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Damage of *Escherichia coli* cells by *t*-butylhydroperoxide involves the respiratory chain but is independent of the presence of oxygen

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The action of *t*-butylhydroperoxide (tBOOH) on *Escherichia coli* cells has been studied as a model system for organic peroxide toxicity. Exposure of *E. coli* cells to tBOOH led to progressive and irreversible impairment of the respiratory function, an effect which was dependent on the availability of substrate. The effect of tBOOH on growth of *E. coli* with different carbon sources and alternative terminal electron acceptors was investigated. It was found that the sensitivity of *E. coli* to tBOOH under diverse growth conditions implicating a functional respiratory chain was greater than when the bacterium grew by fermentation. Also the mutant *E. coli* SASX76, which requires exogenous 5-aminolevulinic acid to synthesize the cytochromes, was more resistant to tBOOH when lacking a functional respiratory chain. These data point to the respiratory chain as a major target in the in vivo action of tBOOH. Experiments with isolated membranes also showed a tBOOH-induced damage of the respiratory chain monitored by impairment of the NADH oxidase. The effect of tBOOH was produced even under anaerobiosis, indicating that development of cell damage was independent of oxygen and, therefore, that neither oxygen-derived radicals nor lipid peroxidation were involved.

Introduction

The damage inflicted on mammalian cells by radical-producing treatments is a subject of great interest in contemporary biological and biomedical research, because free radicals have been implicated in the development of different pathological conditions or aging [1–5]. Peroxides frequently play an important direct or intermediate role in oxidative injury of cells by radicals. Despite many studies, the exact mechanism involved in peroxide toxicity is still unknown and may be dissimilar in different cell types.

The organic hydroperoxide tBOOH has been extensively used in studies on hydroperoxide metabolism and oxidative damage in a variety of model systems [6–19]. A number of alterations have been shown to occur upon addition of tBOOH to mammalian cells and isolated membrane systems. Some authors suggested that tBOOH-induced perturbations are dependent on glutathione or pyridine nucleotide oxidation resulting from hydroperoxide metabolism through the glutathione peroxidase-glutathione reductase system [9–11]. However, a significant alteration in the oxidation state of glutathione or pyridine nucleotides is not necessarily a direct cause of irreversible damage to cell function by tBOOH [20]. Others have attributed the injury to free radicals arising from the breakdown of the hydroperoxide by interaction with cellular haemoproteins [8,12,13,21]. It was also proposed that radicals are generated through a metal-mediated reaction between an endogenous reductant and the hydroperoxide [22]. The question of which radicals are involved, what are their structural targets and which processes finally cause the death of cells subjected to this injury need to be further investigated. Another question that needs to be elucidated is whether the toxicity of tBOOH is mediated by oxygen-derived reactive species which have been implicated in other oxidant stresses [23–26].

In previously studied mammalian systems, treatment with tBOOH induced peroxidation of membrane unsaturated lipids and several other alterations, including the formation of membrane leaks, SH-oxidation, aggregation of membrane proteins and inactivation of membrane-bound enzymes [14–19]. However, the relationship between lipid peroxidation and the other effects of

Abbreviation: tBOOH, *tert*-butylhydroperoxide.

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tBOOH could not be clearly determined, and the relative importance of lipid peroxidation in tBOOH toxicity is still controversial.

We initiated the study of the action of tBOOH on the bacterium *E. coli* because this is a simple cell system with the potentiality of genetic manipulations which could help to elucidate basic questions on hydroperoxide toxicity. This report describes some new aspects of tBOOH-induced injury, demonstrating that irreversible damage to cell function occurs even in the absence of oxygen and therefore without the implication of oxygen-derived radicals or lipid peroxidation. The data show that tBOOH toxicity in *E. coli* is enhanced under growth conditions which depend on the function of a membrane-bound electron transport chain. In vivo and in vitro studies indicate that the respiratory chain is involved in the action of the hydroperoxide.

Materials and Methods

Bacterial strains and growth conditions. E. coli ML 308225 ($i^-z^-y^+a^+$), obtained from Dr. H.R. Kaback, was used in all the experiments except in that of Fig. 3. This bacterium was grown aerobically at 37 °C in minimal salt medium M9 [27] omitting the $CaCl_2$ and containing 0.5% glycerol as carbon and energy source, except in the experiment of Fig. 2 for which growth conditions are given in the legend. *E. coli* SASX76 (F^-, *hemA*$^-$, *met*$^-$, *trp*$^-$, *lac*$^-$, *str*^{R}) [28] was grown in the complex medium LB (10 g tryptone, 5 g yeast extract and 5 g NaCl per litre) supplemented with either 1% glucose or 12.5 μg/ml of 5-aminolevulinic acid.

Anaerobic growth. Anaerobic conditions in the experiment of Fig. 2 were achieved by adaptation of the technique used for growth of methanogens [29]. The cultures (5 ml) were prepared in 18 × 65 mm glass tubes with serum vial lip to accommodate a thick rubber stopper which was crimped into place with a one-piece aluminum seal. A gassing manifold with a reduced copper column heated at 300 °C (oxygen scrubber) was used for supply of oxygen-free N_2. Salt medium was brought to the boil, cooled in ice under an atmosphere of N_2, and dispensed into the culture tubes which were then bubbled with N_2, sealed and autoclaved. Concentrated solutions of glucose and glycerol were identically processed in separate bottles, from which adequate aliquots were added to culture tubes containing the salt medium. Syringes were used for all transfers. tBOOH previously bubbled with N_2 was added to the anaerobic media just before inoculation with adequated aliquots of anaerobic cultures grown in the same media for 24 h. Cultures were incubated at 37 °C and their optical absorbance was followed by periodic readings at 560 nm. Anaerobiosis was checked by processing in parallel samples of media containing 1 μg/ml of the indicator resazurine, which could not be added directly to the cultures because it interfered with cell growth in medium M9-glycerol-nitrate and also reacted with glucose-containing media, giving coloured products.

Cell suspensions. Cells of *E. coli* ML 308225 at the exponential phase of growth were harvested by centrifugation (6000 × *g*, 10 min) at room temperature, washed three times and suspended in 50 mM sodium phosphate (pH 7.5) to give an optical absorbance of about 0.2 and 560 nm.

Cell viability. The viability of *E. coli* ML 308225 was determined by diluting the cells in the suspension media and plating samples on M9 plus 0.5% glycerol agar. The plates were incubated at 37 °C for 27–48 h before colonies were counted. The viability of *E. coli* SASX76 at different tBOOH concentrations was determined as indicated in the legend of Fig. 3.

Oxygen uptake. The oxygen consumption of *E. coli* cells was measured at 37 °C in a Gilson oxygraph with a Clark electrode.

Membrane preparation. Membranes from *E. coli* ML 308225 cells harvested at the exponential phase of growth were prepared by the lysozyme-EDTA treatment and osmotic lysis as described by Evans [30]. The membranes were suspended in 20 mM Tris-HCl (pH 7.4) containing 1 mM $MgCl_2$ (3–5 mg of protein per ml) and fractionated in small portions which were stored frozen at −60 °C until used.

NADH oxidase. The NADH oxidase activity of *E. coli* membranes was assayed at 37 °C recording the absorbance changes at 340 nm in a Gilford spectrophotometer equipped with a thermoset temperature controller. The reaction was started by addition of 20 μl of NADH (3.5–6.5 mM) to 0.5 ml of membrane suspensions (7–20 μg of protein) in 50 mM sodium phosphate (pH 7.5). Only freshly thawed membranes were employed, since repeated freezing-thawing or storage at 4 °C led to NADH oxidase inactivation.

Results

Data from individual experiments are shown in each figure and table, and are representatives of at least three independent experiments. Fig. 1 shows the effect of different concentrations of tBOOH on the aerobic growth of *E. coli*. With 0.25 mM tBOOH, growth stopped for several hours but finally the culture reached the same turbidity as the control. Complete growth inhibition was obtained with tBOOH concentrations of 0.5 mM or higher. Addition of tBOOH to cultures in the exponential phase of growth led to a progressive decrease in the rate of oxygen uptake (not shown). We studied this effect in more detail with cells suspended in buffer in order to simplify the system for analysing the influence of the incubation conditions on tBOOH toxicity. As shown in Table I, when *E. coli* cells suspended in phosphate buffer with glycerol as respirable substrate

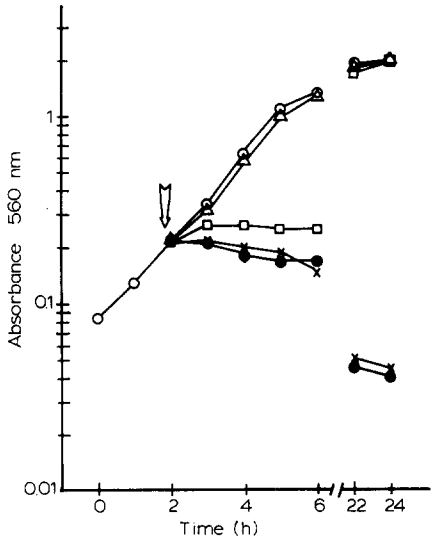

Fig. 1. Growth inhibition by tBOOH. A culture at the exponential phase of growth was fractionated into five aliquots to which 0 (○); 0.1 (△); 0.25 (□); 0.5 (×); and 1 (●) mM tBOOH was added at the point indicated by the arrow. Growth was followed spectrophotometrically by measuring cell turbidity at 560 nm.

were exposed to tBOOH, a time-dependent decrease in oxygen consumption was induced. When after 30 min of tBOOH addition the cells were washed thoroughly and then suspended in fresh medium without added hydroperoxide, the respiration rate remained decreased (not shown). Thus, exposure to tBOOH resulted in irreversible impairment of the respiratory function.

To investigate whether the effect of tBOOH was dependent on metabolic conditions, we performed the experiment of Table II, with cells previously starved in phosphate buffer at 37 °C for 2 h in order to consume endogenous substrates. As can be seen in Table II, pre-incubation of starved cells with tBOOH in the absence of added substrates had no effect on the oxygen consumption of *E. coli* clels, measured with either glycerol or succinate as respirable substrate. On the contrary, pre-inducation of starved cells with tBOOH in the presence of glycerol or succinate led to a subsequent

TABLE I

Time-dependent effect of tBOOH on the oxygen consumption of E. coli

A cell suspension was divided in two fractions to which glycerol (final concentration 0.4%) was added. tBOOH at a final concentration of 1 mM was added to one of the fractions. Both samples were incubated in a shaker at 37 °C and aliquots of 2 ml were taken at 0, 15, and 30 min after the addition of tBOOH to determine their oxygen uptake, which is expressed as a percentage of the control without tBOOH.

Time (min)	Oxygen consumption (%)
0	100
15	76
30	20

TABLE II

Substrate-dependent effect of tBOOH on oxygen consumption and cell viability

A cell suspension was starved for 2 h in a shaker at 37 °C, and then divided in six fractions which were pre-incubated 30 min at 37 °C in the absence and the presence of 2 mM tBOOH under the following conditions: without added substrate, with 0.4% glycerol or with 10 mM succinate. At the end of the pre-incubation the cells were washed twice (by centrifugation at room temperature) and resuspended in phosphate buffer. Aliquots of each suspension were taken to measure oxygen consumption with glycerol or succinate as exogenous substrate and to determine cell viability. Data are expressed as a percentage of the controls without tBOOH.

Pretreatment	Oxygen consumption (%)		Cell viability (%)
	with glycerol	with succinate	
tBOOH alone	100	100	100
tBOOH plus glycerol	18	28	8
tBOOH plus succinate	25	38	15

decrease in the oxygen uptake measured with either of the two substrates. The viability of starved *E. coli* cells was not affected by pre-incubation with tBOOH in the absence of added substrates, showing that no vital cell function was damaged by the hydroperoxide under these conditions. However, pre-incubation with tBOOH in the presence of glycerol or succinate resulted in a decrease of 92% or 85% in cell viability, respectively. The data of Table II indicated that the toxicity of tBOOH was dependent on the availability of substrate.

In order to evaluate further the involvement of the respiratory chain in the toxicity of tBOOH in vivo, we investigated the effect of the hydroperoxide on *E. coli* cells growing by fermentation or by respiration. *E. coli* is a facultative anaerobe which adapts its metabolism to the conditions of the media. It can grow anaerobically either using a fermentable substrate or using an oxidizable substrate in the presence of an adequate final electron acceptor such as nitrate. It can also grow aerobically oxidizing a substrate with oxygen as the final electron acceptor. Therefore, *E. coli* develops an aerobic respiratory chain which uses oxygen as final electron acceptor or an anaerobic respiratory chain in the presence of an alternative final electron acceptor such as nitrate [33–36]. In the absence of respiratory chain oxidants, *E. coli* can grow by fermentation. Glycerol is a non-fermentable substrate which supports the growth of *E. coli* as the sole carbon and energy source in the presence of oxygen, nitrate or fumarate as the terminal electron acceptor. On the other hand, glucose is a fermentable substrate which supports the growth of *E. coli* in the absence of a final electron acceptor, but it can also be oxidized with participation of a respiratory chain in the presence of oxygen or nitrate. As shown in Fig. 2, 0.5 mM tBOOH prevented

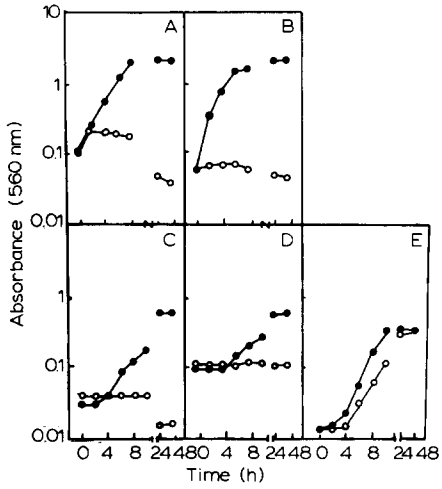

Fig. 2. Growth of *E. coli* in the absence (●) or in the presence (○) of 0.5 mM tBOOH under diverse conditions. *E. coli* ML 308225 was grown aerobically (A, B, upper panels) or anaerobically (C, D, E, lower panels) in minimal salt medium M9 (omitting the $CaCl_2$) with either 0.5% glycerol (A and C) or 1% glucose (B, D and E) as the sole carbon and energy source. In C and D the medium was supplemented with 100 mM sodium nitrate as final electron acceptor and 1 μM ammonium molybdate required for nitrate reductase.

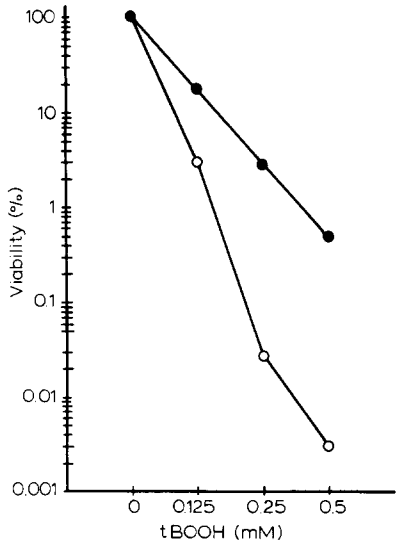

Fig. 3. Sensitivity of *E. coli* SASX76 to tBOOH in medium LB-glucose (●) or LB-levulinic acid (○). The bacterium was grown overnight aerobically at 37° C in LB supplemented with either 1% glucose or 12.5 μg/ml of 5-aminolevulinic acid. Each one of these inoculates was serially diluted in LB and samples were spread on agar plates of the same medium in which the inoculate had been grown, containing the tBOOH concentrations indicated in the abscissa. The plates were incubated at 37° C for 48–72 h before colonies were counted.

the growth of *E. coli* under the four conditions in which a respiratory chain was functioning (panels A, B, C and D), independently of whether the substrate was glycerol or glucose and whether the final electron acceptor was oxygen or nitrate. On the contrary, *E. coli* was able to grow in the presence of 0.5 mM tBOOH when fermenting glucose (panel E). These results indicate that the toxicity of tBOOH in *E. coli* is greater under conditions of respiratory metabolism, and therefore the respiratory chain seems to be a major target in the action of the hydroperoxide in vivo. The data of Fig. 2 also demonstrate the lack of involvement of molecular oxygen or oxygen-derived radicals in the damage induced by tBOOH, since the growth of *E. coli* was inhibited by the hydroperoxide even under anaerobiosis with nitrate as final electron acceptor (panels C and D).

E. coli SASX76 is a mutant affected in the *hemA* gene and deficient in the biosynthesis of 5-aminolevulinic acid, an intermediate in heme synthesis. This mutant has been used in analysis of the composition and function of the aerobic electron transport chain [35]. In media supplemented with 5-aminolevulinic acid, *E. coli* SASX76 synthesizes cytochromes and can grow by respiration, but in the absence of exogenous 5-aminolevulinic acid this bacterium lacks a functional respiratory chain. This mutant is unable to grow in LB medium unless it is supplemented with either 5-aminolevulinic acid or glucose. As shown in Fig. 3, the sensitivity of *E. coli* SASX76 to tBOOH is about 100-fold higher in LB + levulinic acid than in LB + glucose, indicating that the toxicity of tBOOH is enhanced in cells with a functional respiratory chain and suggesting that cytochromes might be involved in the action of the hydroperoxide.

In *E. coli,* the components of the electron transport chain are bound to the cytoplasmic membrane. To test whether the hydroperoxide was able to interact directly with the respiratory chain, we studied the effect of tBOOH on the NADH oxidase activity of isolated

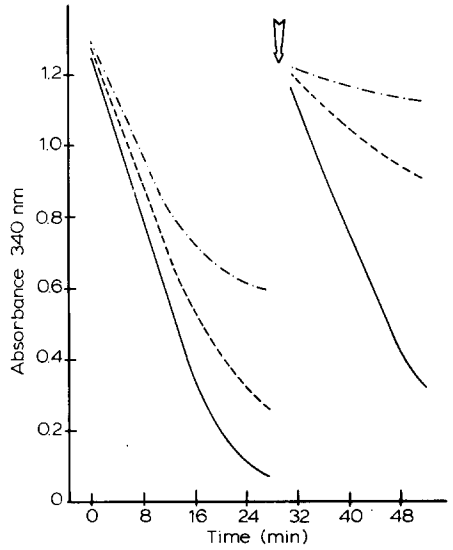

Fig. 4. Time-dependent inhibition of NADH oxidase by tBOOH. NADH oxidase was assayed in the presence of the following concentrations of tBOOH: 0 (———); 1 (– –); or 2 (– · – · –) mM. At the point indicated by the arrow, more NADH was added to each sample to give the same final concentration in all of them (monitored by the absorbance at 340 nm).

membranes. NADH oxidase of *E. coli* membranes involves all the components of the electron transport chain between the NADH dehydrogenase and oxygen as final electron acceptor [31]. As can be seen in Fig. 4, tBOOH induced a time-dependent decrease in the NADH oxidase activity of the membranes. This effect was dependent on the presence of NADH (Fig. 5), since preincubation of the membranes with tBOOH alone had no significant effect on the NADH oxidase, whereas preincubation in the presence of both tBOOH plus NADH led to decreased NADH oxidase activity. The results with isolated membranes shown in Figs. 4 and 5 are consistent with the impairment of the respiratory function induced by tBOOH in the in vivo experiments shown in Tables I and II, and suggest that the effect of tBOOH is dependent on the availability of reduced equivalents. Considering the accumulating evidence that peroxide toxicity may be mediated by transition metals [22,38,39], we examined this possibility in the experiment shown in Fig. 6. As can be seen, the effect of tBOOH on the NADH oxidase of isolated membranes was blocked by 0.1 mM of the chelator EDTA, indicating that this effect is mediated by a metal ion. The tBOOH-induced impairment of the NADH oxidase was also prevented in the presence of 1 mM of the radical scavenger, thiourea (not shown). It is improbable that this effect of thiourea can be explained by its known activity as a hydroxyl radical scavenger, since 50 mM mannitol, another OH· scavenger, had no effect at all (not shown). More likely, thiourea interacts with another

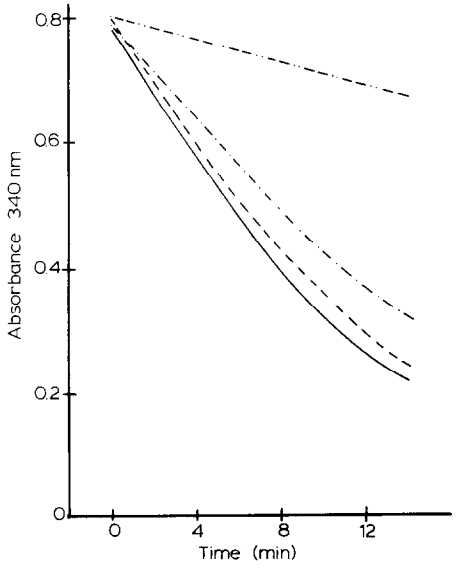

Fig. 5. The inhibition of NADH oxidase by tBOOH is dependent on the presence of NADH. Membranes were pre-incubated 15 min at 37 °C in phosphate buffer without additions (———); with 2 mM tBOOH (–·–·–); with 0.12 mM NADH (– – –) or with 2 mM tBOOH plus 0.12 mM NADH (– – ··). Then, the membranes were washed once and resuspended in phosphate buffer before assaying their NADH oxidase activity.

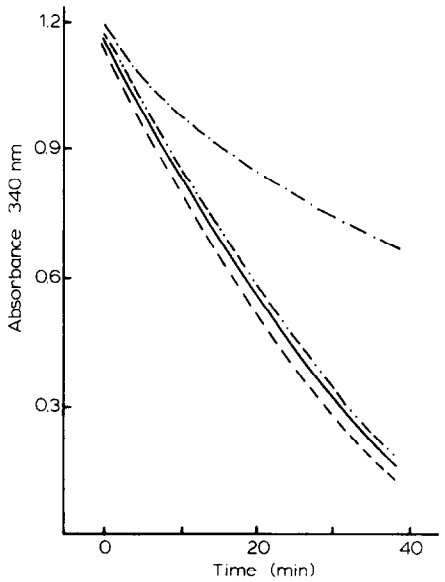

Fig. 6. The effect of tBOOH on the NADH oxidase is blocked by the chelator EDTA. NADH oxidase was assayed without additions (———) or in the presence of 0.1 mM EDTA (– –), 2 mM tBOOH (–·–·–), or 0.1 mM EDTA plus 2 mM tBOOH (– – ··).

tBOOH-derived radical, presumably the *t*-butoxy radical as proposed by others [15], since thiourea is readily oxidized by alkoxy radicals [32]. The effect of tBOOH on the NADH oxidase of isolated membranes was not influenced by the presence of 200 μg/ml of superoxide dismutase (not shown), indicating that superoxide radicals are not involved in the phenomenon.

Discussion

Exposure of *E. coli* cells to tBOOH led to impairment of the respiratory function (Table I), an effect which was dependent on the availability of substrate (Table II). Starvation protected *E. coli* not only against the tBOOH-induced respiratory injury but also against viability loss, as shown in Table II. It was recently reported [37] that starvation induces cross-protection against heat or H_2O_2 challenge in *E. coli*. Thus, the enhanced resistence of starved cells to tBOOH may result from the molecular changes that *E. coli* cells undergo during starvation, which appear to make them more hardy with respect to several different stresses. On the other hand, the greater sensitivity of metabolizing cells to tBOOH might be due to the requirement of available reducing equivalents to convert the hydroperoxide into a toxic oxidant. It was reported [38] that DNA damage by hydrogen peroxide in *E. coli* depends on the availability of both reducing equivalents and an iron species, which together mediate a Fenton reaction in which ferrous iron reduces H_2O_2 to a reactive radical.

E. coli mutants defective in DNA repair are highly sensitive to H_2O_2 [38,39] and tBOOH [40], and therefore a major portion of the toxicity of these peroxides is

attributed to DNA damage. In this report, we show that the sensitivity of *E. coli* to tBOOH is greater under growth conditions which depend on a functional respiratory chain than when the bacterium grows by fermentation (Figs. 2 and 3). Therefore, our data point to the respiratory chain as a major target in the in vivo action f tBOOH. In *E. coli* cells wild-type for DNA repair, peroxide-induced lesions in the DNA can be repaired [41], and under these conditions probably other sites of injury may become more important in determining cell death at tBOOH concentrations which do not overcome the DNA repair systems.

The in vitro experiments with isolated membranes indicated that tBOOH is able to interact with membrane-bound components of the respiratory chain, leading to a time-dependent inactivation of the NADH oxidase (Fig. 4). Since this effect required the presence of the reductant NADH (Fig. 5) and was blocked by either the metal chelator EDTA (Fig. 6) or the radical scavenger thiourea (not shown, see Results), it seems to be mediated by a metal ion-catalysed redox reaction generating toxic free radicals. It has been proposed [22] that tBOOH induces radical production in mitochondria through a metal-mediated reaction between a reductant and the hydroperoxide. A transition metal ion and reduced equivalents have also been implicated in mode-one killing of *E. coli* by hydrogen peroxide [38,39]. Further investigations are needed to elucidate the mechanism of tBOOH-induced damage in *E. coli* membranes, and it also remains to be determined whether the data obtained with isolated membranes are applicable to the in vivo mechanism of action of the hydroperoxide.

Since growth inhibition by tBOOH occurred even in anaerobiosis (Fig. 2), oxygen-derived radicals were not involved in this effect. Nor could an oxygen consuming process of lipid peroxidation be implicated. In hepatic cells, tBOOH induces an initial fall in oxygen consumption followed by a recovery phase leading to respiratory rates higher than the control values [8]. The overshoot in liver respiration was attributed to increased utilization of oxygen for lipid peroxidation, induced by free radicals arising from the interaction of the hydroperoxide with cellular haemoproteins [8]. Exposure of *E. coli* cells to tBOOH resulted in inhibition of respiration with no subsequent overshoot in oxygen consumption, which is consistent with the absence of lipid peroxidation in this system. The membranes of *E. coli*, unlike those of eukaryotic cells, do not contain polyunsaturated fatty acids [42] and should therefore be less susceptible to lipid peroxidation. For this reason, this is an instructive model for analysing other important aspects of hydroperoxide toxicity without the additional complication of lipid peroxidation which occurred in mammalian systems.

Acknowledgements

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