EFFECTS OF RIBOFLAVIN DEFICIENCY ON LIPID PEROXIDATION OF RAT LIVER MICROSOMES

Misako Taniguchi

Division of Food and Nutrition, Nakamura Gakuen College,
Fukuoka 814, Japan

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Summary  Increases in cytochrome P-450 and cytochrome b$_{5}$ and a decrease in NADPH-cytochrome c (P-450) reductase were generally brought about by feeding the riboflavin-deficient diet to young rats (50–120 g body weight) for 5 weeks, whereas no significant changes in these enzymes were observed with rats of 220 g body weight by feeding for 2 weeks. Amounts of lipid peroxides in the serum or the liver tissue and microsomes increased significantly after feeding the deficient diet for 5 weeks, in comparison with the respective control rats. On the other hand, NADPH-dependent lipid peroxidation in the presence of ferric ion and pyrophosphate, assayed as malondialdehyde, was decreased drastically in the liver microsomes of all groups of riboflavin-deficient rats irrespective of the period or body weight of animals. Lipid peroxidation could be detected by addition of EDTA-ferric ion or ferricyanide to the incubation medium, though the formation of malondialdehyde was less than that expected by the activity of NADPH-cytochrome c reductase. NADPH-reduction of nitroblue tetrazolium with liver microsomes was decreased in riboflavin deficiency, and was almost able to be correlated with the activity of NADPH-cytochrome c reductase. Following intraperitoneal injection of riboflavin into the deficient rats, NADPH-dependent lipid peroxidation in the presence of ferric ion and pyrophosphate recovered only to 10% of the control rates at 40 hr after the injection, when cytochrome P-450, cytochrome b$_{5}$ and NADPH-cytochrome c reductase levels were restored to those of their respective controls.

Activities of drug-metabolizing enzymes, aminopyrine demethylase and aniline hydroxylase were decreased by initiation of feeding from the weanling stage, but the activities changed only slightly by feeding from the 120 g of body weight stage.

Keywords  riboflavin deficiency, microsomal lipid peroxidation, microsomal enzymes, NADPH-dependent lipid peroxidation, riboflavin-deficient rat liver enzymes, lipid peroxidation, riboflavin-deficient rat liver

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Morphological changes in hepatic cells were revealed in riboflavin deficiency, and enlargement of sizes and discontinuity of membrane of mitochondria and increase of endoplasmic reticulum were shown in rats (1) and mice (2, 3). Lipid peroxide is regarded as one of the causes of damage to the cytomembrane, and riboflavin is a constituent of NADPH-cytochrome c reductase which has been reported to function in vitro in lipid peroxidation with microsomes (4, 5). In this paper, the amount of lipid peroxide and change in the activity of the reductase were studied with liver microsomes or riboflavin-deficient rats. The presence of EDTA was required for NADPH-dependent lipid peroxidation in addition to ferric ion and pyrophosphate with purified NADPH-cytochrome c reductase (4), while the maximum rate was obtained in the absence of EDTA with microsomal preparations (6). In the present study, the presence and absence of EDTA were compared for NADPH-dependent lipid peroxidation with riboflavin-deficient liver microsomes. It was found that the lipid peroxidation was completely impaired by the riboflavin deficiency in the absence of EDTA. Some lipid peroxidation was observed upon the addition of EDTA, although the rate was about one third to one fifth of the control lipid peroxidation.

Activities of microsomal drug-metabolizing enzymes were also determined with riboflavin-deficient rat liver microsomes in association with the changes in the activity of NADPH-cytochrome c reductase and lipid peroxidation. Cytochrome P-450 and cytochrome b₅ in the liver microsomes were additionally determined, since these enzymes are members of the electron transport systems in the microsomes.

MATERIALS AND METHODS

Riboflavin-deficient diet and maintenance of rats. Ten to twenty male rats of various ages of Wistar-King strain were maintained on a riboflavin-deficient diet as reported previously (7). For the control, rats were fed on the diet to which riboflavin had been added in the amount of 40 mg/kg of the basal deficient diet. Each rat was placed in a separate cage and allowed to eat the diet ad libitum. For the recovery experiment, riboflavin dissolved in sterilized 0.9% (w/v) NaCl solution was daily injected intraperitoneally into the deficient rats at a concentration of 10 mg per 100 g of body weight under light ether anesthesia. As recovery control, rats which had been fed on the control diet were injected with 0.9% (w/v) NaCl solution intraperitoneally.

Preparation of liver microsomes. The rats were killed by decapitation, and each liver was perfused with cold 1.15% (w/v) KCl solution. The livers were weighed and homogenized with 4 volumes of the KCl solution containing 10 mM EDTA, and adjusted to pH 7.4 with 50 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 10,000 g for 20 min, and the supernatant for 105,000 g for 1 hr. The pellet was suspended in two volumes of 1.15% (w/v) KCl solution and the suspension was recentrifuged at 105,000 g for 30 min. The pellet
consisting of microsomes was suspended in the cold 1.15% (w/v) KCl solution to yield a protein concentration of 15 to 20 mg per ml.

Determination of microsomal lipid peroxidation. NADPH-dependent lipid peroxidation was assayed by incubation of microsomes (0.2 to 0.5 mg of protein) at 33°C in a tube containing a reaction mixture of 0.1 M Tris-HCl buffer (pH 7.2), 0.012 mM FeCl₃, 0.075 M phosphate buffer (pH 7.2), and 0.02 mM pyrophosphate in a final volume of 1 ml, the reaction being initiated by the addition of 0.3 μmole of NADPH. At various time intervals of the incubation, 1 ml of 10% (w/v) trichloroacetic acid solution and 2 ml of 0.67% (w/v) thiobarbituric acid were added. The tube was placed in a boiling water bath for 15 min. After cooling, the flocculent precipitates were centrifuged at 1,000 g for 10 min, and the absorbance of the supernatant was determined at 535 nm. In one experiment, 0.2 mM potassium ferricyanide replaced the FeCl₃. In another experiment, the microsomes were incubated at 37°C in the reaction mixture containing 0.25 M Tris-HCl (pH 6.8), 0.25 M NaCl, 2 mM ADP, 0.22 mM Fe(NO₃)₃ and 0.05 mM EDTA, essentially according to the method of Pederson et al. (4), and the lipid peroxide formed was determined as described above. Lipid peroxide was expressed as malondialdehyde with a standard made from 1,1,3,3-tetraethoxypropane, and the concentration was calculated using an extinction coefficient of 1.56 × 10⁵ M⁻¹ cm⁻¹ (6).

Determination of lipid peroxide. Rat blood was collected from the carotid artery after decapitation. The serum was used for determination of lipid peroxides within 1 hr after preparation. A portion of the liver homogenate for the microsomal preparation has been set aside, and centrifuged at 3,000 g for 10 min. The supernatant was used for determination of lipid peroxides, determined by the method of Naito and Yamanaka (8). To 3 ml of 0.05 N HCl, 0.3 ml of the serum, liver homogenate or microsomal suspension, and then 1 ml of 0.67% (w/v) thiobarbituric acid were added. The mixture was heated at 95°C for 30 min. After cooling, 4 ml of n-butanol containing 15% (v/v) methanol was added, and the colored substance derived from lipid peroxides was extracted by the butanol layer by shaking. The absorbance was determined at 535 nm, and lipid peroxide was expressed as nmole of malondialdehyde as described above.

Reduction of nitroblue tetrazolium. The rate of reduction of nitroblue tetrazolium was assayed at 25°C by incubation of microsomes (about 0.1 to 0.2 mg of protein) in 3 ml of the reaction mixture whose composition was the same as used in determination of NADPH-dependent lipid peroxidation. The reduction was initiated by the addition of 0.1 μmol of NADPH and 0.7 μmol of nitroblue tetrazolium chloride to the incubation mixture, and the rate of reduction was expressed as increase of optical density at 560 nm/mg of protein/min.

Assay of enzymes. The microsomal preparation (about 2 mg of protein) was suspended in 0.1 M phosphate buffer (pH 7.5), containing 20% glycerol (v/v), and cytochrome P-450 was determined from CO difference spectra of dithionite-reduced samples, using a value of 91 cm⁻¹ mm⁻¹ for the molar extinction coefficient between 450 and 490 nm (9, 10). Cytochrome b₅ was determined from
the difference spectra between NADH-reduced and air-saturated microsomal samples, using a value of 185 cm\(^{-1}\) mm\(^{-1}\) for the molar extinction coefficient between 424 and 409 nm (9).

NADPH-cytochrome c reductase was assayed with microsomal suspension in 0.3 M phosphate buffer (pH 7.5) at 25°C by the addition of 75 \(\mu\)M cytochrome c and 0.1 mM NADPH. The reductase activity was expressed as \(\mu\)mol of cytochrome c reduced per min measured at 550 nm using an extinction coefficient of 21.1 cm\(^{-1}\) mm\(^{-1}\) (10).

Aminopyrine demethylase and aniline hydroxylase activities were measured by colorimetric determination of formaldehyde or \(p\)-aminophenol, respectively, which was formed by incubation of microsomes (about 1 to 2 mg of protein) with 2 mM aminopyrine or 1 mM aniline at 37°C for 15 min. The reaction mixture contained 0.5 mM NADP, 5 mM glucose 6-phosphate, 5 mM MgCl\(_2\), 1.5 units of glucose 6-phosphate dehydrogenase and 50 mM Tris-HCl (pH 7.4) in a final volume of 3 ml. For determination of the reaction, 3 ml of 10% (w/v) or 1.5 ml of 20% (w/v) trichloroacetic acid solution was added to the mixture. After sedimentation of proteins, formaldehyde or \(p\)-aminophenol in the supernatant was determined with Nash’s reagent (11) or phenol reagent (12), respectively.

Protein was determined by the method of Lowry et al. (13), using bovine serum albumin V as a standard. Riboflavin was determined fluorometrically as lumiflavin (14), after extraction of microsomes with distilled water at 80°C for 15 min.

Reagents and chemicals. NADPH, NADP (disodium salt), NADH, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were obtained from Boehringer Mannheim GmBH. Cytochrome c (horse heart) was obtained from Sigma Chemical Co. Other chemicals used were of reagent grade. Vitamin-free casein was purchased from Nutritional Biochemical Corporation and contained 0.50 \(\mu\)g of riboflavin per gram, according to the product information from the corporation.

RESULTS

Changes in microsomal enzymes by riboflavin deficiency

Rats of different ages were maintained with a riboflavin-deficient diet for 2 to 5 weeks, and microsomal enzymes were assayed. As shown in Table 1, the effects of the riboflavin deficiency on the rate of growth, and contents or activities of microsomal enzymes were quite apparent in the rats which had been fed the deficient diet from early stages of growth.

Increase in contents of cytochrome P-450 and cytochrome \(b_5\) and decrease in activity of NADPH-cytochrome c reductase were generally brought about in the weanling or young rats by feeding the deficient diet for 5 weeks. However, the changes in aminopyrine demethylase and aniline hydroxylase were not significant when rats of initial body weight of about 120 g were fed the deficient diet, whereas

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Table 1. Changes of the rat liver microsomal enzymes by feeding riboflavin-deficient diet.

| Exp. no. | Group | Number | Initial body weight (g) | After period body weight (g) | Cytochrome P-450 (nmol/mg protein) | NADPH-cytochrome P-450 reductase (nmol/mg protein 15 min) | Aminopyrine N-demethylase (nmol/mg protein 15 min) | Aniline hydroxylase (nmol/mg protein 15 min) | Riboflavin (µg/mg protein) |
|----------|-------|--------|-------------------------|-----------------------------|-----------------------------------|----------------------------------------------------------|------------------------------------------------|------------------------------------------|--------------------------|
| I        | Riboflavin deficient | 10     | 50 ± 5                  | 90 ± 5                      | 1.54 ± 0.32*                      | 0.72 ± 0.06*                                              | 31 ± 2*                                      | 140 ± 7*                   | 5.5 ± 1.1*                |
| Control  |       | 5      | 48 ± 5                  | 88 ± 7                      | 0.87 ± 0.06                       | 0.55 ± 0.05                                               | 56 ± 3                                       | 237 ± 21                   | 11.7 ± 1.0                |
| II       | Riboflavin deficient | 10     | 126 ± 6                 | 186 ± 15*                  | 1.43 ± 0.20*                      | 0.72 ± 0.13*                                              | 44 ± 3*                                      | 218 ± 30                   | 11.1 ± 1.4                |
| Control  |       | 5      | 120 ± 15                | 286 ± 27                   | 0.98 ± 0.03                       | 0.49 ± 0.03                                               | 62 ± 3                                       | 226 ± 5                    | 12.8 ± 1.0                |
| III      | Riboflavin deficient | 15     | 115 ± 3                 | 148 ± 12*                  | 1.41 ± 0.19*                      | 0.62 ± 0.09                                               | 40 ± 3*                                      | —                          | —                        |
| Control  |       | 5      | 110 ± 8                 | 252 ± 25                   | 1.03 ± 0.06                       | 0.80 ± 0.02                                               | 62 ± 3                                       | —                          | —                        |
| IV       | Riboflavin deficient | 9      | 218 ± 14                | 274 ± 25                   | 0.93 ± 0.11                       | 0.49 ± 0.06                                               | 55 ± 7                                       | 209 ± 21                   | 8.5 ± 1.1                |
| Control  |       | 2      | 215 ± 6                 | 281 ± 10                   | 0.86 ± 0.08                       | 0.49 ± 0.01                                               | 58 ± 6                                       | 213 ± 34                   | 9.0 ± 1.0                |

Values are average ± SD. * Significantly different from control (p < 0.01).
these enzyme activities were reduced significantly in the rats of initial body weight of 50 g.

By feeding rats of about 210 to 220 g of body weight with the riboflavin-deficient diet for 2 weeks, changes in the contents or activities of enzymes were not significant in comparison with respective control rats. At this stage, the liver microsomal riboflavin content of the deficient diet group did not differ from that of the control rats, as shown in Table 1.

**Lipid peroxide in serum, liver homogenate and microsomes**

Lipid peroxide content was determined with the rats used in the experiment No. III in Table 1. In riboflavin deficiency, lipid peroxides in the serum and liver homogenate and microsomes were all elevated significantly, compared with respective controls (Table 2). The microsomal lipid peroxide content was relatively higher than that of homogenate as expressed per mg of protein. Microsomes contain a large amount of polyunsaturated fatty acids as membrane constituents, and peroxidation would be favoured to occur *in situ*. Since the serum lipid peroxide content was expressed per ml, the values were not comparable with those of the liver homogenate or microsomes, which were expressed as per mg protein.

**Table 2. Lipid peroxides in rat serum, liver tissue and microsomes.**

| Group             | Number | Lipid peroxides                        |
|-------------------|--------|----------------------------------------|
|                   |        | Serum (nmol/ml) | Liver homogenate (nmol/protein mg) | Liver microsomes (nmol/protein mg) |
| Riboflavin deficient | 15    | 5.4 ± 1.2** | 0.55 ± 0.18* | 1.11 ± 0.32* |
| Control           | 5      | 3.7 ± 1.0   | 0.23 ± 0.04  | 0.41 ± 0.09  |

Lipid peroxides in serum, liver tissue homogenate and microsomes were determined with rats fed a riboflavin-deficient or control diet for 5 weeks, whose microsomal enzymes were shown in Table 1, Exp. No. III. Values are average ± SD. Lipid peroxides are expressed as nmol of malondialdehyde. * Significantly different from control (*p < 0.01), ** (*p < 0.05)

**Microsomal lipid peroxidation**

The microsomes prepared from rats (Table 1, No. III) were incubated in the medium which contained ferric chloride and pyrophosphate. As shown in Fig. 1, lipid peroxidation was completely blocked in the rats fed on a riboflavin-deficient diet. However, measurable lipid peroxidation of the same microsomes was observed upon the addition of EDTA or ferricyanide to the incubation medium. Increase in the concentration of ferric chloride from 0.012 to 0.12 mM did not enhance the lipid peroxidation appreciably. The disappearance of the lipid
peroxidation in the presence of ferric chloride and pyrophosphate was observed with microsomes of the rats as early as 2 weeks of feeding of the deficient diet, when no significant differences were demonstrated in the liver microsomal enzymes (Table 1, No. IV).

Initial rates of malondialdehyde formation were determined with liver microsomes of riboflavin-deficient rats (Table 1, No. III) in the presence of various stages of ferric ion. The rate was less than 1% with ferric chloride and pyrophosphate, 25% with EDTA, ferric nitrate and ADP, and 15% with ferricyanide and pyrophosphate, respectively, in comparison with control microsomes (Fig. 1).

It was concluded that the formation of lipid peroxides determined as malondialdehyde was not directly correlated with the activity of NADPH-cytochrome c reductase, since the activity of the reductase was reduced by about 45% in the liver microsomes of the deficient rats (Table 1, No. III).

Reduction of nitroblue tetrazolium

Superoxide anion generated by enzymatic or non-enzymatic systems was found to reduce nitroblue tetrazolium (15); thus, reduction of nitroblue tetrazolium
Table 3. Reduction of NBT by rat liver microsomes.

| Group          | Number | O.D.$_{560}$/protein mg/min |
|----------------|--------|-----------------------------|
| Riboflavin-deficient | 10     | 0.312 ± 0.046               |
| Control        | 5      | 0.557 ± 0.094               |

Values are average ± SD of the rate of reduction of nitroblue tetrazolium (NBT), expressed as increased O.D.$_{560}$ per mg of microsomal protein per min. Number: number of rats.

was assayed with the liver microsomes of riboflavin-deficient rats (Table 1, No. III), in order to reveal whether production of superoxide anion was decreased or whether scavengers of free radicals were increased in the microsomes of riboflavin-deficient rat livers. As shown in Table 3, the reduction of nitroblue tetrazolium was decreased in the microsomes of riboflavin-deficient rats, compared with the controls. It should be noted that the riboflavin deficiency reduced the activity of NADPH-cytochrome c reductase (Table 1), and the reduction of nitroblue tetrazolium was nearly proportional to the activity of the reductase.

Recovery

Twenty weanling rats (50 ± 5 g) had been fed on a riboflavin-deficient diet for 5 weeks, and six of these rats were taken at random and injected with riboflavin intraperitoneally. At 20 and 40 hr after the injection, three of the rats were used for recovery tests of microsomal enzymes and lipid peroxidation upon riboflavin-supplement. Five rats (50 ± 5 g) had been maintained with the control diet, and were used as controls after intraperitoneal injection of 0.9% (w/v) NaCl solution in place of riboflavin solution. The contents of cytochrome P-450 and cytochrome b$_{5}$ recovered nearly to the control levels at 20 hr after the administration of riboflavin. Normalization of NADPH-cytochrome c reductase was delayed and recovered to the control level at 40 hr (Fig. 2). On the other hand, recovery of NADPH-dependent microsomal lipid peroxidation in the presence of ferric chloride and pyrophosphate was greatly retarded, and only 10% of the control malondialdehyde value was observed at 40 hr after injection of riboflavin, when contents of riboflavin in microsomes had nearly recovered to normal levels (Fig. 3). From these results, it was concluded that microsomal NADPH-dependent lipid peroxidation in the presence of ferric chloride and pyrophosphate was the most susceptible to the riboflavin deficiency.

DISCUSSION

Increases in lipid peroxides were observed in the serum and liver of the rats which had been fed on a riboflavin-deficient diet for 5 weeks. The causes of increase

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Fig. 2. Recovery of microsomal enzymes after injection of riboflavin. Points represent mean values of enzyme concentration or activity, with their standard deviations represented by vertical bars. Riboflavin was injected intraperitoneally into the rats which had been fed on a riboflavin-deficient diet for 5 weeks. Cytochrome P-450 (○), cytochrome bs (●) and NADPH-cytochrome c reductase (●) were determined at 20 and 40 hr after the injection of riboflavin, and are shown as percent of respective controls. In the control rats, the concentration of cytochrome P-450 and cytochrome bs were 0.88 ± 0.10 nmol per mg of protein, and 0.55 ± 0.05 nmol per mg of protein, and activity of NADPH-cytochrome c reductase was 58 ± 4 nmol per min per mg of protein. Numbers of the rats are: riboflavin-deficient, 5; control, 5. At 20 hr after riboflavin injection, 3; at 40 hr, 3.

Fig. 3. Recovery of NADPH-dependent lipid peroxidation. Points represent mean values of lipid peroxide assayed as malondialdehyde (●) or content of riboflavin (○), with their standard deviations represented by vertical bars. Rats and numbers of animals are the same as shown in Fig. 2.

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in lipid peroxides in the liver could be accounted for by; i) acceleration of lipid peroxidation by enzymatic or nonenzymatic processes, and/or increase in polyenoic fatty acid levels as the substrates for lipid peroxidation, and ii) decrease in degradation in situ or removal of lipid peroxides from the liver.

In the previous report (7), the contents and composition of fatty acids in lipids of rat liver microsomes were shown to be altered by riboflavin deficiency; an increase in linoleic acid (control: 7.6 ± 0.7%; deficient: 13.1 ± 1.1%) and a decrease in arachidonic acid (control: 32.0 ± 1.8%; deficient: 23.0 ± 1.0%) were shown. In the present experiments, lipid peroxides were determined by conversion to malondialdehyde, and by the reaction of the latter with thiobarbituric acid to produce a colored substance. It was reported by Ohkawa et al. (16) that the hydroperoxide of linoleic acid produced no color with thiobarbituric acid in acidic conditions, whereas those of linolenic and arachidonic acids gave a pigment. Therefore, the decrease in arachidonic acid levels in the liver microsomes of riboflavin-deficient rats would provide less substrates for lipid peroxidation, as detected by the thiobarbituric acid assay, although this could not explain the entire disappearance of the formation of malondialdehyde.

Though NADPH-dependent lipid peroxidation was not detected in the liver microsomes of riboflavin-deficient rats in the presence of ferric chloride and pyrophosphate, this was demonstrated upon the addition of ferric ion in a complexed state, such as EDTA-ferric ion or ferricyanide, to the incubation medium. Pederson et al. (4) showed that purified NADPH-cytochrome c (P-450) reductase catalyzed NADPH-dependent lipid peroxidation. The addition of EDTA was necessary for this reaction in addition to ferric ion and ADP, while microsomal NADPH-dependent lipid peroxidation occurred without the addition of EDTA. Benedetto and Slater (17) observed no correlation between lipid peroxidation in the presence of NADPH, ADP and ferrous ion and activity of NADPH-cytochrome c reductase in microsomes of the various tissues of rat, and they postulated a role of a thiol group-dependent protein in NADPH-dependent lipid peroxidation. From the present experimental results, it was suggested that ferric or ferrous ion in the chelated state would be a prerequisite for radical formation, and consequently for the lipid peroxidation.

Lai and Piette (18) applied spin traps to the studies of NADPH-dependent lipid peroxidation and reported that the OH· radical produced via the classic Fenton reaction (a) was an initiator of the lipid peroxidation.

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}· \quad \text{(a)}
\]

They postulated that OH· was generated during microsomal lipid peroxidation by the following schemes:

\[
\text{Oxidized P-450 reductase} + \text{NADPH} \rightarrow \text{reduced P-450 reductase} + \text{NADP}^+ \quad \text{(b)}
\]

\[
\text{Reduced P-450 reductase} + \text{O}_2 \rightarrow \text{oxidized P-450 reductase} + \text{O}_2^- \quad \text{(c)}
\]

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\[ O_2^+ + H^+ \rightarrow HO_2 \]  
\[ O_2^+ + HO_2 \rightarrow O_2 + H_2O_2 + OH^- \]  
\[ O_2^+ + H_2O_2 \rightarrow O_2 + OH^- + OH^+ \]

Provided that OH· is concerned with lipid peroxidation as postulated by Lai and Piette (18), the step to yield \( O_2^+ \) may be accomplished by oxidation of the reduced reductase in scheme (c). On the other hand, Svingen et al. objected to the mechanism of OH·-mediated lipid peroxidation (19). They proposed two sequential radical steps, initiation and propagation; the former was suggested as \( O_2^+ \)-dependent, and the latter as lipid hydroperoxides-dependent, formed during initiation and catalyzed by EDTA-Fe³⁺ or ferric cytochrome P-450. The reaction yielding OH· or the propagated reaction of lipid hydroperoxides would require a thiol group-dependent protein (17) or a factor isolated from intact hepatic microsomes which acts as an iron chelator (20). Therefore an iron complex such as EDTA-ferric ion or ferricyanide is required for lipid peroxidation by purified NADPH-cytochrome c reductase. If the thiol group-dependent protein or the iron chelator is deficient in liver microsomes of riboflavin-deficient rats, the diminished NADPH-dependent lipid peroxidation in the presence of ferric ion alone would be explained.

Upon feeding rats with a riboflavin-deficient diet for 5 weeks, an increase in cytochrome P-450 and a decrease in NADPH-cytochrome c reductase were observed in the present experiments as well as in the studies reported by Yang (21). Drug-metabolizing enzymes which consist of cytochrome P-450 and NADPH-cytochrome P-450 reductase were shown to be variously affected by riboflavin deficiency, depending on the period of feeding; however, the activities of enzymes did not disappear as did lipid peroxidation. Aniline hydroxylase seemed to be more susceptible than aminopyrine demethylase, and these results were contrary to those obtained by Miltenberger and Oltersdorf (22). The discrepancy might be accounted for by the differences in the contents of riboflavin in the diet and method of enzyme preparation. In the latter studies, 1.5 mg of riboflavin per kg of the diet and the supernatant after centrifugation at 9,000 g were used respectively, whereas 150 μg of riboflavin per kg which were originally contained in the commercial vitamin-free casein, and isolated microsomes were used in these experiments.

Hirokata et al. (5) showed that NADH could support the lipid peroxidation of rat liver microsomes in the presence of EDTA, ADP and ferric ion, and one electron was assumed to be transferred via NADH-cytochrome \( b_5 \) reductase and cytochrome \( b_5 \). In the present experiment, cytochrome \( b_5 \) was slightly increased in riboflavin deficiency. NADH-mediated lipid peroxidation might have contributed to the increase in lipid peroxide in the livers of riboflavin-deficient rats. However, mechanisms for the electron transfer in the cells corresponding to that for EDTA-ferric ion in purified NADPH-cytochrome P-450 reductase or NADH-cytochrome \( b_5 \) reductase have not been elucidated. The contribution of NAD(P)H-dependent
lipid peroxidation in the liver microsomes, especially in the riboflavin-deficient rats, is obscure in the intact livers. From the present results, it was difficult to correlate the activity of lipid peroxidation enzymes with the amounts of peroxides in whole livers, and the author has been continuing the work to examine whether superoxide radical formation or decomposition might be the rate-limiting factors in the intact tissues.

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