LIPID PEROXIDATION DURING ENZYMATIC IODINATION OF RAT LIVER ENDOPLASMIC RETICULUM.[1]

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Summary: During enzymatic iodination of rat liver endoplasmic reticulum the peroxidation of endogenous lipids and the loss of cytochrome P_{450} has been observed. This lipid peroxidation can be inhibited by the inclusion of a low concentration of butylated hydroxytoluene in the iodination mixture. This results in increased incorporation of $^{125}I^-$ and stabilization of cytochrome P_{450}.

Introduction: Phillips and Morrison have recently suggested a technique for examining the spatial arrangement of proteins in membranes using lactoperoxidase, H_2O_2 and $^{125}I^-$ and have applied the technique to study the human erythrocyte membrane (1,2). Poduslo et al. (3) have applied this technique to the study of the surface of mouse fibroblasts. The value of this technique is that it involves labeling membrane proteins using a catalyst which cannot diffuse through the membrane and must act at its surface. We have been using enzymatic iodination to study the spatial arrangement of proteins in the rat liver endoplasmic reticulum (microsomes) and have noted that lipid peroxidation occurs during the iodination reaction. Peroxidation of membrane lipids has been associated with increased permeability of many membranes including those of red blood cells (4,5,6) and such subcellular organelles as mitochondria (7,8,9), microsomes (10), and lysosomes (11,12). To prevent lipid peroxidation, Phillips and Morrison (1,2) suggested that H_2O_2 be added to the iodination mixture at very low concentrations (8 μM) at intervals. We have found that a significant accululation of peroxides still occurs when this procedure is applied to

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microsomes. We also observed a loss of cytochrome P_{450} with no accumulation of cytochrome P_{420}. The inclusion of butylated hydroxytoluene (BHT) in the iodination mixture prevents lipid peroxidation, preserves cytochrome P_{450} and increases the incorporation of $^{125}I^-$.

Methods: The total microsomal fraction was isolated from the livers of rats pretreated with phenobarbital as previously described (13). Before use, the membranes were washed using 0.3 M sucrose containing 0.1 M sodium pyrophosphate (14). This procedure removed 80% of the RNA and catalase and 30% of the protein from the membranes while increasing the specific activity of NADPH-cytochrome c reductase, NADH-ferricyanide reductase, cytochrome b_5, and cytochrome P_{450}.

Washed membranes were suspended to a protein concentration of 0.5 mg/ml in 0.1 M Tris, pH 7.5, 10^{-6} M KI, and 5.0×10^{-7} M lactoperoxidase at 25°C. Iodination was initiated with 5 μM H_2O_2 at 1 minute intervals over a three minute reaction period. The reaction was terminated by dilution with cold Tris buffer and centrifugation at 105,000 xg for 90 minutes at 0-5°C. The pelleted microsomes were suspended into buffer for enzyme assays.

Microsomal NADPH-cytochrome c reductase (15), NADH-ferricyanide reductase (16), cytochrome b_5 (17), cytochrome P_{450} (17), and aminopyrine demethylase (13) were assayed by previously published methods. Lipid peroxidation was assayed by incubating microsomes with 2 mM ADP and 0.12 mM $Fe(NO_3)_2$ to decompose all peroxides present in the membrane before assaying for malondialdehyde by the thiobarbituric acid method (18).

Results and Discussion: When iodination of rat liver endoplasmic reticulum was carried out using multiple additions of a low concentration of H_2O_2, as suggested by Phillips and Morrison (1,2), lipid peroxidation, as measured by malondialdehyde content, was shown to occur (Table I). Iodination in the presence of several agents known to inhibit lipid peroxidation either by chelating iron or acting as an antioxidant, indicated that a very low concentration of BHT combined the desirable effects of completely inhibiting lipid peroxidation while

Table I: Effect of inhibitors of lipid peroxidation on the formation of malondialdehyde and incorporation of ^{125}I into microsomes.

Additions	Malondialdehyde formation nmoles/mg protein	^{125}I incorporation Total cpm/mg protein
None	22.0	800,000
40 mM Dithiothreitol	1.3	0
2 mM EDTA	6.1	860,000
0.0001% BHT	0.0	1,610,000

ble II: Effect of iodination on lipid peroxidation, cytochromes, and enzymatic tivities in the endoplasmic reticulum. Microsomes were incubated for 3 minutes ler the following conditions, centrifuged at 105,000 xg for 90 min. and resus- nded in buffer for assays. 1. Microsomes incubated at 25°C in 0.1 M Tris, 7.5. 2. Microsomes incubated with 0.0001% BHT. 3. Microsomes incubated in e iodination mixture + 0.0001% BHT but without KI. 4. Microsomes incubated in e iodination mixture + 0.0001% BHT. 5. Microsomes incubated in the iodination cture only.

Lipid Peroxidation[a]	P_{450}[b]	P_{420}[b]	b_5[b]	NADPH-cytochrome c reductase[c]	NADH-ferricyanide reductase[c]	Aminopyrine demethylase[d]
4.5	1.21	0.14	0.68	0.24	4.50	7.5
0	2.28	0.25	0.75	0.33	5.10	9.7
0	2.15	0.22	0.83	0.31	4.72	10.3
0	1.86	0.2	0.97	0.28	0.15	6.7
10	0.64	0.0	0.86	0.26	0.16	4.1

moles malondialdehyde/mg protein

moles/mg protein

moles/min/mg protein

moles formaldehyde/min/mg protein

doubling the amount of $^{125}I^-$ incorporated into microsomes during an iodination (Table I).

Experiments designed to measure the effect of iodination on several enzymatic activities and cytochromes present in rat liver microsomes emphasized the importance of carrying out iodination in the presence of BHT. Iodination in the absence of BHT resulted in the loss of 75% of the cytochrome P_{450} and

65% of the aminopyrine demethylase activity (Table II). The loss of cytochrome P_{450} could not be accounted for as cytochrome P_{420} nor by the presence of endogenous carbon monoxide that would interfere with the assay. When iodination was carried out in the presence of BHT, only 15% of the cytochrome P_{450} and 35% of aminopyrine demethylase activity were lost, presumably due to the iodination reaction. Under conditions where no lipid peroxidation was occurring, of the enzymes assayed, iodination most significantly affected NADH-ferricyanide reductase activity. This enzyme (also termed NADH-cytochrome b_5 reductase) has been shown by Strittmatter (19) to contain a tyrosine residue in its active site which may be available for iodination by lactoperoxidase.

Lipid peroxidation has been reported to cause inactivation of enzymes and destruction of cytochromes (20). There has also been a suggested correlation between lipid peroxidation and loss of cytochrome P_{450} in microsomes (17). Direct evidence for such a correlation is offered in Table III. Microsomes incubated aerobically showed an increasing malondialdehyde content and decreasing concentration of P_{450} vs. time. However when microsomes were incubated with 0.005% BHT no lipid peroxidation occurred and cytochrome P_{450} was preserved. In this and other experiments where a loss of cytochrome P_{450} was noted (Table II) very little cytochrome P_{420} was observed suggesting that if cytochrome P_{450} is converted to its altered form, P_{420}, it is quickly converted to other degradation products.

Anaerobic trypsin treatment of iodinated microsomes at 25°C for 60 minutes indicated that proteolytic digestion released 50% of the protein from microsomes which were not protected with BHT during the iodination but only 34% of the protein from microsomes protected from lipid peroxidation. This suggests that the lipid peroxidation occurring during the iodination is indeed changing the properties of the membrane and emphasizes the importance of carrying out the iodination in the presence of BHT.

While Phillips and Morrison (1,2) suggest that the use of a low concentration of H_2O_2 is suitable to prevent lipid peroxidation during enzymatic iodina-

Table III: The correlation between loss of cytochrome P_{450} and the peroxidation of microsomal lipid. Washed microsomes were suspended to a protein concentration of 5 mg/ml in 0.2 M Tris, pH 7.5 and aerobically incubated at 37°C. Samples were removed at various times and made 0.005% with BHT to stop lipid peroxidation and assayed. To one sample 0.005% BHT was added initially and this sample was incubated in parallel at 37°C for 90 minutes.

Time incubated	malondialdehyde nmoles/mg	b_5 nmoles/mg	P_{450} nmoles/mg	NADPH-cytochrome c reductase μmoles/min/mg	NADH-ferricyanide reductase μmoles/min-mg
5 min	2.0	1.0	2.1	0.22	3.6
30 min	7.8	1.0	1.7	0.24	4.0
60 min	14.0	1.0	1.3	0.24	3.4
90 min	16.4	0.9	0.4	0.22	3.7
90 min + BHT	0.5	1.0	2.0	0.21	3.7

tion of the erythrocyte membrane, we have found that this technique is not applicable to microsomal membranes. It seems likely that different membranes may have different sensitivities toward lipid peroxidation. The results of these studies suggest the general use of the antioxidant BHT during enzymatic iodination of membranes to assure protection from lipid peroxidation. Very low concentrations of BHT will also protect cytochrome P_{450} and thereby preserve drug hydroxylation activity. The use of BHT to protect cytochrome P_{450} during isolation, storage, etc. of microsomes is suggested.

1. Phillips, D. R. and Morrison, M., Biochem. Biophys. Res. Commun., 36, 545 (1969).
2. Phillips, D. R. and Morrison, M., Biochemistry 10 (10), 1766 (1971).
3. Poduslo, J. F., Greenberg, C. S., and Glick, M. C., Biochemistry 11 (14), 2616 (1972).
4. Mengel, C. E., Kann, H. E., Jr., and Meriwether, W. D., J. Clin. Invest., 46, 1715 (1967).
5. Mengel, C. E. and Kann, H. E., Jr., J. Clin. Invest., 45, 1150 (1966).
6. Dodge, J. T., Cohen, G., Kayden, H. F., and Phillips, G. B., J. Clin. Invest., 46, 357 (1967).
7. Hunter, F. E., Jr., Scott, A., Hoffsten, P. E., Guerra, F., Weinstein, J., Schneider, A., Schutz, B., Fink, J., Ford, L., and Smith, E., J. Biol. Chem., 239, 604 (1964).
8. Hunter, F. E., Jr., Gebicki, J. M., Hoffsten, P. E., Weinstein, J., and Scott, A., J. Biol. Chem., 238, 828 (1963).
9. Hunter, F. E., Jr., Scott, A., Hoffsten, P. E., Gebicki, J. M., Weinstein, J., and Schneider, A., J. Biol. Chem., 239, 614 (1964).
10. Robinson, J. D., Arch. Biochem. Biophys., 112, 170 (1965).
11. Wills, E. D. and Wilkinson, A. E., Biochem. J., 99, 657 (1966).

12. Desai, I. D., Sawant, P. L., and Tappel, A. L., Biochim. Biophys. Acta, 86, 277 (1964).
13. Pederson, T. C. and Aust, S. D., Biochem. Pharmacol., 19, 2221 (1970).
14. Sachs, H., Biochim. Biophys. Acta, 21, 188 (1956).
15. Omura, T. and Takesue, S., J. Biochem. 67, 249 (1970).
16. Mihara, K. and Sato, R., J. Biochem. 71, 725 (1972).
17. Omura, T. and Sato, R., J. Biol. Chem., 239, 2379 (1964).
18. Bernheim, F., Bernheim, M. L., and Wilbur, K. M., J. Biol. Chem., 174, 257 (1948).
19. Strittmatter, P., Fed. Proc., 24, 1156 (1965).
20. Tappel, A. L., Vitam. Horm., 20, 493 (1962).