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Effects of Lipid Peroxidation on Membrane-Bound Enzymes of the Endoplasmic Reticulum

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1. Induction of the formation of lipid peroxide in suspensions of liver microsomal preparations by incubation with ascorbate or NADPH, or by treatment with ionizing radiation, leads to a marked decrease of the activity of glucose 6-phosphatase. 2. The effect of peroxidation can be imitated by treating microsomal suspensions with detergents such as deoxycholate or with phospholipases. 3. The substrate, glucose 6-phosphate, protects the glucose 6-phosphatase activity of microsomal preparations against peroxidation or detergents. 4. The loss of glucose 6-phosphatase activity is not due to the formation of hydroperoxide or formation of malonaldehyde or other breakdown products of peroxidation, all of which are not toxic to the enzyme. 5. All experiments lead to the conclusion that the loss of activity of glucose 6-phosphatase resulting from peroxidation is a consequence of loss of membrane structure essential for the activity of the enzyme. 6. In addition to glucose 6-phosphatase, oxidative demethylation of aminopyrine or *p*-chloro-*N*-methylaniline, hydroxylation of aniline, NADPH oxidation and menadione-dependent NADPH oxidation are also strongly inhibited by peroxidation. However, another group of enzymes separated with the microsomal fraction, including $NAD^+/NADP^+$ glycohydrolase, adenosine triphosphatase, esterase and NADH–cytochrome *c* reductase are not inactivated by peroxidation. This group is not readily inactivated by treatment with detergents. 7. Lipid peroxidation, by controlling membrane integrity, may exert a regulating effect on the oxidative metabolism and carbohydrate metabolism of the endoplasmic reticulum *in vivo*.

Suspensions of liver microsomal preparations incubated with ascorbate or NADPH, or subjected to ionizing radiation, rapidly form lipid peroxide (Wills, 1969*a*). Formation of peroxide results in loss of capacity of this subcellular fraction to carry out hydroxylation reactions such as the hydroxylation of aniline or the oxidative demethylation of aminopyrine. Treatment of microsomal suspensions with deoxycholate also inhibits hydroxylation, and peroxide formation is believed to cause loss of membrane structure essential for the functioning of the electron-transport chain involved in the hydroxylation process (Wills, 1969*b*; Wills & Wilkinson, 1970). The fact that peroxidation leads to loss of structure of certain phospholipids of the membrane, primarily as a result of attack on the unsaturated fatty acids of the β-position, has been demonstrated by May & McCay (1968).

In the present investigation the relationship of peroxidation to enzyme activity has been studied more closely and extended to many other membrane-bound enzymes of the endoplasmic reticulum. Attempts have been made to establish whether loss of activity of these enzymes is a direct consequence of peroxide formation or is due to subsequent loss of membrane structure, and whether peroxidation is selective in causing loss of activity of some enzymes but not of others.

MATERIALS AND METHODS

Materials

The sources of most materials, preparation of microsomal fraction of rat liver, methods of determination of lipid peroxide, rate of oxidative demethylation of aminopyrine and *p*-chloro-*N*-methylaniline, have been described (Wills, 1969*b*; Wills & Wilkinson, 1970). In addition the following sources of materials and methods were used.

Phospholipase A prepared from *Crotalus terr. terr.* was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K., and contained 1 mg of protein (200 units)/ml.

Phospholipase D (cabbage) was obtained from Sigma (London) Chemical Co. Ltd., London S.W.5, U.K. and

contained 50mg of protein/ml. Ethanol dehydrogenase was obtained from Boehringer Ltd.

Tween 20 and Tween 65, cetylpyridinium bromide and sodium deoxycholate were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Triton X-100 was obtained from Sigma (London) Chemical Co., and sodium dodecyl sulphate from British Drug Houses Ltd., Poole, Dorset, U.K.

Enzyme assays

Glucose 6-phosphatase (*EC* 3.1.3.9). This was determined by the method based on that described by Bergmeyer (1963); 0.3ml of 0.1M-sodium citrate buffer, pH6.5, 0.3ml of 0.1M-glucose 6-phosphate, 0.9ml of 0.25M-sucrose or 125mM-KCl and microsomal suspension (1.5mg of protein in 0.5ml) were incubated at 37°C. Samples (0.5ml) were removed at intervals for 10min and transferred to 2.0ml of 10% (w/v) trichloroacetic acid. After centrifuging, the phosphate hydrolysed was determined in the supernatant (1ml) by adding 3ml of 0.25% (w/v) ammonium molybdate in 0.25M-H_2SO_4 followed by ascorbic acid solution (1ml containing 1mg). The blue colour was read after 30min at 700nm and compared with a series of phosphate standards.

Adenosine triphosphatase (*EC* 3.6.1.3). This was determined essentially as described for glucose 6-phosphatase, but 0.1M-ATP replaced the glucose 6-phosphate and 0.2ml of 50mM-$MgCl_2$ was also added.

$NAD^+/NADP^+$ glycohydrolase (*EC* 3.2.2.6). The activity of this enzyme was measured by a method based on that of Clark & Pinder (1969). For this 10mM-NAD^+ (0.2ml), 0.3ml of 0.2M-sodium phosphate buffer, pH7.0, and microsomal suspension (1.0ml, containing 3.0mg of protein) were incubated at 37°C. Samples (0.2ml) were removed at intervals for 10min and added to 0.5ml of 0.15M-HCl. The mixture was boiled at 100°C for 1min, then 0.5ml of 0.15M-NaOH and 0.3ml of 0.25M-glycylglycine buffer, pH7.6, were added to neutralize and the precipitate was centrifuged down. To 1ml of supernatant 0.8ml of 0.25M-glycylglycine buffer, pH9.0, was added, followed by ethanol (0.1ml) and ethanol dehydrogenase (0.1ml, containing 0.1mg of protein). The increase in E_{340} was measured in a Hilger–Gilford reaction-kinetics spectrophotometer compared with a blank with the supernatant replaced by water. The method was calibrated against a series of standard NAD solutions.

For experiments in which NADP was substrate the method was essentially the same but the pH was adjusted to 7.0, sodium isocitrate (10mM) replaced the ethanol and isocitrate dehydrogenase (0.05ml) (Sigma, type IV) replaced the ethanol dehydrogenase.

This determination actually measures two enzymes, the glycohydrolase and pyrophosphatase, but the activity of pyrophosphatase constitutes only a very small percentage (approx. 5% of the total NAD-ase activity in rat liver; Clark & Pinder, 1969). The low percentage activity of pyrophosphatase was confirmed in the present investigation, and a small (5%) correction made to give a true indication of the glycohydrolase activity.

Esterase activity. This was measured by two methods. (i) Arylesterase (EC 3.1.1.2), by a method based on that of Huggins & Lapides (1947). In a cuvette 0.2ml of 0.2M-sodium phosphate buffer, pH7.0, *p*-nitrophenol acetate (16μg in 0.4ml), microsomal suspension (0.1ml, containing 0.075mg of protein), and 0.25M-sucrose (1.3ml) were mixed and the rate of increase of E_{420} was measured at 25°C in a Hilger–Gilford reaction-kinetics spectrophotometer. Standard solutions of the product of the reaction, *p*-nitrophenol, were prepared in the phosphate buffer, pH7.0, and the E_{420} values determined. (ii) Carboxylesterase (EC 3.1.1.1), by a continuous titration method. For this 7.8ml of 0.25M-sucrose, 2.0ml of a saturated aqueous solution of ethyl butyrate and microsomal suspension (0.2ml, containing 1.5mg of protein) were magnetically stirred in a reaction vessel kept at 25°C by circulating water at this temperature. The pH was maintained at pH7.0 by continuous addition of 0.01M-NaOH and the linear rate of hydrolysis measured over 5min.

Oxidation of NADPH. To measure this, 0.2ml of 0.40mM-NADPH, 0.2ml of 0.2M-sodium phosphate buffer, pH7.0, microsomal suspension (0.1ml in 0.25M-sucrose containing 1.5mg of microsomal protein) and 1.5ml of 0.25M-sucrose were mixed in the cuvette and the rate of decrease of E_{340} was measured for 2min in a Hilger–Gilford reaction-kinetics spectrophotometer.

For menadione-dependent NADPH oxidation, menadione (50μM) was also added and the increase of the rate of oxidation of NADPH over and above that of NADPH alone was calculated.

NADH–cytochrome c *reductase* (*EC* 1.6.99.3). To assay this, 1.3ml of 0.25M-sucrose, 0.2ml of 1mM-NADH, 0.2ml of 0.5mM-cytochrome *c*, 0.2ml of 0.2M-sodium phosphate buffer, pH7.0, and 0.1ml of microsomal suspension containing 0.6mg of protein were incubated at 25°C and the rate of increase of E_{550} was measured in a Hilger–Gilford reaction-kinetics spectrophotometer for periods of 1–2min.

Iodometric determination of hydroperoxide. Peroxide was determined by a method based on that of Swoboda & Lea (1958). To 8ml of acetic acid–chloroform (3:2, v/v), 2ml samples of microsomal suspension were added and mixed, and the phases were separated by centrifugation. Then 3ml of the chloroform phase was removed and N_2 was bubbled through it for 1min, after which KI solution (0.1ml containing 0.12g) was added. The mixture was left in the dark for 30min at 20°C. Then 6ml of 0.5% cadmium acetate was added, and the mixture shaken and centrifuged to separate the phases. The E_{350} was then measured in the aqueous layer and the method calibrated by using a standard solution of benzoyl peroxide in chloroform.

Irradiation

Microsomal suspensions were irradiated by using a ^{60}Co source of γ-rays (Wills, 1970). The suspensions were stirred in open vessels during irradiation and kept at 20°C by circulating water in a surrounding jacket.

Unless otherwise stated, all experimental values are means of four experiments.

RESULTS

Effect of lipid peroxidation on the activity of glucose 6-phosphatase. Incubation of suspensions of liver microsomal preparation with ascorbate for periods of 10–60min causes extensive formation of

Table 1. *Glucose 6-phosphatase activity of microsomal suspensions after incubation with ascorbate*

Liver microsomal suspensions (2.5ml.) containing 3.0mg of protein/ml were incubated in 0.25M-sucrose containing 50mM-tris–HCl buffer, pH 7.0, under conditions specified below. After incubation, a sample (0.5ml) was removed for determination of lipid peroxide and other samples (0.5ml) were removed for the determination of glucose 6-phosphatase activity. Activities are expressed as a percentage of the mean control rate, which for 30 experiments was 55.8nmol of phosphate/min per mg of protein.

Incubation temp.	Incubation time (min)	Ascorbate added (0.5mM)	Increase in lipid peroxide (nmol of malonaldehyde/mg of protein)	Glucose 6-phosphatase activity (% of control)
0°C	30	–	0	100
	30	+	0.27	102
37°C	30	–	0.17	97
	10	+	2.01	64
	30	+	6.50	20

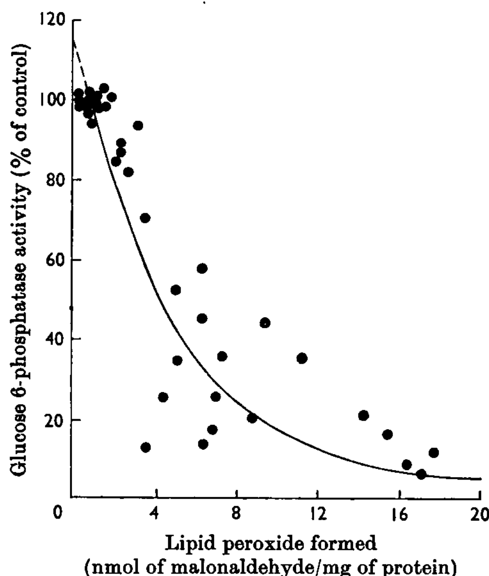

Fig. 1. Effect of lipid peroxidation on glucose 6-phosphatase activity of microsomal suspensions. Microsomal preparations (3.0mg of protein/ml) were suspended in 0.25M-sucrose containing 50mM-tris–HCl buffer, pH 7.0 (total vol. 2.5–5.0ml), and incubated with ascorbate (0–0.5mM) or NADPH (40μM) for various periods between 0 and 60min at 37°C or were subjected to γ-rays (5–50krd). After incubation or irradiation, samples (0.5ml) were removed for determination of lipid peroxide, and other samples (0.5ml) were taken for determination of glucose 6-phosphatase activity.

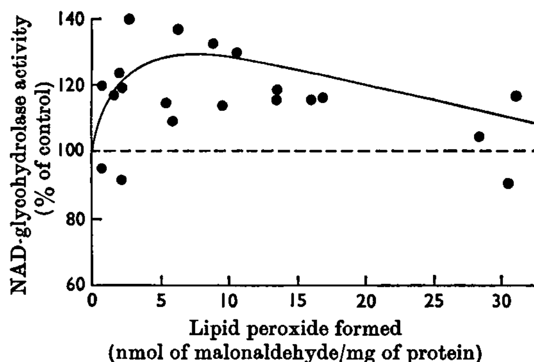

Fig. 2. Effect of lipid peroxidation on NAD glycohydrolase activity of microsomal suspensions. Experimental details are as described for Fig. 1 but samples were taken for determination of NAD glycohydrolase activity.

lipid peroxide (Wills, 1969*a*). This was accompanied by a sharp fall in the activity of the membrane-bound glucose 6-phosphatase. Incubation at 37°C with no ascorbate added, or incubation with ascorbate at 0°C, caused very little peroxide formation and the activity of the glucose 6-phosphatase was little affected (Table 1).

Production of lipid peroxide in microsomal suspensions by incubation with either ascorbate or NADPH for different times and under different conditions caused the formation of lipid peroxide, which was always accompanied by decrease in the activity of glucose 6-phosphatase. Peroxidation was also induced by subjecting microsomal suspensions to γ-rays (5–50krd) from a ^{60}Co source (Wills, 1970). A summary of all results is shown in Fig. 1. The curve may be extrapolated to zero lipid peroxide concentration, which gives an activity for glucose 6-phosphatase nearly 20% greater than that normally found despite the fact that control preparations were kept at 0°C and used as soon as possible after preparation.

Effects of peroxidation on other membrane-bound enzymes of the endoplasmic reticulum. After formation of lipid peroxide in microsomal suspensions

Table 2. *Effects of lipid peroxidation and deoxycholate on the activity of enzymes of microsomal suspensions*

For peroxidation, microsomal suspensions were treated as described in Fig. 1 and the activities of the enzymes, expressed as a percentage of a control suspension kept at 0°C, were plotted against amount of lipid peroxide formed. Activities are compared with the controls kept at 0°C, after the formation of 10nmol of malonaldehyde/mg of protein. For deoxycholate treatment, microsomal suspensions were incubated in the presence of sodium deoxycholate (1mM) for 15min at 37°C.

		Enzyme activity (% of control)	
	Enzyme activity	After peroxidation	After deoxycholate
Sensitive enzymes	Glucose 6-phosphatase	18	6
	Oxidative demethylation		
	(i) Aminopyrine	20	38
	(ii) p-Chloro-N-methylaniline	36	56
	Hydroxylation of aniline	60	40
	NADPH oxidation	60	55
	Menadione-dependent NADPH oxidation	56	8
Insensitive enzymes	NAD–NADP glycohydrolase	122	105
	Adenosine triphosphatase	103	97
	Esterase	85	100
	NADH–cytochrome c reductase	82	102

the activity of some enzymes, unlike glucose 6-phosphatase, was unaffected and the activity of $NAD^+/NADP^+$ glycohydrolase was even increased to a small extent (Fig. 2). Further studies of several other enzymes showed that they were either very sensitive to peroxidation, like glucose 6-phosphatase, or insensitive like $NAD^+/NADP^+$ glycohydrolase.

Oxidative demethylation, hydroxylation, NADPH oxidation and menadione-dependent NADPH oxidation were found to belong to the sensitive group, and adenosine triphosphatase, esterase activity and NADH–cytochrome c reductase were much less sensitive to peroxidation (Table 2).

Comparison of different methods of inducing peroxide formation. To test the possibility that the incubation with ascorbate or NADPH was involved in the enzyme inactivation a comparison was made of the activity of glucose 6-phosphatase after induction of peroxidation by NADPH or ascorbate and of the oxidative demethylation of aminopyrine after induction of peroxidation by ionizing radiation. Whichever method was used for inducing peroxide formation the activity of both enzymes clearly depended on the formation of peroxide and not on the method of inducing its formation (Fig. 3).

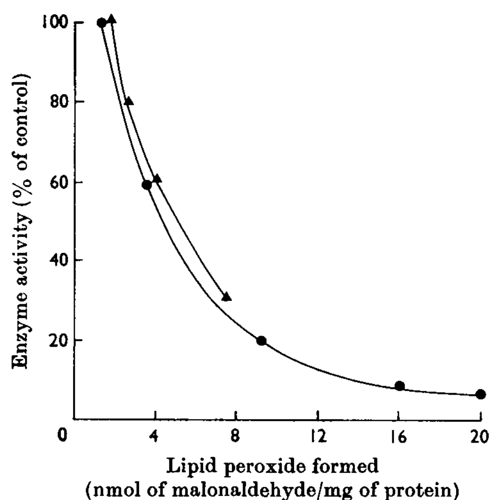

Fig. 3. Comparison of effect of peroxidation on oxidative demethylation and glucose 6-phosphatase activity of microsomal suspensions. For glucose 6-phosphatase activity experimental details are as described for Fig. 1. Peroxidation was induced by incubation with ascorbate or NADPH. For oxidative demethylation of aminopyrine, microsomal suspensions (3.0mg of protein/ml) were irradiated with doses (5–50krd) of γ-rays from a ^{60}Co source before determination of lipid peroxide and oxidative demethylation ●, Glucose 6-phosphatase; ▲, oxidative demethylation.

Mechanism of inactivation of enzymes of the endoplasmic reticulum. Inactivation of the membrane-bound enzymes is independent of the addition of ascorbate or NADPH because it can be induced by irradiation (Fig. 3) and ascorbate is not effective at 0°C (Table 1).

During lipid peroxidation, hydroperoxides are believed to be formed by oxidation of the unsaturated fatty acids in the β-position of the membrane phospholipids. These break down rapidly to form malonaldehyde and other products

(May & McCay, 1968; Tam & McCay, 1970), and this in turn causes membrane breakdown (Wills, 1969*b*). Enzyme inactivation could therefore be a result of (*a*) inactivation by hydroperoxide formation, (*b*) inactivation by breakdown of products of peroxide formation such as malonaldehyde or (*c*) membrane breakdown as a result of peroxidation. To try to establish the nature of the mechanism the following investigations were made.

Effects of detergents. It has been established (Stetten, Malamed & Federman, 1970) that glucose 6-phosphatase is inactivated readily by sodium deoxycholate at 37°C but is stable to deoxycholate at 0°C. This study of the effect of deoxycholate was extended to the other enzymes of the endoplasmic reticulum. Enzyme inhibition increased both with peroxide formation and deoxycholate concentration (Fig. 4) and the enzymes inactivated by peroxidation were also those inactivated by deoxycholate. Other enzymes were stable to peroxidation and deoxycholate (Table 2).

Several other detergents were similar to deoxycholate, causing strong inhibition at 37°C and having little effect at 0°C, but sodium dodecyl sulphate and cetylpyridinium bromide also inactivated after they were added to the suspension

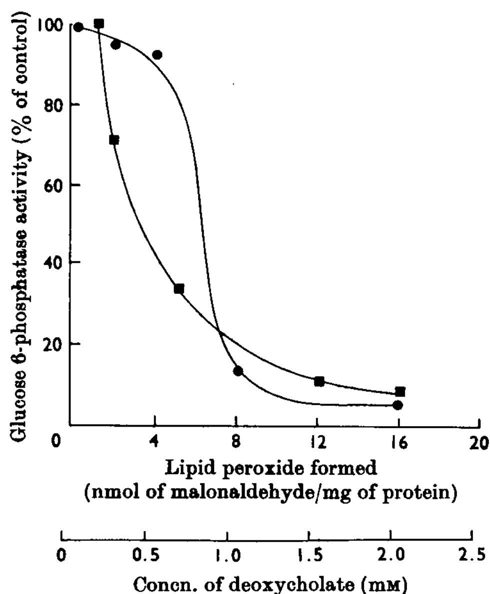

Fig. 4. Comparison of the effects of peroxidation and deoxycholate on glucose 6-phosphatase activity. For peroxidation, experimental details are as described for Fig. 1. For deoxycholate treatment, microsomal suspensions were incubated for 15min at 37°C in the presence of the specified concentration of deoxycholate. ■, Peroxidation; ●, effect of deoxycholate.

at 0°C (Table 3). These results stress the importance of the lipid membrane structure in the maintenance of glucose 6-phosphatase activity.

Effect of addition of substrate. Stetten *et al.* (1970) demonstrated that the activity of the membrane glucose 6-phosphatase could be stabilized against detergent inactivation by addition of glucose 6-phosphate. This substrate also protected glucose 6-phosphatase in microsomal suspensions in which extensive peroxidation had been induced by incubation with ascorbate, and this was despite the fact that peroxide formation was increased when glucose 6-phosphate was added (Table 4).

Effects of phospholipase. If peroxidation inactivates by causing the breakdown of membrane phospholipids it should be possible to imitate these effects by incubating with phospholipase. Suspensions of microsomal preparations were incubated with phospholipase A, which hydrolyses the fatty acids from the β-position, and phospholipase D, which causes hydrolytic cleavage of the terminal phosphate diester bond. After incubation the activities of glucose 6-phosphatase and esterase were compared. The glucose 6-phosphatase was strongly inactivated by phospholipase A treatment, but much less affected by phospholipase D, whereas esterase activity, which is much more stable to peroxidation, was less affected by phospholipase treatment (Table 5). Phospholipase A treatment therefore imitated the effects observed after peroxidation.

Effect of breakdown products of peroxidation. Products formed by cleavage of unsaturated fatty acids as a consequence of peroxidation may cause inactivation of enzymes studied, and this possibility

Table 3. *Effect of detergents on glucose 6-phosphatase activity at* 0°C *and at* 37°C

Microsomal suspensions (3.0mg of protein/ml) were incubated in 0.25M-sucrose containing 50mM-tris–HCl buffer for 10min at 0°C or 37°C in the presence of the detergent specified at a final concentration of 1mM. Enzyme activity is expressed as a percentage of that of similar suspensions incubated at 0° with no detergent present.

	Glucose 6-phosphatase activity (% of control)	
Detergent	Incubated at 0°C	Incubated at 37°C
Deoxycholate	122	6
Triton X-100	132	17
Tween 20	100	17
Tween 65	100	112
Sodium dodecyl sulphate	30	16
Cetylpyridinium bromide	4	2

Table 4. *Protective effect of the substrate on glucose 6-phosphatase activity of microsomal suspensions after peroxidation or treatment with deoxycholate*

Experimental details were as described for Fig. 1 and Table 4. The incubation time was 20min at 37°C.

Treatment of suspension	Glucose 6-phosphate (10mM) added	Lipid peroxide formed (nmol of malonaldehyde/ mg of protein)	Glucose 6-phosphatase activity (% of control)
None (control)	–	0.2	100
Incubated at 37°C for	–	4.55	28
20min with ascorbate (0.5mM)	+	7.30	55
Incubated at 37°C for	–	0.48	17
20min with deoxycholate 1 mM)	+	0.89	64

Table 5. *Effects of phospholipase A and phospholipase D on glucose 6-phosphatase and esterase activity of microsomal suspensions*

Microsomal suspensions in 0.25M-sucrose (7.5mg of protein/ml) were incubated with phospholipase A (50μg/ml) in 50mM-tris–HCl buffer, pH 7.0, or with phospholipase D (2.5mg/ml) in 50mM-sodium acetate buffer, pH 5.8, for 30min at 37°C. Activities of glucose 6-phosphatase and esterase were measured and compared with activities of microsomal suspensions kept at 0°C with no addition.

Treatment	Glucose 6-phosphatase activity (% of control)	Esterase activity (% of control)
None (control)	100	100
Incubation at 37°C for 30min	107	92
Incubation with phospholipase A	8	44
Incubation with phospholipase D	63	82
Incubation with ascorbate (0.5mM)*	24	84

* Lipid peroxide = 8nmol of malonaldehyde/mg of protein.

was tested in three series of experiments. In the first series, suspensions of microsomal preparations were incubated at 20°C or at 37°C with malonaldehyde, one of the breakdown products of peroxidation of unsaturated fatty acids. Malonaldehyde added in a much greater concentration than that produced during peroxidation (Figs. 1 and 3; 25–70nmol/mg of protein) did not affect the activity of oxidative demethylation of aminopyrine or the activity of glucose 6-phosphatase.

In a second series of experiments, effects of peroxidized fatty acids on glucose 6-phosphatase were studied by inducing peroxidation in emulsions of pure unsaturated fatty acids. Emulsions (1–10mM) of oleic acid, arachidonic acid and linolenic acid were prepared as described by Wills (1965) and arachidonic acid and linolenic acid emulsions were incubated in the presence of haemoglobin (1μM) for 30min at 37°C to induce peroxidation. Addition of emulsions of all fatty acids caused some inhibition of glucose 6-phosphatase when added in high concentration (>0.5mM) but inhibition did not depend on the extent of peroxidation and was not significantly greater than that caused by unoxidized oleic acid emulsion containing negligible concentrations of peroxide, even when the concentration of malonaldehyde added was 33nmol/ mg of protein. Addition of emulsions in low concentration (0.1mM) did not affect glucose 6-phosphatase activity whether or not the emulsions contained high concentrations of peroxide.

In the third series of experiments, microsomal suspensions incubated with ascorbate to induce peroxide formation were added to untreated suspensions and the glucose 6-phosphatase activity of untreated suspensions was compared with that of the mixed suspensions and of the incubated suspensions. If toxic degradation products had been formed, it would be expected that a decrease in the activity of the untreated suspensions would have resulted. The enzyme activity was, however, the sum of the two components, indicating that after incubation peroxidized suspensions gave no products that were toxic to glucose 6-phosphatase (Table 6).

Effect of hydroperoxide formation. It is not possible to dissociate completely hydroperoxide formation from malonaldehyde formation by

Table 6. *Effect of adding peroxidized microsomal suspensions to untreated suspensions on the activity of glucose 6-phosphatase*

Microsomal suspensions (3.0mg of protein/ml) in 0.25M-sucrose in 50mM-tris–HCl buffer, pH 7.0, were incubated with 0.5mM ascorbate for 10min and for 30min. At the end of this incubation glucose 6-phosphatase was measured on incubated suspensions alone and after mixing with equal volumes of control suspensions kept at 0°C. Glucose 6-phosphatase activity in parentheses denotes the activity if no change in activity occurs after mixing.

	Treatment	Lipid peroxide (nmol formed of malonaldehyde/mg of protein)	Glucose 6-phosphatase activity (% of control)
A	Control (30min at 0°C)	1.33	100
B	Incubation with ascorbate for 10min	5.90	60
C	Incubation with ascorbate for 30min	11.9	25
2A		1.33	208 (200)
A+B		3.62	163 (160)
A+C		6.62	127 (125)

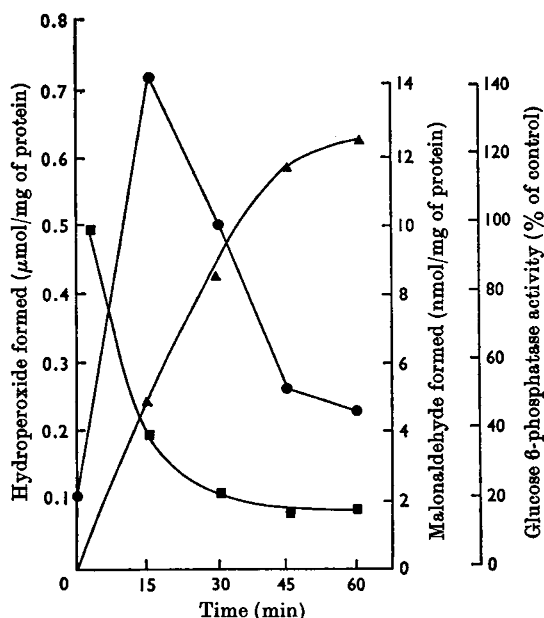

Fig. 5. Relation between hydroperoxide formation, malonaldehyde formation and glucose 6-phosphatase activity of microsomal suspensions. Microsomal suspensions (3.0mg of protein/ml) were incubated at 37°C in the presence of 0.5mM-ascorbate. Samples (2.0ml) were removed at intervals for the determination of hydroperoxide (●) by the iodometric method, 0.5ml samples were removed for determination of malonaldehyde (▲) and 1.0ml samples for the determination of glucose 6-phosphatase activity (■). Each value is the mean of four determinations.

microsomal phospholipid, because under most conditions disintegration of peroxidized unsaturated fatty acids, with malonaldehyde formation, begins almost as soon as the hydroperoxides are formed (Tam & McCay, 1970). Measurement of hydroperoxide, malonaldehyde and glucose 6-phosphatase activity of incubated microsomal suspensions, however, indicates that inactivation of glucose 6-phosphatase occurs with malonaldehyde formation rather than with hydroperoxide formation (Fig. 5). Some differential formation of hydroperoxide and malonaldehyde may be produced by aging microsomes at different temperatures. When this is done, it is clear (Table 7) that as during short-incubation experiments, malonaldehyde formation, rather than hydroperoxide formation, parallels enzyme inhibition.

DISCUSSION

Lipid peroxidation in the microsomal membrane leads to loss of membrane structure, which results in decreased activity of the electron-transport chain involved in hydroxylation of drugs and steroids (Wills, 1969*b*). Peroxidation has now been shown to cause a loss of activity of other membrane-bound enzyme systems of the endoplasmic reticulum listed in Table 2. The very close correspondence (Fig. 3) between the inactivation of oxidative demethylation and glucose 6-phosphatase indicates that it is very likely that the same membrane, or a membrane of very similar structure, is involved in the binding of the electron-transport chain and glucose 6-phosphatase. All enzymes other than glucose 6-phosphatase examined, which are sensitive to peroxidation, are directly or indirectly involved in the electron-transport chain from NADPH of the endoplasmic reticulum (Table 2).

The fact that enzyme inactivation is a result of membrane breakdown and not a result of other factors or additions used in the generation of

Table 7. *Comparison of effects of hydroperoxide formation and malonaldehyde formation on glucose 6-phosphatase*

Microsomal suspensions (3.0mg of protein/ml) in 0.25M-sucrose were stored for 20h at 4°C or −10°C before measurement of hydroperoxide, malonaldehyde and glucose 6-phosphatase activity. Enzyme activity is expressed as a percentage of that of suspensions measured immediately after preparation.

Storage conditions	Hydroperoxide formed (μmol/mg of protein)	Malonaldehyde formed (nmol/mg of protein)	Glucose 6-phosphatase activity (% of control)
4°C	1.39	16.5	49
−10°C	3.21	4.1	91

peroxides or caused by disintegration products is strongly supported by the experimental evidence.

Hydroperoxides themselves do not appear to be toxic to glucose 6-phosphatase (Fig. 5 and Table 7) and neither is malonaldehyde nor other components that are produced in peroxidation (Table 6).

Other methods of causing membrane breakdown, by treatment with detergents (Table 2; Fig. 3) or phospholipases (Table 5), also cause enzyme inactivation and correlate well with peroxidation effects, some enzymes being inactivated by both peroxidation and detergents, whereas other enzymes that are resistant to peroxidation are also resistant to detergents. The fact that incubation with phospholipase A caused much more inactivation than did phospholipase D (Table 5) stresses the importance of the fatty acid of the β-position of phospholipids in the maintenance of membrane structure and it is likely that the unsaturated fatty acids of the β-position are also those most sensitive to peroxidation. It is possible that fatty acids or lysolecithins released from membrane phospholipids by phospholipase A may be responsible for some inhibition of the membrane-bound enzymes. However, fatty acid emulsions added to untreated microsomal preparations in a concentration expected to be formed by phospholipase treatment caused little inhibition, and therefore it is unlikely that inhibition due to fatty acids hydrolysed from phospholipids would be significant. Furthermore, breakdown products of membrane components produced by peroxidation containing lysolecithins or related components and fatty acids are not toxic to glucose 6-phosphatase (Table 6).

The fact that several enzymes are resistant to detergents and to peroxidation (Table 2) indicates that the membrane is of much less importance in the maintenance of the conformation of an active structure of these enzymes. Some detergents, such as sodium dodecyl sulphate and cetylpyridinium bromide, caused inactivation of enzymes unaffected by sodium deoxycholate but this is likely to be a result of the denaturing effect of these compounds on the enzyme protein, which has long been known (Wills, 1954).

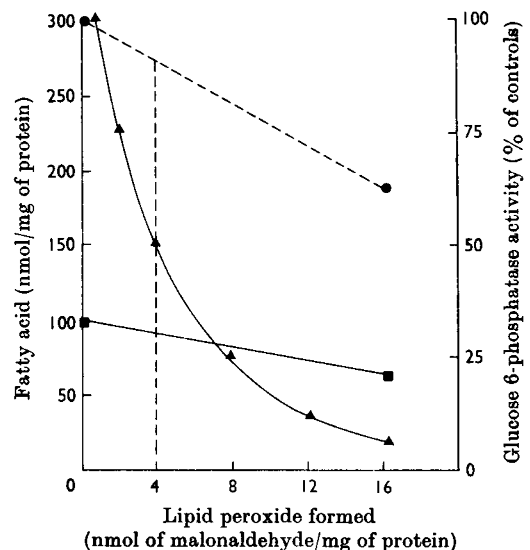

Fig. 6. Relationship between loss of unsaturated fatty acids of phospholipids of the endoplasmic reticulum and the activity of glucose 6-phosphatase. Values for disintegration of arachidonic acid (●) and $C_{22:6}$ acid (■) as a result of incubation of microsomal suspensions with NADPH are calculated from data of May & McCay (1968). ▲, Glucose 6-phosphatase activity.

From the results of May & McCay (1968), who related malonaldehyde formation in microsomal suspensions mainly to the disappearance of the arachidonic acid ($C_{20:4}$) and the $C_{22:6}$ acid from the β-position of membrane phospholipids as a result of peroxidation, a calculation can be made of the importance of the integrity of unsaturated phospholipids to enzyme activity. Fig. 3 shows that approximately 50% inactivation of oxidative demethylation and glucose 6-phosphatase activity results from the formation of 4nmol of malonaldehyde/mg of protein. May & McCay (1968) calculated that approx. 12% of the unsaturated fatty acids destroyed by peroxidation formed malonaldehyde, and this value is used in calculating the relationship

between malonaldehyde production and fatty acid loss. When the results of May & McCay (1968) and those of the present investigation are superimposed it will be observed (Fig. 6) that 50% of the enzyme inactivation results from a destruction of only 6nmol (8%) of the $C_{22:6}$ acid and 30nmol (10%) of arachidonic acid. These results emphasize the important role played by unsaturated fatty acids in the maintenance of membrane integrity and how a relatively small amount of disintegration of unsaturated fatty acids in the β-position of phospholipids can lead to extensive loss of enzyme activity.

These findings may be of considerable practical importance to those engaged in the study of metabolism of the endoplasmic reticulum because it is clear (Figs. 1 and 3) that even when all reasonable precautions are taken to preserve stability most 'control' microsomal preparations had already lost about 15% of their original activity before the experiments began.

The precise physiological role of these changes in the normal cell *in vivo* is at present a matter for speculation but as the NADPH electron-transport chain may be switched readily to peroxidation a potential mechanism of membrane remodelling and control of enzyme activity is clearly possible. In pathological conditions, and especially if protection of unsaturated fatty acids of phospholipids by natural anti-oxidants became impaired or inadequate, serious loss of membrane-enzyme activity could follow as a consequence of loss of membrane structure.

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