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MITOCHONDRIAL LIPID PEROXIDATION BY CUMENE HYDROPEROXIDE AND ITS PREVENTION BY SUCCINATE

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Rat liver mitochondria form lipid hydroperoxides when they are incubated aerobically with cumene hydroperoxide. The rate of reaction is dependent on the initial concentration of the latter and involves the consumption of oxygen. Gradient-separated and cytochrome *c*-depleted mitochondria, mitoplasts and submitochondrial fractions also undergo this peroxidation. Mitochondrial lipid peroxidation by cumene hydroperoxide is strongly inhibited by SKF52A (an inhibitor of cytochrome *P*-450), by antioxidants and to a lesser extent by the enzymes superoxide dismutase and catalase. Consersely, rotenone and *N*-ethylmaleimide stimulate the reaction. Succinate protects against the lipid peroxidation and in some mitochondrial fractions the associated oxygen uptake is also inhibited. This protection by succinate is prevented by malonate but not by *N*-ethylmaleimide or antimycin. Lipid hydroperoxides present in previously peroxidised mitochondria are partly lost on reincubation with succinate and this reaction is also unaffected by *N*-ethylmaleimide but inhibited by both malonate and antimycin. The results suggest that reduction of mitochondrial ubiquinone may prevent the generation of lipid hydroperoxides but that their subsequent removal may require reduction at or beyond cytochrome *b*.

Introduction

Rat liver mitochondria are subject 'in vitro' to the peroxidation of their membranes by reagents such as oxygen and ultraviolet light [1], Fe^{2+} (with [2] or without [3] ADP), ascorbate [4], GSH [5], dihydroxyfumarate [6], CCl_4 [7] and chaotropic agents [8]. The subject has been recently reviewed [9] and it is clear that this peroxidation may be of physiological significance, since there are reports that it also occurs in vivo after treating experimental animals in various ways [10–12].

A number of substances protect mitochondria as well as other subcellular particles from this peroxidation including superoxide dismutase [13], vitamin E [14,15], flavonoids [6] and phenols [16,44]. However, mitochondria are already more resistant to peroxidation in vitro than other particles [9,19]. This may be partly due to their lower lipid/protein ratio but it is also possible that they may possess their own endogenous protective systems. In particular, the involvement of GSH and glutathione peroxidase has been proposed. Thus, mitochondria from foetal rats are low in their content of this enzyme and at the same time unusually susceptible to peroxidation [17]. Mitochondria swollen to allow loss of glutathione peroxidase similarly show an inverse relationship between enzyme content and ease of peroxidation [18].

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Abbreviations: butylated hydroxyanisole, 2-(*tert*-butyl)-4-methoxyphenol + 3-(*tert*-butyl)-4-methoxyphenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

A new group of peroxidising agents which do not impair primary mitochondrial functions are the organic hydroperoxides. These substances have been used extensively to give oxidant pulses in perfused organs, in whole cells (such as erythrocytes and hepatocytes) and also in subcellular fractions [19–21].

We have studied lipid peroxidation induced in mitochondria by cumene hydroperoxide and now describe some properties of the reaction. Our evidence, obtained using this reagent, is consistent with the presence of at least two protective mechanisms which are operative in vitro.

Materials and Methods

Superoxide dismutase and catalase were purchased from Sigma Ltd. and SKF525A from Smith, Kline and French Ltd., Milan.

Rat liver mitochondria were isolated in 0.25 M sucrose buffered with 10 mM Tris-HCl at pH 7.4 [22]. Some preparations were also obtained by a method which minimises microsomal contamination [23]. Mitochondria were washed three times in this sucrose solution then once in 0.125 M KCl containing 25 mM Tris-HCl, pH 7.4, and EDTA (1 mM). The washed mitochondria were finally dispersed as a concentrated suspension (50–60 mg/ml) in this solution at 0°C and aliquots (4 mg/ml) subsequently dispensed into the same medium ('incubation medium') for the incubation experiments to be described in Results. Mitoplasts were prepared from mitochondria by the swelling method [23]. Mitochondria freed from microsomes were isolated [24] in linear sucrose gradients using the vertical Sorvall rotor SV288 in a Sorvall RC-5B refrigerated centrifuge. Submitochondrial particles were obtained after ultrasonic disruption of hypotonically swollen mitochondria [25]. Cytochrome *c*-depleted mitochondria were prepared according to the method of Jacobs and Sanadi [26].

Proteins were determined by a biuret method [27] and oxygen with a Clark type oxygen electrode [28]. Malondialdehyde was assayed by the thiobarbituric acid method as described by Buege and Aust [29] (this assay is unaffected by the presence of cumene hydroperoxide), lipid hydroperoxides by iodide oxidation [29] and conjugated dienes by the difference spectrum of peroxidised versus non-peroxidised extracted phospholipids at 232 nm [29]. Cumene hydroperoxide was determined with GSH [30] in the presence of glutathione peroxidase using enzyme prepared from rat liver [31].

Results

Formation of lipid hydroperoxides

When rat liver mitochondria are incubated without substrate at 30°C in aerated buffer at pH 7.4 containing cumene hydroperoxide, there is a progressive increase with time in the formation of lipid hydroperoxides. These substances are not formed if cumene hydroperoxide is omitted or nitrogen replaces oxygen in the incubation medium (Fig. 1A). As previously described, some of the added cumene hydroperoxide is consumed under these conditions; after a large first minute burst its rate of loss than roughly corresponds, on a 1:1 basis, with the rate of formation of these hydroperoxides (Fig. 1 of Ref. 30). The latter also increase progressively as the initial concentration of cumene hydroperoxide is raised (Fig. 1B). Accompanying the generation of hydroperoxides, there is a parallel increase in related parameters such as malondialdehyde (as already reported by Sies and Sommer [19]) and conjugated dienes (Fig. 1C). The reaction is independent of pH in the range 6.5–8.5 and boiled mitochondria do not exhibit any peroxidation in the presence of cumene hydroperoxide (data not shown).

The effect of some inhibitors at concentrations customarily used for in vitro studies (see Introduction) on the rate of formation of lipid hydroperoxides has been investigated (Table I). The most striking inhibition is obtained with the inhibitor of cytochrome *P*-450-dependent reactions, SKF 525A [32]. The antioxidants, tocopherol and butylated hydroxyanisole, are also good inhibitors but superoxide dismutase and catalase are less so. Peroxidation reactions are usually enhanced by metal ions [2,4,35,38] and EDTA in large amounts has frequently been used to quench them [8,34,36]. However, in cumene hydroperoxide-supported lipid peroxidation, EDTA has no appreciable effect (data not shown) except that it improves the reproducibility of the assays. Among electron-transport inhibitors (Table II), antimycin has some inhibi-

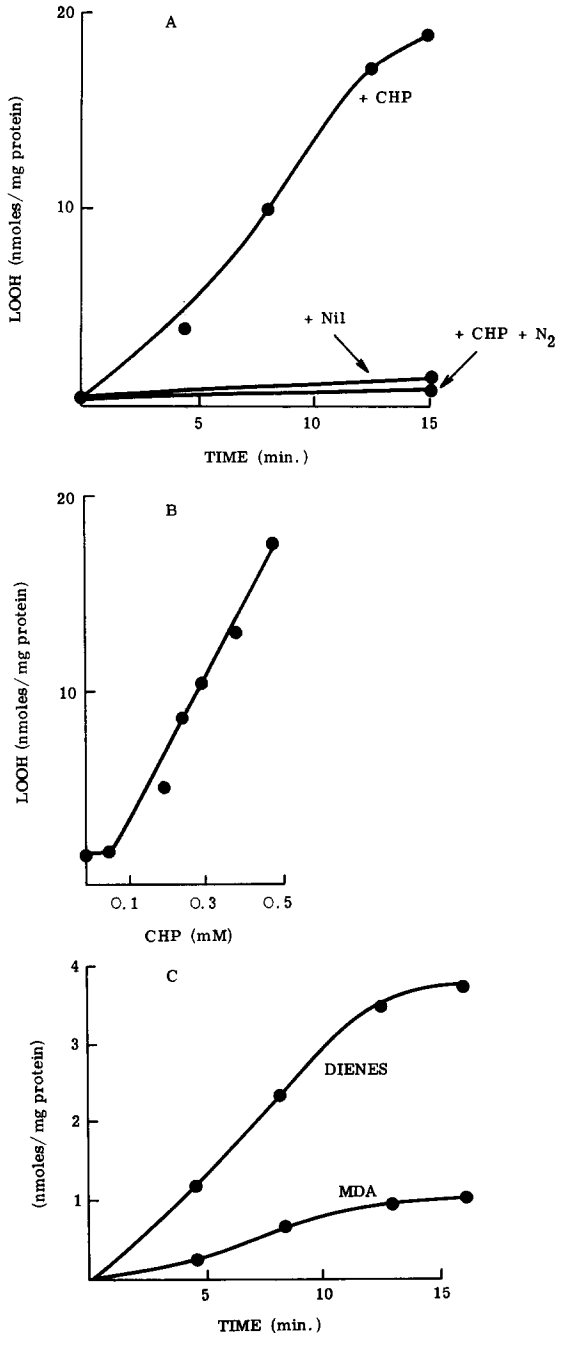

Fig. 1. The dependence of the formation of lipid hydroperoxides (LOOH) in mitochondria on (A) the presence of cumene hydroperoxide (CHP) and oxygen, (B) the initial concentration of cumene hydroperoxide (determinations were made after 15 min of incubation), (C) the associated formation of malondialdehyde (MDA) and conjugated dienes. A suspension of rat liver mitochondria, 4 mg protein/ml of the incubation medium (see Materials and Methods), was shaken with addition of cumene hydroperoxide (0.5 mM or as indicated) and samples (1 ml) taken at intervals for assay of lipid hydroperoxides or (as in C) conjugated dienes and malondialdehyde. Oxygen depletion was performed in A by bubbling the medium with N_2 before cumene hydroperoxide addition and performing the incubation in closed vessel filled with N_2.

tory effect. On the other hand, rotenone and malonate show a considerable stimulation and the thiol reagent, *N*-ethylmaleimide, especially after a short preincubation before adding the cumene hydroperoxide, an even bigger one. Cyanide, an inhibitor of both cytochrome oxidase and cytochrome *P*-450, has a significant but small inhibitory effect at higher concentrations but the uncoupler FCCP is inert.

Since cumene hydroperoxide is a known substrate for cytochrome *P*-450 [33,34], mitochondria were freed from any microsomal contamination by gradient centrifugation. After such treatment, however, they retain part of their capacity for peroxidation (Table III, column 2) consistent with the reported presence of this cytochrome in rat liver mitochondria [35–38]. As also shown, mitoplasts and cytochrome *c*-depleted mitochondria also sustain peroxidation to much the same extent as the mitochondria from which they are derived; however, the rate of lipid peroxidation is considerably increased in submitochondrial particles though it is still much less than the rate obtained with microsomes. The experiments were performed in the presence of cyanide to allow the oxygen consumption due only to the peroxidation of the particles by cumene hydroperoxide to be measured (Table III, column 3). The amount of oxygen consumed per mole of malondialdehyde produced is higher in mitochondria than in microsomes but it is lowered in the former either by gradient centrifugation or by subfractionation. Indeed, submitochondrial particles despite their high peroxidation rate nevertheless consume proportionately the least amount of oxygen.

Protection against lipid peroxidation

Succinate, although ineffective in preventing peroxidation by cumene hydroperoxide in microsomes, when added to mitochondrial suspensions significantly inhibits the reaction as measured by

TABLE I

EFFECT OF SOME PEROXIDATION INHIBITORS ON THE CUMENE HYDROPEROXIDE-INDUCED FORMATION OF LIPID HYDROPEROXIDES

A suspension of rat liver mitochondria (4 mg protein) in 1 ml of incubation medium containing cumene hydroperoxide (0.5 mM) and the amount of addition specified was incubated at 30°C for 10 min. The sedimented pellet was washed by resuspension in 1 ml of the medium and lipid hydroperoxides (or malondialdehyde) determined on the resedimented pellet as described in Materials and Methods. Values are given as a percentage of the control (value found without the addition). Control values were 12.6 ± 1.6 (8) nmol/mg protein. Mean ± S.D. is given and brackets enclose number of estimations each with a different batch of mitochondria. Student *t*-test values [53] are calculated relative to the control.

Substance added	Amount	Hydroperoxides (% control)	*P*
Superoxide dismutase	10 μg (30 U)	61 ± 1.5 (3)	<0.01
Catalase	10 μg (110 U)	77 ± 5.5 (4)	<0.01
Butylated hydroxyanisole	20 nmol	27 ± 2.0 (3)	<0.01
α-Tocopherol	2 μmol	48 ± 15.0 (3)	<0.01
SKF525A	1 μmol	30 ± 11.0 (3)	<0.01

assay by either hydroperoxides (Fig. 2), malondialdehyde (Table III, column 4) or conjugated dienes (data not shown). This protection is also found in mitoplasts, submitochondrial particles and cytochrome *c*-depleted mitochondria. In general, the oxygen consumption accompanying peroxidation by cumene hydroperoxide follows the same pattern. Thus, it is unaffected by succinate in microsomes but decreased in all the other fractions. Unexpectedly, the consumption in untreated mitochondria is unaffected by succinate but this may be due to cyanide-insensitive respiration and to the presence of some contaminating microsomes (Table III, column 5). Succinate is known to be able to reduce cumene hydroperoxide and other organic hydroperoxides in mitochondria [30] and such an effect would inhibit the formation of lipid hydroperoxides. However, succinate does not act primarily in this way but gives direct protection against lipid peroxidation. This is established by adding an uncoupler which has been previously shown to prevent the reductive loss of cumene hydroperoxide [30]. Under these conditions, i.e., when both cyanide and uncoupler are added, cumene hydroperoxide levels remain high but the protection by succinate remains unimpaired (Fig. 2). In addition to uncouplers, the reduction of cumene hydroperoxide is also inhibited by *N*-ethylmaleimide [29] and this substance likewise does not greatly affect the protection by succinate

TABLE II

EFFECT OF SOME INHIBITORS OF MITOCHONDRIAL FUNCTIONS ON THE CUMENE HYDROPEROXIDE-INDUCED FORMATION OF LIPID HYDROPEROXIDES

Experimental conditions and units as in the legend to Table I. n.s., not significant.

Substance added	Amount	Hydroperoxides (% control)	*P*
Rotenone	4 μg	137 ± 15 (3)	<0.01
Antimycin	4 μg	68 ± 26 (5)	<0.05
Cyanide	1 μmol	86 ± 9 (4)	n.s.
Cyanide	2 μmol	71 ± 8 (3)	<0.01
FCCP	0.5 nmol	103 ± 17 (3)	n.s.
Malonate	15 μmol	121 ± 15 (6)	<0.05
N-Ethylmaleimide [a]	200 μmol	208 ± 34 (3)	<0.01

[a] Preincubation for 2 min before adding cumene hydroperoxide.

TABLE III

COMPARISON OF THE EFFECT OF CUMENE HYDROPEROXIDE ON MALONDIALDEHYDE FORMATION AND ASSOCIATED OXYGEN UPTAKE IN VARIOUS SUBCELLULAR FRACTIONS FROM RAT LIVER MITOCHONDRIA WITH AND WITHOUT ADDED SUCCINATE (5 mM)

Fractions containing 4 mg protein (except microsomes, 1 mg) in 1 ml medium were incubated with cumene hydroperoxide (0.5 mM) at 30°C. Oxygen consumed and malondialdehyde (MDA) formed were measured after 10 min. Values are expressed as nmol/mg protein. For methods of preparation of fractions see Materials and Methods.

Fraction	Extent of peroxidation and oxygen consumption			
	No succinate		With succinate	
	MDA	O_2	MDA	O_2
Mitochondria [a]	1.45	57.25	1.00	57.21
Gradient-separated mitochondria [a]	0.83	14.00	0.36	9.00
Mitoplasts	1.63	37.50	0.72	15.38
Cytochrome *c*-depleted mitochondria	1.30	35.00	0.32	31.00
Submitochondrial particles	3.73	33.00	0.90	3.96
Microsomes	12.00	27.90	12.36	27.30

[a] Depleted of substrate by prior incubation for 12 min at 20°C with 1 μM FCCP.

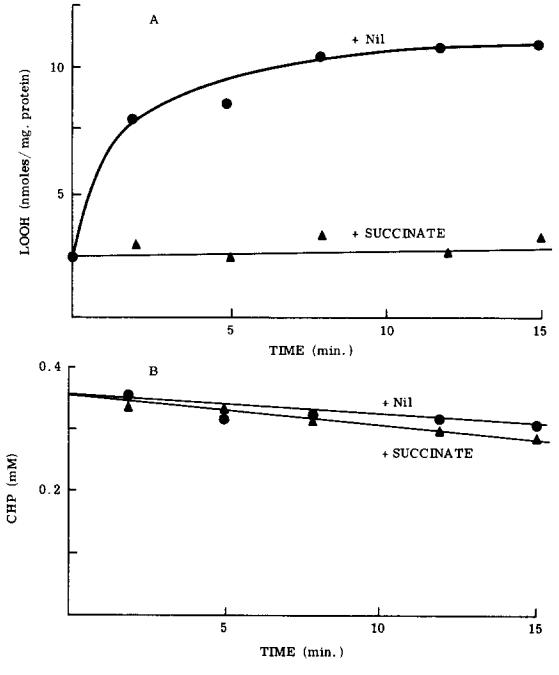

Fig. 2. Effect of succinate on (A) the production of lipid hydroperoxides (LOOH) induced by cumene hydroperoxide (CHP) and (B) the associated concentration of cumene hydroperoxide during the reaction. Rat liver mitochondria (4 mg protein/ml) were incubated at 30°C in the incubation medium containing cumene hydroperoxide (0.35 mM) with (▲) or without (●) the addition of succinate (5 mM) and in the presence of NaCN (1 mM) and FCCP (0.4 μM). 1 ml samples were taken at intervals for the assay of lipid hydroperoxides and remaining cumene hydroperoxide.

if both are added together with cumene hydroperoxide (Table IV). The succinate protection is not prevented by rotenone or antimycin but it is significantly inhibited in the presence of 15 mM malonate (Table IV). The protection is not unique to succinate alone but is shown to much the same extent with some other oxidisable substrates including 3-hydroxybutyrate, isocitrate, malate, glutamate, 2-oxoglutarate and pyruvate (data not shown).

Removal of preformed hydroperoxides

The stability of lipid hydroperoxides is conveniently studied by first generating them with cumene hydroperoxide as described (see Materials and Methods) then sedimenting them and washing them free of the oxidant. They can then be resuspended in the same buffer and reincubated (Table V). Without any other additions there is found to be a slight further increase (about 6%) in the concentration of these lipid hydroperoxides which is not diminished by inclusion of respiratory inhibitors in the resuspension medium (Table V, column 3).

However, when succinate is added after resuspension, there is a striking loss in the amount of lipid hydroperoxides such that following reincubation for 10 min almost half of those previously

TABLE IV

ACTION OF SOME INHIBITORS ON THE PROTECTION OF MITOCHONDRIA FROM PEROXIDATION IN THE PRESENCE OF SUCCINATE

Mitochondria (4 mg protein) incubated with 1 ml medium containing cumene hydroperoxide (0.5 mM), succinate (5 mM), cyanide (2 mM), FCCP (0.4 μM) and the addition noted at 30°C for 10 min. Peroxidation was measured by assay of lipid hydroperoxides. The control did not contain succinate or inhibitor. See legend to Table I, n.s., not significant.

Addition	Amount	Peroxidation (% control)	*P*
None	–	35 ± 12 (6)	
Rotenone	4 μg	38 ± 23 (7)	n.s.
Antimycin	4 μg	35 ± 11 (5)	n.s.
Malonate	15 μmol	68 ± 14 (3)	<0.01
N-Ethylmaleimide	0.2 μmol	54 ± 12 (2)	n.s.

present are lost. The removal of preformed hydroperoxides by succinate still occurs when cyanide or rotenone are also added to the reincubation stage. *N*-Ethylmaleimide and antimycin give the same effect to a less marked extent but malonate completely neutralises the hydroperoxide-depleting action of succinate (Table V, column 4).

Discussion

The peroxidation of subcellular particles by cumene hydroperoxide has previously been reported [19,39]. In this study we were concerned in investigating more fully the reaction with mitochondria. There are several unusual features. EDTA (1 mM) does not affect the rate of peroxidation whereas when this is induced by chaotropic agents [8], cysteine [39], ADP plus Fe^{2+} [40] or ascorbate [41] it is inhibitory. The reaction is also not accompanied by swelling (not shown), a normal concomitant of peroxidation by e.g., ascorbate [42], Fe^{2+} plus ADP [4,43] and GSH [44].

Superoxide dismutase and catalase are not very effective inhibitors whereas antioxidants such as vitamin E and butylated hydroxyanisole are. These facts suggest that oxygen activation by cumene hydroperoxide does not primarily entail the production of $HO_2^{\cdot}$ or H_2O_2 as also implied by a recently proposed mechanism [45]. Cytochrome *P*-450, which can act as a peroxidase towards cumene hydroperoxide and other hydroperoxides [46], may be involved in the mitochondrial lipid peroxidation perhaps by acting in a Fenton-type reaction that produces primarily alkoxy radicals

TABLE V

CHANGES IN THE CUMENE HYDROPEROXIDE-INDUCED LIPID HYDROPEROXIDE CONCENTRATION OF MITOCHONDRIA AFTER SUBSEQUENT INCUBATION WITH OR WITHOUT SUCCINATE AND IN THE PRESENCE OF VARIOUS ADDITIONS

Mitochondria were first incubated in medium (1 ml) containing only cumene hydroperoxide (0.5 mM) for 7 min at 30°C then sedimented, washed with 1 ml medium, resuspended in 1 ml and reincubated for 10 min in medium without cumene hydroperoxide but with or without succinate (5 mM) and one of the additions shown. They were then resedimented and used for the assay of lipid hydroperoxides remaining (see Materials and Methods). Values are expressed as percentage of the lipid hydroperoxides present in controls (i.e., reincubated without any addition) at the end of the reincubation (10.10 ± 1.4 (4) nmol/mg protein). The amounts present immediately after the preincubation with cumene hydroperoxide but before the reincubation were about 6% lower. Student's *t*-test values were obtained by the orthogonality test [53] between the couples 'no succinate' versus 'succinate'. n.s., not significant.

Addition during reincubation	Amount	Lipid hydroperoxide (% control) [b]		*P*
		No succinate	With succinate	
None	–	100.0 ± 13 (4)	56.6 ± 18 (12)	<0.01
Rotenone	4 μg	113.4 ± 34 (5)	57.4 ± 22 (5)	<0.01
Antimycin	4 μg	98.1 ± 27 (6)	81.1 ± 27 (6)	n.s.
Cyanide	1 μmol	95.0 ± 17 (4)	57.2 ± 8 (4)	<0.05
Malonate	15 μmol	117.6 ± 18 (5)	106.2 ± 48 (5)	n.s.
N-Ethylmaleimide	200 μmol	106.2 ± 4 (4)	75.0 ± 12 (4)	<0.05

[47,48]. This is suggested by the prevention of peroxidation by SKF525A, a known inhibitor of reactions utilising this cytochrome [49]. This substance has other effects on mitochondrial functions as well [42] but cytochrome *P*-450 is known to be a component of rat liver mitochondria [35–38] and has been reported to be localised in the inner mitochondrial membrane [36], consistent with the peroxidation found in mitoplasts and submitochondrial particles. This consideration is especially important for interpreting the protective effect of succinate and other oxidisable substrates.

Succinate has previously been found to have no effect if peroxidation is induced with ascorbate [42] but to be protective if induced by chaotropic agents [8] or Fe^{2+}/ADP [2,52]. In the last two cases, however, succinate could have acted indirectly by using up the oxygen necessary for peroxidation. By preventing this loss with cyanide and also the previously documented succinate-induced loss of cumene hydroperoxide [30], we have been able to observe that succinate has a direct inhibitory effect independent of these parameters.

This effect of succinate (and other oxidisable substrates) may explain why mitochondria (which always contain some endogenous intermediates) are less prone to peroxidation than other particles [9,19] and why the extent is increased in the presence of rotenone.

There are at least two ways by means of which succinate affects peroxidation by cumene hydroperoxide. It prevents it from occurring and it also removed some of the lipid hydroperoxides once they have been formed. The first of these inhibitions is also found with submitochondrial particles, hence soluble mitochondrial components are not essentially involved. The protection is inhibited by malonate but not by antimycin, suggesting that endogenous ubiquinol or its semiquinone radical [51] may be the actual protective agent. Externally added ubiquinol has been shown to inhibit peroxidation [50] and the high mitochondrial concentration of this substance supports the proposed explanation. Ubiquinol formation has also recently been proposed as the mechanism for an inhibition of peroxidation by succinate in beef heart submitochondrial particles [52]. The second inhibition mechanism is shown by using mitochondria already peroxidised with cumene hydroperoxide and then reincubated without the oxidant. There is a small continued rise in their lipid hydroperoxide content if no substrate is present (presumably due to autocatalysis) but when succinate is added, not only is this increase prevented but there is also a fall of about a third in their concentration before the reincubation. This action of succinate shows one difference from its effect in preventing the initial lipid hydroperoxide formation, i.e., it is partially inhibited by antimycin as well as malonate, suggesting that the succinate-induced fall which presumably entails a transfer of reducing equivalents to the hydroperoxides themselves may involve the region of the electron-transport chain subsequent to the antimycin block. However, participation of the Q cycle is also possible as shown by recent studies of antimycin on the reduction of the *b* cytochromes [54]. The action of succinate in both inhibiting lipid peroxidation and removing preformed lipid hydroperoxides is unaffected by *N*-ethylmaleimide and hence mitochondrial GSH is probably not involved in either protective pathway. It remains possible, nevertheless, that GSH or other thiol groups could constitute an alternative protective mechanism because this would account for the large increase in hydroperoxide formation found after preincubation with *N*-ethylmaleimide in the absence of succinate.

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