EFFECT OF RIBOFLAVIN AND RIBOFLAVIN 2',3',4',5'-TETRABUTYRATE ON RAT LIVER MICROSOMAL LIPID PEROXIDATION¹

Kyoko TAHARA, Shigeru MATSUOKA, and Hirobumi OHAMA²

Section of Biochemistry, Tokyo Tanabe Research Laboratories, Kita-ku, Tokyo

(Received January 31, 1974)

Effect of riboflavin and riboflavin 2', 3', 4', 5'-tetrabutryate on microsomal lipid peroxidation *in vitro* was studied. Riboflavin-tetrabutryate decreased the lipid peroxide formation measured by thiobarbituric acid reaction and oxygen uptake, and prevented enzyme inactivation at the same time. Riboflavin showed a similar effect, but the effect was less than that of riboflavin-tetrabutryate. The antioxidative and enzyme-protective effects of flavins were discussed in connection with lipid metabolism *in vivo*.

Recently, a number of papers reported that rat liver microsomes have enzymic and nonenzymic lipid peroxidizing systems under various conditions. HOCHSTEIN and ERNSTER (1), and MAY and MCCAY (2) observed NADPH-dependent microsomal lipid peroxidation in the presence of ADP which was postulated as the reaction coupled to NADPH oxidase. Recently, PEDERSON and AUST (3) confirmed that the enzyme involved in the peroxidizing reaction in microsomes is a flavoprotein, NADPH-cytochrome *c* reductase [EC 1.6.2.3]. Ascorbate-induced peroxidation in rat liver mitochondria was reported by BERNHEIM *et al.* (4) and OTTOLENGHI *et al.* (5). Microsomal lipid peroxidation induced by ascorbate was also reported by HOCHSTEIN and ERNSTER (1). Further, carbon tetrachloride was shown to stimulate the lipid peroxidation in rat liver microsomal suspension (6, 7). These observations definitely show that lipid peroxide-producing systems exist in living cells. Since marked toxicity of lipid peroxides is known, their removal or inhibition of lipid peroxidation are important problems in the medical field. Natural and synthesized antioxidants are effective for these objectives but these substances are also toxic when large doses are administered as drugs. OHAMA and YAGI (8) reported that a lipophilic flavin derivative, riboflavin 2', 3', 4', 5'-tetrabutryate

¹ Abbreviations: FR, riboflavin (free form); R-BUT, riboflavin 2', 3', 4', 5'-tetrabutryate; TBA, 2-thiobarbituric acid; PG, polyethylene glycol 400.

² 田原京子, 松岡 繁, 大浜宏文

(R-BUT) had an antioxidative and lipid peroxide-removing activity. This material does not exhibit any observable toxicity. The present paper deals with an antioxidative effect of R-BUT on microsomal lipid peroxidation induced by NADPH, ascorbate, or carbon tetrachloride, *in vitro*.

MATERIALS AND METHODS

Materials. NADPH, ADP, glucose-6-phosphate, and ascorbic acid were purchased from the Sigma Chemical Company, St. Louis, Missouri and 2-thio-barbituric acid from Kokusan Kagaku Co., Ltd., Tokyo.

Preparation of microsomes. Male Wistar rats weighing 150 to 200 g fed on normal diet (CLEA Japan, Inc., Tokyo) were sacrificed by decapitation. Microsomes were prepared from perfused rat liver according to OMURA *et al.* (9). Microsomes equivalent to 1 g wet weight of original liver were finally suspended in 1.0 ml of ice-cold 0.15 M KCl solution and used as a stock solution.

Methods. NADPH-coupled peroxidation of microsomal lipids *in vitro* was performed according to the method of ERNSTER and NORDENBRAND (10) with a slight modification. The reaction mixture consisted of 0.15 M KCl, 2 mM Tris-HCl buffer (pH 7.5), 50 μ M NADPH, 1 mM ADP, 40 μ M FeCl_3 , 20% PG, and microsomes containing 7–10 mg protein in a final volume of 3.0 ml. This mixture was incubated at 30°C for 15 min and interrupted by the addition of 0.2 ml of 50% trichloroacetic acid solution containing 45 mM EDTA. For examination of ascorbate-induced microsomal lipid peroxidation, 0.1 mM ascorbate and 0.2 mM FeCl_3 were replaced with NADPH, ADP and 40 μ M FeCl_3 .

Oxygen consumption by the lipid peroxidation was measured with a galvanic cell with a diaphragm (Bioxygraph, Kyusui Kagaku Kenkyusho, Tokyo) at 30°C in an air-tight electrode chamber. The reaction mixture (2.5 ml) contained the same components as those in the NADPH-induced peroxidation, however the concentration of microsomal protein decreased to about 0.5 mg in 2.5 ml. On addition of NADPH solution and ADP solution containing FeCl_3 , oxygen uptake was recorded on a recorder. In spite of an inhibitory effect on the NADPH-coupled microsomal lipid peroxidation, as shown in Fig. 1, PG was used for increasing the solubility of R-BUT in the reaction mixture. Malonaldehyde produced from lipid peroxides was determined by the TBA reaction reported by BERNHEIM *et al.* (11) and modified by ERNSTER and NORDENBRAND (10). After the addition of 1.7 mM EDTA in the final concentration, the solution was heated with TBA to 80°C for 40 min, instead of at 100°C for 15 min, because the reaction of TBA with PG was minimum at 80°C, as in the case of sugars and carbohydrates (12). Malonaldehyde concentration was expressed as nmoles/mg of microsomal protein using the molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at 535 nm (13). The activity of glucose-6-phosphatase [glucose-6-phosphate phosphohydrolase, EC 3.1.3.9] was assayed by the method of BELFIELD and GOLDBERG (14). Protein

content of microsomes was determined by the method of LOWRY *et al.* (15).

RESULTS

NADPH-coupled peroxidation of microsomal lipids of rat liver, as measured by the initial oxygen consumption rate, was observed in the presence of PG, but the extent was reduced to about one-half when the concentration of PG was raised to 20%, as shown in Fig. 1. R-BUT inhibited oxygen uptake by lipid peroxidation, while FR was less effective (Table 1). The inhibitory effect of R-

Fig. 1. Effect of PG on the initial oxygen consumption rate due to NADPH-coupled microsomal lipid peroxidation.

Table 1. Effect of FR and R-BUT on initial oxygen consumption rate due to NADPH-coupled microsomal lipid peroxidation.

Flavins	PG (%)	Experiment 1		Experiment 2	
		Concentration of flavins (M)	Oxygen uptake (μmoles/min/mg protein)	Concentration of flavins (M)	Oxygen uptake (μmoles/min/mg protein)
None	None	—	0.44	—	—
None	20	—	0.24	—	0.46
FR	20	3×10^{-5}	0.20	5×10^{-5}	0.42
R-BUT	20	3×10^{-5}	0.16	5×10^{-5}	0.26

BUT on the peroxidation was also demonstrated by malonaldehyde formation which is known to be produced by the degradation of polyunsaturated fatty acid peroxides. Figure 2 shows typical results obtained by the effect of R-BUT and FR on both NADPH-coupled and ascorbate-induced microsomal lipid peroxidation. R-BUT was suppressive against both peroxidation systems, while the effect of FR was less than that of R-BUT. The preventive effect of flavins on the lipid peroxidation was reproduced exactly in different series of experiments, though

degrees of stimulation and inhibition of lipid peroxide formation in microsomes were different. This might be ascribed to a difference among the endogenous potentials of peroxidation.

Fig. 2. Effect of FR and R-BUT on NADPH-coupled and ascorbate-induced microsomal lipid peroxidation measured by malonaldehyde formation. Flavins were added at the concentration of 4×10^{-5} M. Results are expressed as a mean of three experimental data.

Figure 3 shows the time course of the ascorbate-induced peroxidation in the presence and absence of R-BUT. It is evident from the figure that the suppressive effect of R-BUT is observed only in the initial rate of lipid peroxidation and not in a subsequent phase.

The peroxidation of microsomal lipid was induced, moreover, by an addition of carbon tetrachloride to the final concentration of 0.17% into the microsomal suspension, even in the absence of NADPH, ADP and ferric ion (see Fig. 4). However, increasing the concentration of carbon tetrachloride did not accelerate further peroxide formation. SLATER and SAWYER (7) reported carbon tetrachloride-induced peroxidation in rat liver microsomes *in vitro* and concluded that the stimulation of the peroxidation requires NADPH in the reaction mixture. The result shown in Fig. 4, therefore, indicates that the peroxide formation depended on endogenous NADPH in the microsomal preparations and that the reaction ceased with the disappearance of NADPH.

GHOSHAL and RECKNAGEL (16), and WILLS (17) reported that inactivation of glucose-6-phosphatase accompanied the microsomal lipid peroxidation, since

phospholipids are essential for the enzyme activity (18), and peroxidation leads to the destruction of membrane structure which is necessary for its function. A

Fig. 3. Ascorbate-induced microsomal lipid peroxidation in function of time. ○, In the presence of R-BUT; ●, in the absence of R-BUT.

Fig. 4. The peroxidation of lipid in microsomes in the presence of carbon tetrachloride. The reaction mixture consisted of 0.15 M KCl, 2 mM Tris-HCl buffer (pH 7.5), microsomes (8 mg of microsomal protein), and carbon tetrachloride in a final volume of 3.0 ml. Incubation was performed at 30°C for 15 min.

preventive effect of R-BUT on the inactivation of glucose-6-phosphatase by microsomal peroxidation might be explained by taking into account the results reported by the above workers. A typical result of the effect of R-BUT on the inactivation of the enzyme is shown in Table 2, in comparison with that of FR. The lipo-peroxide formation was clearly inhibited by the addition of R-BUT and no

inactivation of the enzyme was observed, while FR did not produce any appreciable inhibitory effect.

Table 2. Effect of FR and R-BUT on inactivation of glucose-6-phosphatase due to carbon tetrachloride-induced peroxidation of microsomal lipids.

Flavins	Malonaldehyde formed (nmoles/mg protein)		Glucose-6-phosphatase (%)	
	Incubation time 5 min	Incubation time 15 min	Incubation time 5 min	Incubation time 15 min
None	0.16 ^a		100 ^a	
None	0.19	0.34	58.8	58.8
FR (4×10^{-5} M)	0.31	0.29	64.9	60.2
R-BUT (4×10^{-5} M)	0.06	0.13	98.1	100.0

^a Values before incubation.

DISCUSSION

It is evident from the present study that R-BUT and FR equally exhibit a certain amount of inhibitory effect on microsomal lipid peroxidation *in vitro*. However, the effect of R-BUT was greater than that of FR. Since R-BUT is more lipophilic than FR, the difference observed between the two flavins may be due to their affinity or accessibility to lipids present in the microsomal membranes. The fact that both esterified and nonesterified riboflavins are effective on the microsomal lipid peroxidation may suggest that the suppressing effect might be ascribed to the isoalloxazine moiety of R-BUT. OHAMA and YAGI (8) suggested the possibility that R-BUT reacts with lipid peroxides formed and represses their accumulation in tissue cells. Our results showing that R-BUT inhibits oxygen uptake in the process of peroxidation further supports the view that R-BUT can be an "antioxidant" in its wide meaning.

R-BUT inhibited both NADPH-coupled and ascorbate-induced microsomal lipid peroxidation. It is known that these two peroxidation systems have different mechanisms by which microsomal lipids are peroxidized; the former is an enzymic process coupled with a microsomal electron transporting chain *via* NADPH, NADPH-cytochrome *c* reductase and cytochrome P-450, and the latter is non-enzymic (1-3). R-BUT, therefore, seems to exhibit its antioxidative action at or after the hydrogen atom is abstracted as a free radical from an active methylene group of polyunsaturated fatty acids during the process of enzymic oxidation-reduction reaction. PEDERSON and AUST (3) postulated that a superoxide anion produced by NADPH-cytochrome *c* reductase is involved in the process of lipid peroxidation. Further studies should be made concerning which step of the reaction R-BUT reacts.

It was reported from many laboratories that R-BUT clinically decreases serum triglyceride and cholesterol levels (19, 20). It seems possible to speculate

that R-BUT might improve the metabolism of lipids in patients suffering from atherosclerosis, diabetes, fatty liver and so on through the inhibition of lipid peroxide, resulting in the decrease of the elevated serum lipid. COMPORTI *et al.* (21) demonstrated that polyunsaturated fatty acids, *e.g.* C_{18:2}, C_{20:4}, and C_{22:6} are decreased by the carbon tetrachloride-induced peroxidation of microsomal phospholipids *in vivo*. Considering the fact that unsaturated fatty acids might play an important role in lipid metabolism, prevention of their degradation by peroxidation may improve abnormal metabolism. It was reported that carbon tetrachloride- and ethanol-induced disturbance of lipid metabolism in the liver may be correlated to peroxide formation and that antioxidants such as α -tocopherol protect the living body from the intoxication by lipoperoxide (22-24). The effect of R-BUT to repress the peroxidation and to prevent the inactivation of the enzymes requiring lipids for their function, such as glucose-6-phosphatase, may be worth mentioning from the clinical point of view. Furthermore, it was reported that ω -oxidation of fatty acids in microsomes is associated with the electron transport chain from NADPH to cytochrome P-450 (25-27) and competes with oxidative demethylation of drugs (28). These observations led us to postulate that NADPH-coupled peroxide formation competes with ω -oxidation of fatty acids in microsomes, resulting in some disorder in lipid metabolism. It can also be supposed that the lipoperoxide formed in turn inactivates some enzymes including lipid metabolizing enzymes.

The authors are grateful to Prof. K. Yagi of University of Nagoya for his valuable suggestion and to Dr. M. Iwasaki of our laboratory for his useful discussion.

REFERENCES

- 1) HOCHSTEIN, P. and ERNSTER, L., *Biochem. Biophys. Res. Commun.*, **12**, 388 (1963).
- 2) MAY, H. E. and MCCAY, P. B., *J. Biol. Chem.*, **243**, 2296 (1968).
- 3) PEDERSON, T. C. and AUST, S. D., *Biochem. Biophys. Res. Commun.*, **48**, 789 (1972).
- 4) BERNHEIM, F., WILBUR, K. M., and KENASTON, C. B., *Arch. Biochem. Biophys.*, **38**, 177 (1952).
- 5) OTTOLENGHI, A., BERNHEIM, F., and WILBUR, K. M., *Arch. Biochem. Biophys.*, **56**, 157 (1955).
- 6) GHOSHAL, A. K. and RECKNAGEL, R. O., *Life Sci.*, **4**, 1521 (1965).
- 7) SLATER, T. F. and SAWYER, B. C., *Biochem. J.*, **123**, 805 (1971).
- 8) OHAMA, H. and YAGI, K., *J. Vitaminol.*, **16**, 297 (1970).
- 9) OMURA, T., SIEKEVITZ, P., and PALADE, G. E., *J. Biol. Chem.*, **242**, 2389 (1967).
- 10) ERNSTER, L. and NORDENBRAND, K., *Methods in Enzymology*, **10**, 574 (1967).
- 11) BERNHEIM, F., BERNHEIM, M. L. C., and WILBUR, K. M., *J. Biol. Chem.*, **174**, 257 (1948).
- 12) FORTNEY, S. R. and LYNN, W. S., Jr., *Arch. Biochem. Biophys.*, **104**, 241 (1964).
- 13) SINNHUBER, R. O., YU, T. C., and YU, T. C., *Food Res.*, **23**, 626 (1958).
- 14) BELFIELD, A. and GOLDBERG, D. M., *Life Sci.*, **8**, Part II, 129 (1969).
- 15) LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
- 16) GHOSHAL, A. K. and RECKNAGEL, R. O., *Life Sci.*, **4**, 2195 (1965).

- 17) WILLS, E. D., *Biochem. J.*, **123**, 983 (1971).
- 18) DUTTERA, S. M., BYRNE, W. L., and GANOZA, M. C., *J. Biol. Chem.*, **243**, 2216 (1968).
- 19) IMAMURA, M., HENMI, H., HAYASHI, T., and AOKI, I., *Igaku no Ayumi*, **84**, 222 (1973).
- 20) YAMASAKI, S., HARAGUCHI, Y., KAWAGUCHI, K., ISHIHARA, A., FUKUMOTO, T., ONO, T., and SATO, Y., *Kiso to Rinsho*, **7**, 2483 (1973).
- 21) COMPORTI, M., LANDUCCI, G., and RAJA, F., *Experientia*, **27**, 1155 (1971).
- 22) KALISH, G. H. and DI LUZIO, N. R., *Science*, **152**, 1390 (1966).
- 23) COMPORTI, M. and BENEDETTI, A., *Biochem. Pharmacol.*, **21**, 418 (1972).
- 24) DI LUZIO, N. R., *Life Sci.*, **3**, 113 (1964).
- 25) ICHIHARA, K., KUSUNOSE, E., and KUSUNOSE, M., *Biochim. Biophys. Acta*, **176**, 704 (1969).
- 26) LU, A. Y. H. and COON, M. J., *J. Biol. Chem.*, **243**, 1331 (1968).
- 27) WADA, F., SHIBATA, H., GOTO, M., and SAKAMOTO, Y., *Biochim. Biophys. Acta*, **162**, 518 (1968).
- 28) ORRENIUS, S. and THOR, H., *Eur. J. Biochem.*, **9**, 415 (1969).