ascorbate-induced MA formation at 37°C involving the lengthened lag period as shown in Fig. 3.

Judging from such divergent effects of phosphate on the mitochondrial lipid peroxidations induced by Fe^{++} and ascorbate, there seems to be a linear correlation between logarithmic values of the induction period and the molarity of phosphate in a certain range as illustrated in Fig. 4.

Effect of pH on MA formation in phosphate buffer: Effect of pH on the lipid peroxidations incubated at 37°C in KP-medium was tested. In Fe^{++}-induced lipid peroxidation, the onset of peroxidation was early in alkaline medium but MA was produced more aboundantly at a later time in the range of pH 6 to 7 (Fig. 5). On the other hand, ascorbate-induced lipid peroxidation was optimum at pH 6, and hardly reacted in alkaline solution (Fig. 6).

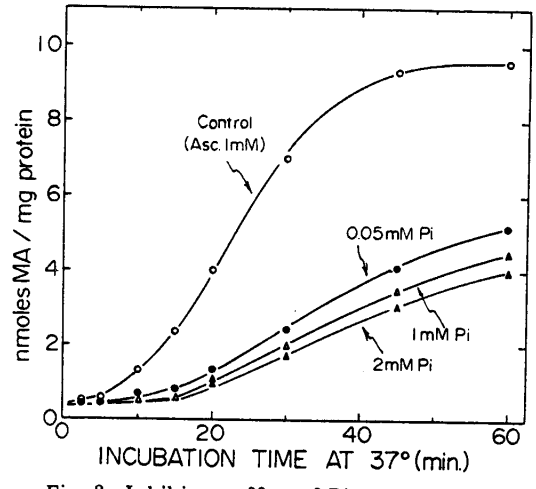

Fig. 3. Inhibitory effect of Pi on the lipid peroxidation induced by ascorbate (1 mM) in KT-medium at 37°.

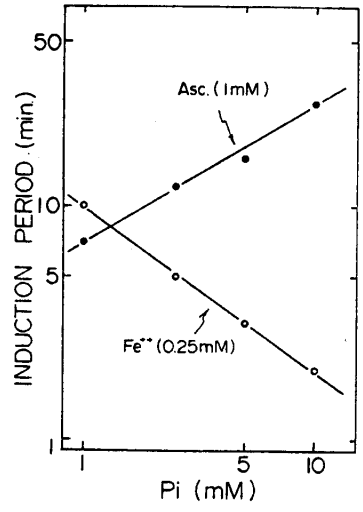

Fig. 4. Relation between the concentration of Pi and the induction period of Fe^{++}-and ascorbate-induced lipid peroxidation of mitochondria. Experimental conditions were identical with those in Figs. 1 and 3.

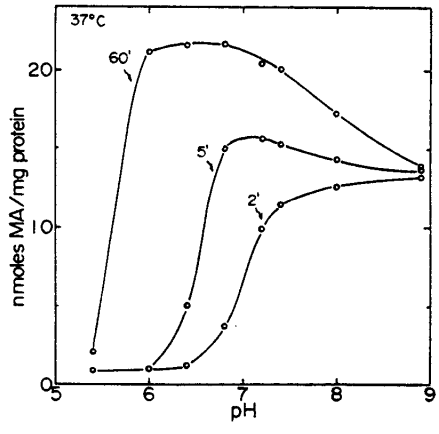

Fig. 5. Effect of pH on Fe^{++}-induced lipid peroxidation of mitochondria. The incubation system was composed of KP (0.15M KCl, 0.01M Na-K-phosphate buffer)-medium at 37°. Produced MAs at 2, 5 and 60 min after addition of 0.25 mM Fe^{++} are graphed respectively.

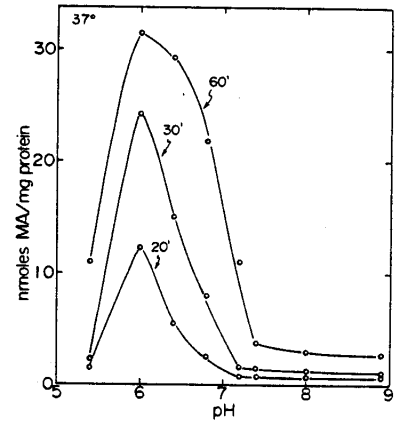

Fig. 6. Effect of pH on ascorbate-induced lipid peroxidation of mitochondria. The incubation system was composed of KP-medium at 37°. Produced MAs at 20, 30 and 60 min after addition of 1 mM ascorbate are graphed respectively.

Effect of temperature on MA formation: McKNIGHT *et al.* (21) reported that the inhibitory effect of Fe^{++} at high concentration in producing lag period before the onset of peroxidation in the KCl-malate medium was abolished at 0°C. This fact suggests non-enzymatic process of peroxidation in mitochondria. Peroxidation of mitochondria and heat-treated mitochondria was, therefore, compared at 4°C and 37°C in KP-medium, pH 7.4. The heat-treated mitochondria suspended in KT-medium were obtained by heating in a boiling-water bath for two min and resuspended by teflon homogenizer. It can be seen from Fig. 7 that Fe^{++}-induced peroxidation of non-treated mitochondria at 37°C almost allows no lag period, and at 4°C the reaction has a

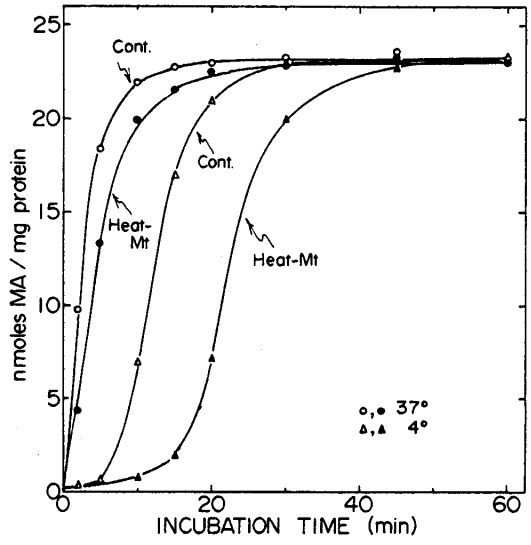

Fig. 7. Fe^{++}-induced lipid peroxidations of mitochondria and heat-treated mitochondria. Incubations were carried out at 37° or in an ice-water bath, and the reaction was started by addition of 0.25 mM Fe^{++} in the system of 0.15 M KCl and 0.01 M Na-K-phosphate buffer, pH 7.4.

lag period. At both temperatures the peroxidation of heat-treated mitochondria occurs later than that of non-treated one. On the other hand, ascorbate-induced peroxidation occurs faster in the heat-treated mitochondria at both temperatures than in non-treated mitochondria, as shown in Fig. 8. Ascorbate-induced reaction of non-treated mitochondria appears to be not producing MA at 4°C in the tested period of time.

Oxygen uptake on mitochondrial lipid peroxidation: In an earlier report it has been demonstrated that there is an extra oxygen consumption during the

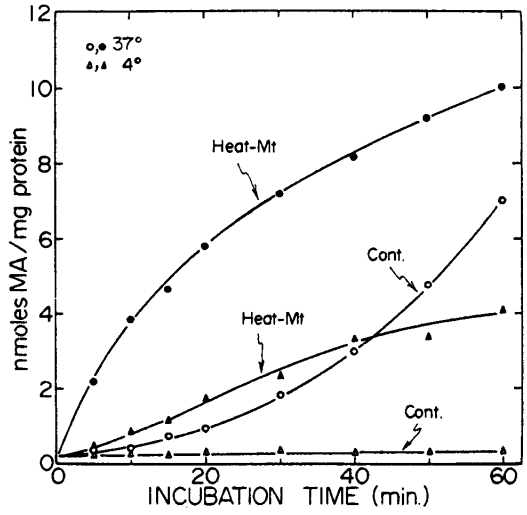

Fig. 8. Ascorbate-induced lipid peroxidations of mitochondria and heat-treated mitochondria. Ascorbate used was 1 mM and other conditions were identical as in Fig. 7.

Fe^{++}-induced lipid peroxidation (12). Oxygen uptake of mitochondria following the lipid peroxidation in the present system was measured at 25°C as recorded in Fig. 9. Endogenous oxygen consumption of mitochondria in KT-

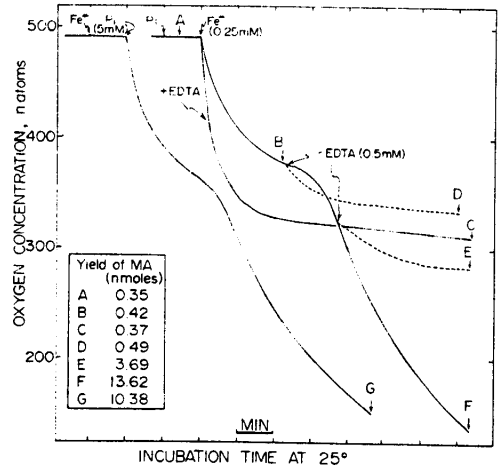

Fig. 9. Oxygen electrode traces involved in the Fe^{++}-induced lipid peroxidation of mitochondria (2.1 mg of protein) in 2 ml of KT-medium at 25°. Concentrations of added reagents are shown in Fig. Respective records are summarized. Dotted lines show the traces after addition of EDTA, and the trace marked +EDTA was recorded in the medium containing 0.5 mM EDTA. The left under table represents the yields of MA at point described by capital letters with arrows in the recorded traces.

medium could hardly be observed. The addition of Fe^{++} or phosphate alone had almost no effect on the oxygen uptake in a short period after the addition. But the addition of Fe^{++} in the presence of phosphate induced immediately a rapid oxygen uptake (Fig. 9). Similar oxygen uptake was observed on the addition of phosphate in the presence of Fe^{++}. The velocity of this initial burst of oxygen uptake was dependent on the concentration of phosphate (Fig. 10), but MA was not produced at the periods (checked by arrow B in

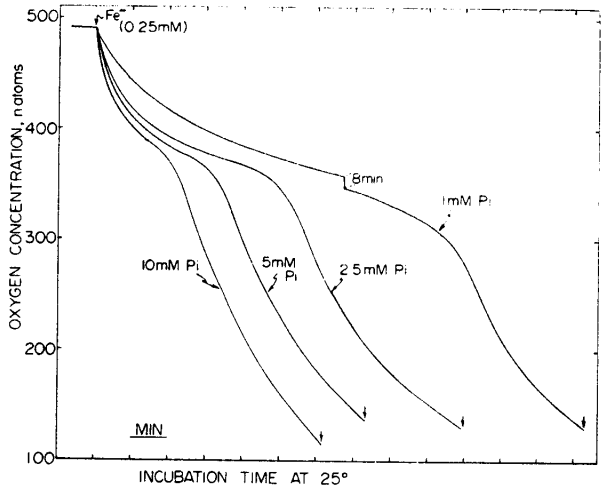

Fig. 10. Changes of oxygen uptake in mitochondrial lipid peroxidation in the presence of Pi at various concentrations. Incubation system at 25° was composed of 2.1 mg protein of mitochondria, KT-medium (pH 7.4), and Pi (pH 7.4, concentration shown in Fig.), total volume 2 ml. Fe^{++} (0.25 mM) was added at point shown by arrow, and respective records are summarized. MAs produced at the end of reactions (shown in Fig. by arrow) are 16.6, 13.6, 12.8 and 13.2 nmoles in 10, 5, 2.5 and 1 mM Pi, respectively.

Fig. 9), revealing that the oxygen uptake occurs during the lag period of induction of peroxidation. After a certain period of such oxygen consumption, the oxygen uptake was again observed in association with the production of MA (arrows G & F). Peroxidation was inhibited rapidly on the addition of EDTA which decreased both oxygen uptake and MA production (arrow D), even in the midst of peroxidation reaction (arrow E). But even in the presence of EDTA, the oxygen uptake of mitochondria was induced on the addition of phosphate and Fe^{++}. The oxygen uptake in such cases was larger than in the absence of EDTA. Needless to say, the oxygen uptake in this instance was not accompanied by MA production (arrow C). The molar ratio of MA produced per oxygen calculated from the consumed oxygen in relation to the MA

production was approximately 0. 05.

DISCUSSION

HUNTER *et al.* (2) state that phosphate at relatively low concentrations prevents the lipid peroxide production induced by GSSG+GSH in mitochondria, and point out some possible role of phosphate. The inhibitory behaviors of phosphate at high concentrations on ascorbate- and Fe^{++}-induced lipid peroxidation were also reported (2, 8). In the present study, the fact that phosphate prevents the ascorbate-induced lipid peroxidation in rat liver mitochondria coincides with other reports (2, 22). Since ascorbate-induced lipid peroxidation was facilitated at high temperature and in heat-treated mitochondria, the structural change of mitochondria could be preferential to lipid peroxidation reaction. However, such a lipid peroxidation was prevented by phosphate, so that phosphate might act as an antioxidant associated probably with oxidation of ascorbate. HUNTER *et al.* (2) suggested that phosphate inhibits GSSG+GSH-induced changes in mitochondria by a direct antioxidant action, possibly in a non-specific manner. It has been pointed out that phosphoric acid may belong to the electron-donating class of antioxidants (23).

The fact that phosphate eliminates the lag period of Fe^{++}-induced lipid peroxidation of mitochondria does not coincide with the other reports (2, 4). From the results of shortening the lag period and of pH dependency, the reaction of phosphate on lipid peroxidation induced by Fe^{++} might differ from that by ascorbate.

A number of interactions of phosphate with Fe^{++}-induced lipid peroxidation may be considered as follows :

(a) Since spontaneous swelling of mitochondria will permit $Fe^{++}$to penetrate into the site of certain unsaturated lipid (8), phosphate-induced swelling might affect the initiation of Fe^{++}-induced lipid peroxidation, but the accelerating action of phosphate can also be observed in heat-treated mitochondria.

(b) MA was formed earlier at a low concentration of Fe^{++} than at its high concentration (8). Practical change of Fe^{++} with phosphate toward insoluble salts might be considered for the lowering the concentration of Fe^{++}. However, yields of MA in a steady state after the onset of lipid proxide formation in various concentrations of phosphate were fairly constant. Thus, a complex salt, if formed, might be of an active form. While, MA-producing velocity of mitochondria after onset of the reaction with or without phosphate is likely to be of the same rate. It is suggested that phosphate affects the initiation of lipid peroxidation reaction chain, and enhances the prereaction such as producing free radical intermediates.

(c) HUNTER *et al.* (8) have described that Fe^{++} has no effect at pH 8. 0,

possibly because of its rapid conversion to Fe^{+++} and precipitation of ferric hydroxide. Fe^{++}-induced MA production in KP-medium occurred readily in an alkaline medium, but that at pH 6-7 was followed by the high rate of its production. Fe^{+++} added to the system eliminates the lag-producing effect in Fe^{++}-induced lipid peroxidation (8), then the rapid conversion of Fe^{++} to Fe^{+++} in alkaline might be considered to be responsible for the initiation of MA production. The mechanism of action of Fe^{+++} is not entirely clear, but it is possible that Fe^{+++} acts by rapidly releasing MA from oxidized fatty acid during incubation (24), and the oxidation-reduction rate in incubation system is an important role.

(d) The yield of MA (TBA chromogen) during peroxidation induced by Fe^{++} is far greater at 0°C than at 25°C (14). However, in KP-medium, the lag period was further lengthened at 4°C than at 37°C. The present experiment has not elucidated whether or not Fe^{++}-induced lipid peroxidation of mitochondria is involved in enzymatic reaction, but phosphate-induced alterations of structure and function in mitochondrial membrane dependent on temperature seem to be specific to the initial step of Fe^{++}-catalized peroxidation.

(e) Incorporation or binding of Fe^{++} in mitochondria is likely performed by both enzymatic and nonenzymatic processes, and the interaction between Fe^{++} and phosphoric base of phospholipid has been proposed in relation to the formation of metal-chelate complex (25, 26). Oxymetric experiments showed that the oxygen uptake in the presence of Fe^{++}, phosphate and mitochondria was observed without MA production, suggesting that it may reflect a metal-chelate complex formation with oxygen also being involved. TBA reaction (27), being employed to detect MA for judging lipid peroxidation, dose not divulge peroxidation reaction probably occurring during the lag period (28). The metal complex formation with oxygen seems to be an important role in peroxidation reaction. These compounds may be a reflection of oxidation of Fe^{++} to Fe^{+++} for the production of free radical intermediates which initiate lipid peroxidation chain reactions.

The mechanisms of interaction of phosphate on lipid peroxidation in mitochondria induced by Fe^{++} and ascorbate seem to be variable as above discussed. Therefore, further detailed experiments are required to clarify a great possibility of cooperative and control mechanisms of lipid peroxidation among Fe^{++}, ascorbate and phosphate in mitochondria.

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