

## Lipid Peroxide Formation in Microsomes

### THE ROLE OF NON-HAEM IRON

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1. Metal ion-chelating agents such as EDTA, *o*-phenanthroline or desferrioxamine inhibit lipid peroxide formation when rat liver microsomes prepared from homogenates made in pure sucrose are incubated with ascorbate or NADPH. 2. Microsomes treated with metal ion-chelating agents do not form peroxide on incubation unless inorganic iron ( $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$ ) in a low concentration is added subsequently. No other metal ion can replace inorganic iron adequately. 3. Microsomes prepared from sucrose homogenates containing EDTA (1 mM) do not form lipid peroxide on incubation with ascorbate or NADPH unless  $\text{Fe}^{2+}$  is added. Washing the microsomes with sucrose after preparation restores most of the capacity to form lipid peroxide. 4. Lipid peroxide formation in microsomes prepared from sucrose is stimulated to a small extent by inorganic iron but to a greater extent if adenine nucleotides, containing iron compounds as a contaminant, are added. 5. The iron contained in normal microsome preparations exists in haem and in non-haem forms. One non-haem component in which the iron may be linked to phosphate is considered to be essential for both the ascorbate system and NADPH system that catalyse lipid peroxidation in microsomes.

The importance of inorganic iron as a catalyst or as a part of a catalytic system for the peroxidation of unsaturated lipids both *in vivo* and *in vitro* has been indicated by many investigations.  $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$  in the presence of ascorbate are powerful catalysts for the oxidation of emulsions of pure linoleic acid or linolenic acid (Wills, 1965), and inorganic (non-haem) iron is considered to be important as a factor in the catalysis of the peroxidation of emulsions of unsaturated fatty acids added to tissue homogenates (Wills, 1966). The rate of lipid peroxidation in mitochondrial suspensions is strongly stimulated by addition of  $\text{Fe}^{2+}$  in the presence of ascorbate (Ottolenghi, 1959; Bernheim, 1963; McKnight, Hunter & Oerhert, 1965), and EDTA strongly inhibits lipid peroxide formation in incubated brain homogenates (Bernheim, 1963; Barber, 1963). Hochstein & Ernster (1963) reported that some nucleotides, and especially ADP, markedly stimulated the rate of lipid peroxide formation in liver microsomes in the presence of NADPH, but later Hochstein, Nordenbrand & Ernster (1964) discovered that the main factor concerned in ADP stimulation was the presence of a relatively high concentration of contaminating iron compounds. In view of the readiness with which the microsomal fraction of liver forms lipid peroxide on incubation with

NADPH or ascorbate, it is likely that inorganic (non-haem) iron is an essential component of the system and an evaluation of the role of iron is made in this investigation.

### EXPERIMENTAL

Most materials used and methods were as described by Wills (1969*a*). The Wistar rats used in the investigation were fed on a stock diet containing 87  $\mu\text{g}$ . of iron/g. of diet, and the microsomes, prepared from rat liver, were normally suspended in 125 mM-KCl (1 ml./g. of liver used for the preparation). This suspension contained  $15.0 \pm 1.5$  mg. of protein/ml. In addition the following materials and methods were used.

**Materials.** ATP, ADP and bathophenanthroline were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.) or from British Drug Houses Ltd. (Poole, Dorset). Adenine and AMP were from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.) and desferrioxamine (Desferal) was from CIBA Laboratories Ltd. (Horsham, Sussex). All other chemicals were obtained from British Drug Houses Ltd.

**Iron determinations.** (a) Free iron. The non-haem iron content of microsomal suspensions was determined by using, with some modification, a method described for serum iron (Henry, Sobel & Chiamori, 1958). Samples of microsomal suspensions (3 mg. of protein/ml.) were diluted with water (1.25 ml.) and 20% (w/v) trichloroacetic acid (0.75 ml.). The mixture was kept at 100° for 15 min., cooled

and centrifuged. To 2.0 ml. of supernatant, 50% (w/v)  $(\text{NH}_4)_2\text{SO}_4$  (2.0 ml.) and a saturated solution of hydrazine sulphate (0.2 ml.) were added and, after thorough mixing, the  $E_{535}$  value was read. Sulphonated bathophenanthroline (0.05%, w/v) (0.6 ml.) was added, and the mixture was left for 15 min. and its  $E_{535}$  value read again. The difference in extinction between the two readings is proportional to the iron concentration and was compared with a series of standards.

(b) Total iron. Microsomal suspensions (7.5 mg. of protein/ml.) were heated at 100° for 1 hr. with  $\text{HNO}_3$  (7.8 M), cooled, made up to 4.0 ml. with water and read against a series of suitable standards in the Perkin-Elmer no. 303 atomic absorption spectrophotometer.

Unless otherwise specified all values are means of three experiments.

## RESULTS

*Effect of metal ion-chelating agents on lipid peroxide formation.* Lipid peroxide formation and oxygen uptake by liver microsomes when incubated in the presence of NADPH or ascorbate were strongly inhibited by EDTA (1 mM) (Table 1). EDTA is a non-specific metal ion chelator, but strong inhibition also resulted from the addition of more specific iron chelators, such as *o*-phenanthroline, 8-hydroxyquinoline or desferrioxamine. Similar inhibitory effects were observed in ascorbate systems and with boiled microsomes (Wills, 1969a).

*Treatment of microsomes with chelating agents and metal ion replacements.* As low concentrations of iron-chelating agents (0.5 mM *o*-phenanthroline) almost completely inhibited lipid peroxide formation in dilute suspensions of microsomes (1.5 mg. of protein/ml.), attempts were made to remove the iron by washing the microsomes immediately after preparation by treating a more concentrated suspension (15 mg. of protein/ml.) with a chelating agent such as *o*-phenanthroline (1 mM). After this treatment the microsomes were centrifuged at 100000g for 40 min. and washed once more with potassium chloride (125 mM). A control microsomal suspension was washed twice with potassium chloride. Peroxidation in washed and unwashed microsomes was measured in the presence of NADPH or ascorbate and found to be virtually unaffected by treatment with this concentration of chelating agent.

If, however, after separation of the mitochondria and nuclei the microsomal suspension was treated with 5 mM-desferrioxamine, 5 mM-*o*-phenanthroline or 5 mM- $\alpha\alpha'$ -bipyridyl, almost complete inhibition of peroxidation resulted (Table 2).

The activity of the microsomes could be restored to and even increased beyond the control by the addition of 0.2 mM- $\text{Fe}^{3+}$  or  $-\text{Fe}^{2+}$ .  $\text{Zn}^{2+}$  was also partially effective after *o*-phenanthroline treatment, but not after desferrioxamine treatment (Table 2). The binding of *o*-phenanthroline to the microsomes

was not strong and a single washing with potassium chloride (125 mM) restored the rate of peroxidation, but washing caused only a small increase in the activity of the microsomes treated with desferrioxamine. The rate of peroxidation in desferrioxamine-treated microsomes was strongly stimulated by the addition of  $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$  and none of the large number of other metal ions tested could replace the requirement for inorganic iron (Table 2). Microsomes suspended in potassium chloride after preparation and treated with *o*-phenanthroline or with desferrioxamine did not form lipid peroxide unless iron was added. Washing with potassium chloride restored the activity to *o*-phenanthroline-treated microsomes, but not to desferrioxamine-treated microsomes, but a rapid rate of peroxidation resulted from the addition of  $\text{Fe}^{3+}$  or  $\text{Fe}^{2+}$  (Table 3).

*Preparation of microsomes in the presence of EDTA and the effects of washing and the addition of metal ions.* When microsomes, prepared from liver homogenates made in 0.25 M-sucrose containing EDTA (1 mM), were drained completely of supernatant but not washed, the rate of lipid peroxide formation in the presence of NADPH or ascorbate was decreased nearly to zero. The rate of peroxidation could be restored to and even increased beyond that of microsomes prepared from homogenates made in sucrose alone by the addition of  $\text{Fe}^{2+}$

Table 1. *Effect of chelating agents on lipid peroxide formation in microsomes*

Suspensions of microsomes (1.5 mg. of protein/ml.) were incubated in a medium containing 20 mM-sodium phosphate buffer, pH 7.0, NADPH (40  $\mu\text{M}$ ), nicotinamide (50 mM) and KCl (90 mM) at 37° in a total volume of 2.0 ml. Ascorbate (0.5 mM) replaced NADPH + nicotinamide in one series of experiments. Samples (0.5 ml.) were removed at intervals for the determination of lipid peroxide by the thiobarbituric acid method (Wills, 1969a).

Addition	Concn. (mM)	Rate of lipid peroxide formation (% of control)	
		NADPH system	Ascorbate system
None	—	100	100
EDTA	0.5	5	2
	1.0	2	0
	2.0	0	0
<i>o</i> -Phenanthroline	0.5	2	6
	1.0	1	0
	2.0	0	0
8-Hydroxyquinoline	0.5	29	8
	1.0	23	3
	2.0	6	2
Desferrioxamine	0.5	20	7
	1.0	0	4
	2.0	0	3

Table 2. *Treatment of microsomes with chelating agents during preparation and effects of metal ions*

After removal of the nuclei and mitochondria from a liver homogenate in 0.25 M-sucrose (Wills, 1969a), the microsomal suspension was divided into three parts. To one *o*-phenanthroline was added to give a final concentration of 5 mM, to a second desferrioxamine was added to a concentration of 5 mM and a third part was untreated control. The suspensions were kept for 10 min. at 0° and then centrifuged for 1 hr. at 100000g. The sedimented microsomes drained completely of supernatant were suspended in 125 mM-KCl (2 ml./g. of liver), a portion of each was removed and further 125 mM-KCl (4 ml./g. of liver) was added to wash each microsomal sediment thoroughly. Each suspension was then centrifuged for 30 min. at 100000g and the sedimented microsomes were suspended in 125 mM-KCl (2 ml./g. of liver). Rates of lipid peroxide formation in the presence of NADPH were measured with and without added metal ions as described for Table 1, but the phosphate buffer was replaced by tris-HCl buffer, pH 7.0.

Chelating agent	Treatment of microsomes	Metal ion added	Concn. (mM)	Lipid peroxide formation (nmoles of malonaldehyde/min.)	
None (control)	Not washed	None	—	3.86	
		Fe <sup>2+</sup>	0.2	4.33	
		Fe <sup>3+</sup>	0.2	6.80	
		Zn <sup>2+</sup>	0.2	4.62	
	Washed	None	—	7.30	
		Fe <sup>2+</sup>	0.2	12.20	
		Fe <sup>3+</sup>	0.2	11.40	
		Zn <sup>2+</sup>	0.2	7.20	
	<i>o</i> -Phenanthroline	Not washed	None	—	1.47
			Fe <sup>2+</sup>	0.2	6.20
			Fe <sup>3+</sup>	0.2	3.98
			Zn <sup>2+</sup>	0.2	1.95
Co <sup>2+</sup>			0.2	1.16	
Washed		None	—	7.29	
		Fe <sup>2+</sup>	0.2	11.32	
			0.1	12.17	
		Fe <sup>3+</sup>	0.2	11.24	
			0.1	10.34	
		Zn <sup>2+</sup>	0.2	6.94	
			0.1	7.95	
Desferrioxamine	Not washed	None	—	0.09	
		Fe <sup>2+</sup>	0.2	4.45	
		Fe <sup>3+</sup>	0.2	1.16	
		Zn <sup>2+</sup>	0.2	0	
	Washed	None	—	1.15	
		Fe <sup>2+</sup>	0.2	10.03	
			0.1	11.90	
		Fe <sup>3+</sup>	0.2	11.45	
			0.1	10.90	
		Zn <sup>2+</sup>	0.2	1.57	
			0.1	1.04	
		Co <sup>2+</sup>	0.2	0.97	
Cu <sup>2+</sup>		0.2	0.79		
Ni <sup>2+</sup>		0.2	0.42		
Pb <sup>2+</sup>		0.2	0.13		
Mn <sup>2+</sup>		0.2	0.19		
Ca <sup>2+</sup>	0.2	0.25			
Mg <sup>2+</sup>	0.2	0.43			

in a very low concentration (Fig. 1). No other metal ion from among those tested, Cu<sup>2+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup> and Zn<sup>2+</sup>, restored completely the rate of lipid peroxide formation when added in a concentration of 0.1 or 0.2 mM, whereas inorganic

iron was effective at 2 μM (Table 4). However, some metal ions, and especially Zn<sup>2+</sup>, restored a percentage of the rate of peroxidation. A small fraction of the iron necessary to restore the rate of lipid peroxide formation appeared to be bound by traces

Table 3. *Effect of Fe<sup>2+</sup> and Fe<sup>3+</sup> on microsomes treated with chelating agents*

A microsomal suspension (15 mg. of protein/ml. in 0.125 M-KCl) was divided into three parts. To one suspension *o*-phenanthroline was added to give a final concentration of 5 mM, then kept for 5 min. at 0°, centrifuged at 100000g for 30 min., washed with 0.125 M-KCl, centrifuged again and resuspended in the original volume of KCl solution. One other portion was subjected to the same process of centrifugation and washing with KCl solution after addition of desferrioxamine (5 mM), and the third was treated with KCl only as a control. Each suspension was then diluted 1:10 and the rate of lipid peroxide formation measured in the presence of NADPH and 0.02 M-tris-HCl buffer, pH 7.4 (Wills, 1969a).

Chelating agent	Treatment of microsomes	Metal ion added (0.2 mM)	Lipid peroxide formation (nmoles of malonaldehyde/min.)		
None (control)	Washed	None	3.48		
	Washed twice	None	4.05		
<i>o</i> -Phenanthroline	Not washed	None	0.28		
		Fe <sup>2+</sup>	7.15		
		Fe <sup>3+</sup>	7.95		
		Zn <sup>2+</sup>	1.07		
	Washed	None	3.93		
		Fe <sup>2+</sup>	6.09		
		Fe <sup>3+</sup>	6.45		
		Zn <sup>2+</sup>	2.46		
		Desferrioxamine	Not washed	None	0
				Fe <sup>2+</sup>	4.53
Fe <sup>3+</sup>	8.05				
Zn <sup>2+</sup>	0.45				
Washed	None		1.26		
	Fe <sup>2+</sup>		6.65		
		Fe <sup>3+</sup>	7.35		
		Zn <sup>2+</sup>	0.65		
		Co <sup>2+</sup>	0		
		Mn <sup>2+</sup>	0.18		

of EDTA left in the preparation, because if Mg<sup>2+</sup>, Ca<sup>2+</sup> or Zn<sup>2+</sup> (0.2 mM) was first added to the microsomes before the inorganic iron then low concentrations of inorganic iron became more effective (Table 4). Mg<sup>2+</sup> and Ca<sup>2+</sup> were virtually without effect when added separately.

Restoration of a large percentage, but not all, of the original rate of peroxidation could also be achieved by washing microsomes, prepared from sucrose-EDTA, once with sucrose alone. EDTA cannot therefore be firmly bound to the metal component of the microsomes. During the washing some of the protein is lost from all microsome preparations and this is accompanied by a proportional decrease in the rate of lipid peroxidation (Table 5). After a washing of microsomes with potassium chloride, however (Table 2), a small increase of rate of peroxidation results.

The rate of peroxidation in microsomes prepared from sucrose-EDTA homogenates and subsequently washed with sucrose was only weakly stimulated by Fe<sup>2+</sup>, 10 μM-Fe<sup>2+</sup> causing a 10% increase in rate, but was strongly inhibited by EDTA (0.1 mM). These experiments indicate that a major portion, at least, of the iron component of the microsomes, essential for the lipid peroxidation system, remains

bound in the microsomes and yet is available to EDTA.

*Iron content of microsomal preparations.* The 'free' (non-haem) and total iron contents of 52 microsome suspensions prepared from sucrose alone were determined. The total iron content was found to be 28.63 ± 9.78 nmoles of iron/mg. of protein and the 'free' (non-haem) iron content 8.27 ± 3.15 nmoles/mg. of protein. Treatment of the homogenates with EDTA (1 mM) before preparation of the microsomes did not significantly alter the measured content of total iron or even that of the 'free' (non-haem) iron of the microsomes. Further, if, after removal of nuclei and mitochondria, suspensions of microsomes in sucrose (0.25 M) were treated with *o*-phenanthroline (5 mM) or with desferrioxamine (5 mM), centrifuged and then washed with potassium chloride (125 mM), no significant decrease in the 'free' (non-haem) content of the microsomes was observed.

*Effect of adenine nucleotides.* A comparison of the effects of adenine, adenosine and several adenine nucleotides showed that the stimulating effect depended on the iron content of the nucleotides and, as found by Hochstein *et al.* (1964), some specimens of ADP used contained large quantities of iron

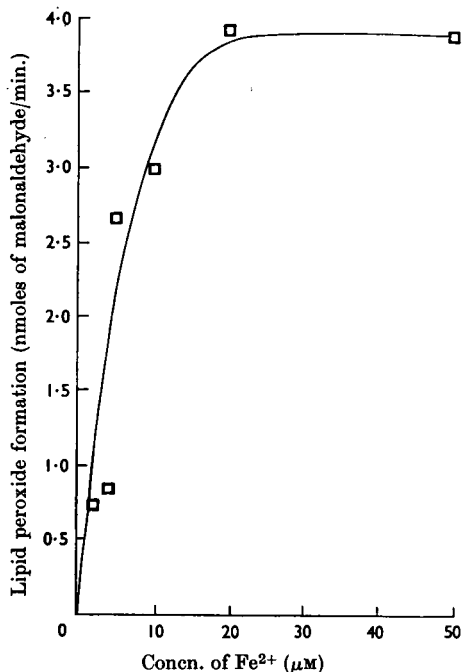


Fig. 1. Effect of addition of inorganic iron to microsomes prepared from homogenates containing EDTA (1 mM). Rat liver (10 g.) was divided into two equal parts. One portion (5 g.) was homogenized in 50 ml. of 0.25 M-sucrose and the other was homogenized in 50 ml. of 0.25 M-sucrose containing EDTA (1 mM). Microsomes were prepared from each homogenate by the same process, resuspended in 125 mM-KCl and the rate of lipid peroxide formation in the presence of NADPH and different concentrations of Fe<sup>2+</sup>, added as FeSO<sub>4</sub>, was measured as described in Table 1.

contaminants and stimulated strongly (Table 6). Other specimens of ADP used in subsequent experiments contained much less iron contaminants and were much less effective in increasing the rate of peroxidation. Pyrophosphate was not effective.

The rate of lipid peroxide formation in microsomes prepared from homogenates containing sucrose + EDTA (1 mM) was also stimulated by adenine nucleotides to an extent that depended on the iron content of the nucleotide, but this stimulation was much less than that resulting from addition of inorganic iron, and the combination of the adenine nucleotide added with inorganic iron was only slightly greater than that of inorganic iron added alone (Table 7).

*Effects of amino acids and dicarboxylic acids.* In model systems several carboxylic acids such as malate or succinate were found to cause small increases in the catalytic activity of inorganic iron for the peroxidation of linoleic acid and linolenic

acid, presumably by the formation of a more active iron complex (Wills, 1965). Addition of succinate, fumarate, malate, malonate, glutamate or aspartate in a concentration of 2 mM to microsomes suspensions did not, however, alter the rate of peroxide formation in the presence of NADPH or ascorbate to a significant extent.

## DISCUSSION

Liver microsomes clearly contain an iron component that is essential for the formation of lipid peroxides in the presence of either ascorbate or of NADPH. The requirement for iron compounds appeared to be absolutely specific and no other metal ions tested could replace it (Tables 2, 3 and 4). Iron is present in the microsome fraction in haem and in non-haem forms. Cytochromes and some haemoglobin will form a large proportion of the haem iron, but the non-haem or 'free' iron is likely to be in several different states of combination. Evidence from studies on the inhibition of lipid peroxide formation in the presence of NADPH by thiol reagents has led to the conclusion that a ferredoxin-type protein may be an essential component of the system, but it may not be involved in ascorbate-induced peroxidation because this system is unaffected by thiol reagents (Wills, 1969a).

Ferritin is another form in which non-haem iron occurs in the microsomal fraction. Purified separated ferritin does not catalyse the peroxidation of emulsions of pure linoleic acid unless the iron is first released by a reducing agent such as ascorbate on the acid side of neutrality (Wills, 1966). In the microsomal system peroxidation occurs at neutral pH or even at pH 8.0 in the presence of NADPH (Wills, 1969a), and, although it is possible that iron released from ferritin is the active component, it is more likely that the active iron exists in some other combination. Different forms of iron may be utilized by the NADPH and ascorbate systems, but the similar pattern of inhibition by chelating agents observed for the two systems (Table 1) provides evidence against this view.

Microsomes prepared in the presence of EDTA or *o*-phenanthroline or treated with these chelating agents after preparation form very little lipid peroxide on incubation with NADPH or ascorbate (Tables 2 and 3). The readiness with which these chelating agents inhibit peroxidation indicates that non-haem iron and not haem iron is the component that is bound. This inhibition could be a result of either the removal of iron or of the binding of iron while it remains *in situ* on a microsomal protein or other component. Experimental evidence is strongly in favour of the latter hypothesis and against the former, and it may be significant that the iron of plant and bacterial ferredoxin is

Table 4. *Effect of metal ions on the rate of lipid peroxide formation in microsomes prepared from liver homogenate made in sucrose-EDTA*

Microsomes were prepared as described in the Experimental section, but for a portion of the liver the sucrose (0.25 M) contained EDTA (1 mM). After preparation the supernatant was drained completely and the microsomes were suspended in 125 mM-KCl (15 mg. of protein/ml.). Rates of peroxide formation were measured as described for Table 1, but with tris-HCl buffer in place of phosphate. For the experiments in which Zn<sup>2+</sup> was added with Fe<sup>2+</sup>, the Zn<sup>2+</sup> was added to the suspension before the Fe<sup>2+</sup>.

Preparation	Metal ion added	Concn. ( $\mu$ M)	Lipid peroxide formation (nmoles of malonaldehyde/min.)
Sucrose	None (control)	0	4.69
Sucrose-EDTA	None (control)	0	0.27
	Cu <sup>2+</sup>	50	0.32
		20	0.48
	Co <sup>2+</sup>	200	0.29
		100	1.15
	Mn <sup>2+</sup>	200	0.32
		100	1.11
	Zn <sup>2+</sup>	100	3.30
		50	2.69
		20	2.12
	Ni <sup>2+</sup>	200	0.92
		100	0.52
	Ca <sup>2+</sup>	200	0.13
		100	0.18
	Mg <sup>2+</sup>	200	0.32
		100	0.25
	Fe <sup>2+</sup>	1	1.77
		2	2.20
		10	4.48
20		4.83	
Zn <sup>2+</sup> (0.2 mM) + Fe <sup>2+</sup>		1	4.98
	2	5.75	
	10	5.90	

generally neither removed nor replaced with great ease (Malkin & Rabinowitz, 1967).

First, values for the 'free' and total iron contents of the microsomes prepared from sucrose-EDTA homogenates, or treated with *o*-phenanthroline, are not significantly different from the values for microsomes prepared from sucrose; secondly, virtually the whole capacity of the microsomes to carry out peroxidation is restored simply by washing (Tables 3 and 5). Activity may also be restored completely by the addition of Fe<sup>2+</sup> or Fe<sup>3+</sup>, but there is no doubt that some of the iron added functions by binding with the chelator and removing it from combination with the iron of the microsomes. Zn<sup>2+</sup> and a few other metal ions probably also restore part of the activity by a binding of similar nature, but they are less effective than inorganic iron (Tables 2 and 3). The excess of iron not bound by the EDTA or *o*-phenanthroline may become attached to vacant iron-binding sites on the microsomal protein or even be active in its inorganic

form. However, the activity of microsomes treated with desferrioxamine is not restored by washing (Table 3), despite the fact that the washed microsomes contain virtually the same quantity of non-haem iron/mg. of protein as do untreated microsomes. This chelator has a very high affinity for ferric iron with a stability constant of 10<sup>31</sup> as compared with 10<sup>14</sup> for copper and 10<sup>11</sup> for zinc (Tripod, 1964).

When microsomes are treated with desferrioxamine this therefore remains firmly bound to the iron and activity can only be restored by ferrous or ferric iron (Tables 2 and 3). The effect of iron is specific and these experiments provide strong evidence for the role that non-haem iron plays in the production of peroxides in microsomes.

Adenine nucleotides, and especially samples of ADP containing relatively high concentrations of contaminating iron compounds, stimulate the rate of peroxide formation (Table 6), in confirmation of the observations by Hochstein *et al.* (1964),

Table 5. *Lipid peroxide formation in microsomes prepared from sucrose and from sucrose-EDTA homogenates*

Microsomes were prepared from liver homogenates made either in sucrose (0.25 M) or in 0.25 M-sucrose containing EDTA (1 mM). A portion of the microsomes was set aside immediately after preparation, a second portion was washed with 0.25 M-sucrose (15 ml./g. of liver) and centrifuged again at 100000g for 1 hr., and a third portion was washed a second time with sucrose as before. All microsome preparations were suspended in 125 mM-KCl (1 ml./g. of liver used for the preparation) and the rate of lipid peroxide formation in the presence of NADPH was measured as described in Table 1.

Preparation	No. of times washed	Protein content of microsome suspension (mg./ml.)	Lipid peroxide formation	
			Rate (nmoles of malonaldehyde/min.)	Amount (nmoles of malonaldehyde/mg. of protein)
Sucrose	0	13.07	4.56	0.355
	1	8.60	2.92	0.340
	2	4.32	2.28	0.526
Sucrose-EDTA	0	11.80	0.30	0.025
	1	8.17	2.38	0.292
	2	4.10	1.92	0.465

Table 6. *Effect of adenine nucleotides on lipid peroxide formation and oxygen uptake by microsomes*

Microsomal suspensions (1.5 mg. of protein/ml.) prepared from sucrose homogenates were incubated with NADPH at 37°, in 0.02 M-tris-HCl buffer, pH 7.4 (Wills, 1969a).

Addition	Iron content (nmoles of Fe/mole)	O <sub>2</sub> uptake (nmoles/min.)	Lipid peroxide formation (nmoles of malonaldehyde /min.)
None (control)	—	84	4.15
ATP (1 mM)	0.41	159	12.00
ADP (1 mM)	4.90	486	32.00
AMP (1 mM)	0.11	131	10.06
Adenine (1 mM)	0.05	96	4.35

but it has been noted (Wills, 1969a) that strong stimulation can also be obtained by the addition of inorganic phosphate in a high concentration. Adenine is ineffective. Adenine nucleotides containing contaminating iron compounds stimulate peroxidation in microsomes prepared from sucrose-EDTA to a very small extent and are much less effective than inorganic iron (Table 7). Nucleotide-iron complexes cannot remove the EDTA that is bound to the non-haem iron in these preparations and clearly they are not able to replace the active iron complex of the microsomes. Nevertheless the powerful stimulation observed when adenine nucleotide-iron complexes are added to normal microsomes (Table 6) and stimulation by phosphate (Wills, 1969a) suggest that some, at least, of the active iron is in a phosphate complex and that adenine or adenosine helps to orientate or stabilize it in an active configuration. It is therefore possible that in the microsome fraction iron may be bound to the phosphate groups of nucleic acids.

Table 7. *Effect of adenine nucleotides on lipid peroxide formation in microsomes prepared from sucrose (0.25 M)-EDTA (1 mM)*

The concentration of nucleotides when added was 1 mM in all experiments. Other experimental details were as described in Table 3.

Nucleotide added	Concn. of Fe <sup>2+</sup> added (μM)	Lipid peroxide formation (nmoles of malonaldehyde/min.)
None	None	0.14
None	200	6.08
	100	4.14
	50	3.75
ATP (0.15 nmoles of Fe/mole)	None	0.50
	200	5.96
	100	4.03
ADP (1.08 nmoles of Fe/mole)	50	3.81
	None	0.83
	200	5.35
ADP (11.60 nmoles of Fe/mole)	100	4.25
	50	4.20
	None	0.72
AMP (1.03 nmoles of Fe/mole)	200	8.88
	100	6.50
	50	5.75
None	None	0.61
	200	4.38
	100	3.37
None	50	1.16

It is doubtful if this iron, which can form loose complexes with chelating agents and is essential for lipid peroxidation induced by either ascorbate or by NADPH, is an essential component of the microsomal electron-transport chain responsible for drug hydroxylation (Wills, 1969b), but it is likely that it exerts an important regulating effect

by providing an alternative pathway of lipid peroxidation and membrane disintegration in the place of hydroxylation.

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