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Oxidative Stress: Damage to Intact Cells and Organs [and Discussion]

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Oxidative stress: damage to intact cells and organs

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Oxidative cell damage can be monitored by detection of (*a*) photoemission of singlet molecular oxygen formed from radical interactions (so-called low-chemical chemiluminescence), (*b*) end products of lipid peroxidation, such as ethane, and (*c*) glutathione disulphide release. These methods, preferably used in a complementary fashion, provide insight into the pro-oxidant–antioxidant balance in the intact cell or organ.

Recent work from this laboratory on the metabolism of hydroperoxides and aldehydes as well as on redox cycling of the quinone menadione is presented. The comparison of GSSG transport systems in liver and heart reveals a limitation of capacity in the latter, thus making GSSG export potentially critical in the heart.

As part of an inter-organ feedback system between extrahepatic tissues and liver, the newly described hormone stimulation of GSH release from liver is also presented.

The exposure of cells to oxidative conditions of a diverse nature can be accompanied by an elevated production of free radicals, which in turn is expressed as an enhanced generation of electronically excited states leading to the production of low-level chemiluminescence. Among others, lipid peroxidation reactions may occur, leading to products that include volatile hydrocarbons. Changes in the cellular glutathione status are linked to the reduction of hydroperoxides which can result from free-radical intermediates.

We give here a survey of recent work on these topics in our laboratory. Several reviews of more general coverage are available (see, for example, Chance *et al*; 1979; Sies 1985). Techniques of monitoring cell damage, for example, the release of enzymes to the extracellular space or the uptake of dyes such as trypan blue, are not within the scope of the present article.

Low-level chemiluminescence

The detection of light emission from biological samples is a useful method for studying oxidative reactions in intact systems (Boveris *et al*. 1979; Cadenas & Sies 1984). The generation of electronically excited states during intracellular oxidative conditions can result from free-radical interactions that may or may not be associated with the peroxidation of membrane fatty acids. Alternatively, the direct generation of excited states can occur in enzyme-catalysed reactions (Cilento 1982), as demonstrated in model systems using peroxidases. The excited states discussed are (*a*) singlet molecular oxygen as measured by photoemission during its decay to the triplet ground state in the monomol (1) or dimol (2) reactions;

$$^1O_2 \rightarrow {}^3O_2 + h\nu \ (1270 \text{ nm}), \quad (1)$$

$$2\ {}^1O_2 \rightarrow 2\ {}^3O_2 + h\nu \ (634 \text{ and } 703 \text{ nm}); \quad (2)$$

and (*b*) excited triplet carbonyls (RO*) exhibiting a weak emission in the blue–green region of the spectrum (reaction (3)) or an indirect emission after energy transfer to a suitable acceptor

A (reaction (4*a*)), thus eliciting sensitized emission (reaction (4*b*));

$$RO^* \rightarrow RO + h\nu, \quad (3)$$

$$RO^* + A \rightarrow RO + A^*, \quad (4a)$$

$$A^* \rightarrow A + h\nu. \quad (4b)$$

Interactions of lipid peroxy radicals can be a source of excited state(s) as in reactions (5*a*,*b*) (Russell 1957; Howard & Ingold 1968). Because peroxy radicals are produced at the final stages of lipid peroxidation, they might be considered as the common mechanism for chemiluminescence (reactions (5*a*,*b*)), shared by different oxidative conditions which promote lipid peroxidation: CCl_4 poisoning, iron overload, oxidative breakdown of hydroperoxides, hyperoxia, etc.

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROH + RO + {}^1O_2, \quad (5a)$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROH + RO^* + O_2. \quad (5b)$$

Free-radical interactions supporting redox cycling, however, elicit chemiluminescence that is not associated with lipid peroxidation. Although O_2^- and $HO^{\bullet}$ are generally produced during the activation of different xenobiotics by redox cycling, a direct link between them and the electronically excited state(s) generated remains to be determined. The fact that redox-cycling-supported photoemission might be inhibited by superoxide dismutase indicates that the O_2^- generated is required at some stage for the generation of photoemission.

The molecular mechanism for the production of singlet oxygen during several peroxidase or peroxidase-like reactions seems to follow the dismutation of hydroperoxides without involving free radical intermediates (Cadenas *et al.* 1983*a*). The production of triplet excited carbonyl compounds during the peroxidase-catalysed oxidation of aliphatic aldehydes to their lower analogues (Cilento 1982) is thought to proceed via the formation of a dioxetane intermediate.

Alkane production

Volatile hydrocarbons such as ethane or pentane (and others) are liberated from membranes after peroxidation and fragmentation of lipids. Possible alternative sources for these alkanes have not yet been characterized. The general route for formation of ethane and pentane includes the formation of free-radical products from hydroperoxide decomposition (Tappel 1980). A pentyl free radical is formed on cleavage of an ω-6 fatty-acid chain on the side of the peroxy group closest to the methyl end (reaction (6)). Hydrogen abstraction by the resulting free radical forms pentane (reaction (7)). These mechanisms suggest that the most likely precursor for pentane and ethane formation is an alkoxy radical ($RO^{\bullet}$).

$$CH_3{-}(CH_2)_3{-}CH_2{-}\underset{\displaystyle O^{\bullet}}{\underset{|}{CH}}{-}CH_2{-}R \rightarrow CH_3{-}(CH_2)_3{-}CH_2^{\bullet} + HOC{-}CH_2{-}R, \quad (6)$$

$$CH_3{-}(CH_2)_3{-}CH_2^{\bullet} + H^{\bullet} \rightarrow CH_3{-}(CH_2)_3{-}CH_3 \quad (7)$$

The methodology for the assay of alkanes with intact animals or with isolated organs has been described by Müller & Sies (1984). The monitoring of alkanes as products of lipid peroxidation was introduced by Riely *et al.* (1974) into the field of biochemical toxicology. Alkane

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measurements from experimental models such as isolated liver microsomes, isolated hepatocytes, and the isolated perfused rat liver afford the possibility for the direct study of metabolic influences and mechanistic aspects with respect to lipid peroxidation.

Glutathione and oxidative stress

Glutathione and reactive oxygen species: GSSG *release and oxidative stress*

The well described function of glutathione peroxidases (GSH Px) is that of reducing hydroperoxides at the expense of GSH thiol equivalents, thus leading to the formation of glutathione disulphide, GSSG (see figure 1). GSSG is then reduced back to GSH by GSSG reductase at the expense of NADPH, thus allowing the maintenance of flux through GSH Px.

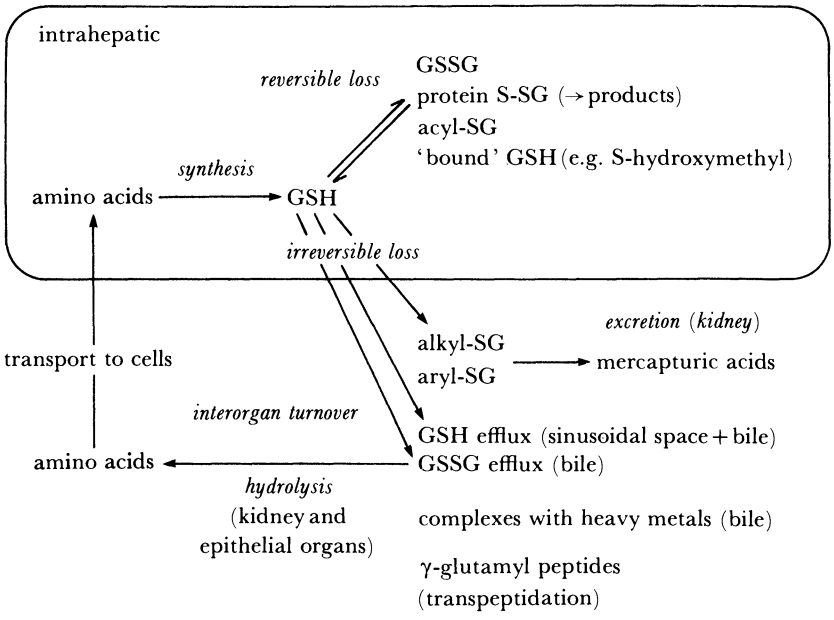

Figure 1. Processes (chemical and translocation) affecting intrahepatic and extrahepatic glutathione status. Modified from Sies *et al.* (1983*a*).

Interestingly, about 3% of the flux through GSH Px is not going back to GSH but rather is dealt with by an efflux system. Thus, for every 1000 nanomoles of hydroperoxide reduced per gram of liver per minute about 30 nanomoles of hydroperoxide reduced per gram of liver per minute about 30 nanomoles of GSSG are released per minute per gram of liver (Sies & Summer 1975). This apparently is a means of keeping the GSSG level from rising, and so GSSG efflux may serve a protective purpose. The efflux of GSSG occurs selectively into bile (Sies *et al.* 1978), and the correlation between the rise in intracellular (cytosolic) GSSG with the rate of GSSG efflux across the canalicular membrane (Akerboom *et al.* 1982*a*) suggest the existence of a transport system in the plasma membrane of the canalicular area of the hepatocyte. Carrier-mediated transport of GSSG has recently been detected in canalicular membrane vesicles (Akerboom *et al.* 1984*b*).

The export of GSSG can be competitively inhibited by other glutathione derivatives, such as glutathione S-conjugates (Akerboom *et al.* 1982*b*). It was found that when

1-chloro-2,4-dinitrobenzene was infused into the portal vein to form S-dinitrophenyl glutathione in the hepatocytes (Wahlländer & Sies 1979), the release of GSSG into bile was inhibited. This was accompanied by an increase in intracellular GSSG, as would be expected if the rate of GSSG formulation remained unchanged during the formation of the S-conjugate. However, we detected an intracellular accumulation of GSSG even above that of the value expected by the inhibition of transport across the canalicular membrane, suggesting that the partition ratio of 0.03 for efflux:GSSG reduction was altered. It was found that the accumulation of S-conjugate in the cytosol led to an inhibition of GSSG reductase (Bilzer *et al.* 1984). This was verified with the isolated GSSG reductase, the K_i being about 30 μM for the enzymes from yeast or from human erythrocytes. Further, in an X-ray crystallographic analysis of the binding of S-dinitrophenyl glutathione it was found that the conjugate binds to the enzyme in the active site, largely but not completely overlapping with the binding arrangement for GSSG (Bilzer *et al.* 1984).

More recently, it was also found that binding of S-dinitrophenyl glutathione occurs to another enzyme, and by this can exert an effect of regulatory interest. Kondo *et al.* (1984) have shown that S-conjugate plays a role in stimulating erythrocyte GSH synthesis, presumably by releasing the feedback inhibition of GSH on the γ-glutamyl cysteine synthetase.

In the state of selenium deficiency, the amount of GSH Px selenium decreases to very low levels (2–5% of control values). Burk *et al.* (1978) observed that while hydrogen peroxide infusion did not lead to an increase in GSSG release in this condition, the infusion of an organic hydroperoxide such as t-butyl hydroperoxide (t-BuOOH) still did; the latter hydroperoxide is a substrate for the non-selenium GSH Px activity of glutathione S-transferases, notably isozyme B. It is interesting that in Se deficiency the plasma concentrations of GSH were found to be double that of controls fed a selenium-adequate diet (Hill & Burk 1982; see also next section).

A listing of the response of difference cells and tissues to cellular oxidative conditions coupled to a release of glutathione disulphide into the extracellular space was given, together with a critical survey on the methodology currently used for the detection of GSH, GSSG, and mixed disulphides (Akerboom & Sies 1981; Sies & Akerboom 1984).

Glutathione release into plasma: GSH *release and hormonal responses*

Hepatic efflux of GSH, initially described by Bartoli & Sies (1978), substantially accounts for the turnover of the tripeptide in this organ (for a review, see, for example, Sies 1983; Meister & Anderson 1983). A system of transport of GSH across hepatocyte sinusoidal plasma membrane vesicles has been described (Inoue *et al.* 1984). Factors that govern GSH release under physiological conditions that are not well known. Recently, we observed that GSH efflux across the sinusoidal plasma membrane in isolated perfused rat liver was stimulated by the addition of hormones such as vasopressin, phenylephrine and adrenaline, whereas glucagon or dibutylryl cyclic AMP were without effect (Sies & Graf 1985). As shown in figure 2, the addition of adrenaline (epinephrine) leads to an increase in thiol release. Such hormonal response of the GSH transport system may explain the known loss of GSH during conditions of experimental shock (traumatic or endotoxin) and stress. For example, hepatic GSH content decreases to half that of controls in peripheral inflammation (Bragt & Bonta 1980). Because reactive oxygen species are known to be involved in the process of inflammation (for a review, see for example, Flohé *et al.* 1985), the newly discovered hormone dependence of GSH release from the liver (Sies & Graf 1985) may be part of a servo system to maintain the thiol redox

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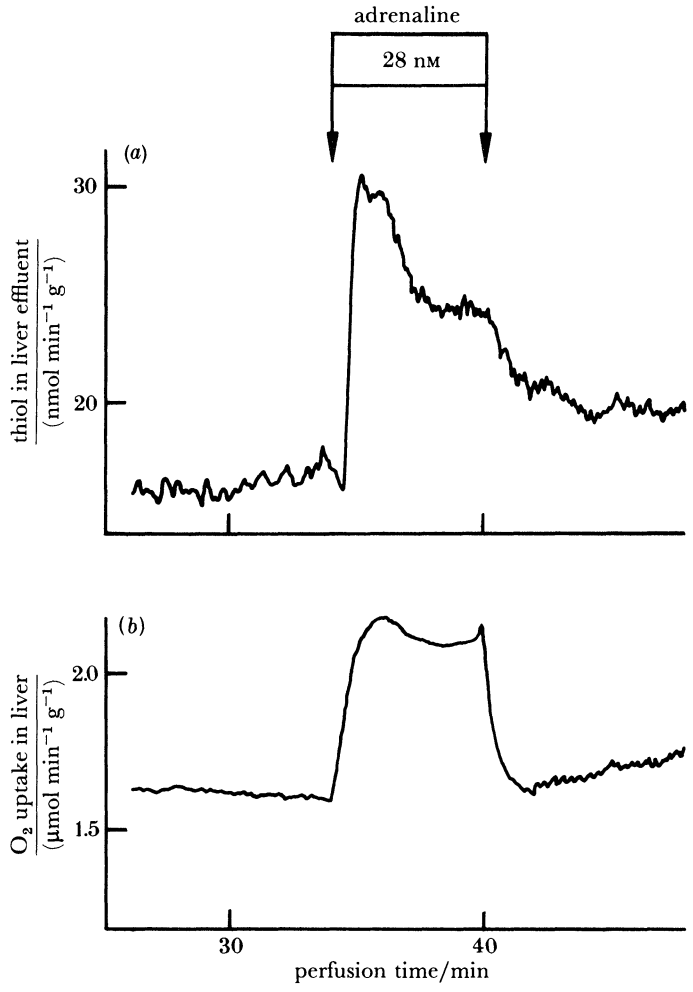

FIGURE 2. Epinephrine (adrenaline) stimulation of thiol efflux from isolated perfused rat liver (*a*) and stimulation of O_2 uptake (*b*). Modified from Sies & Graf (1985).

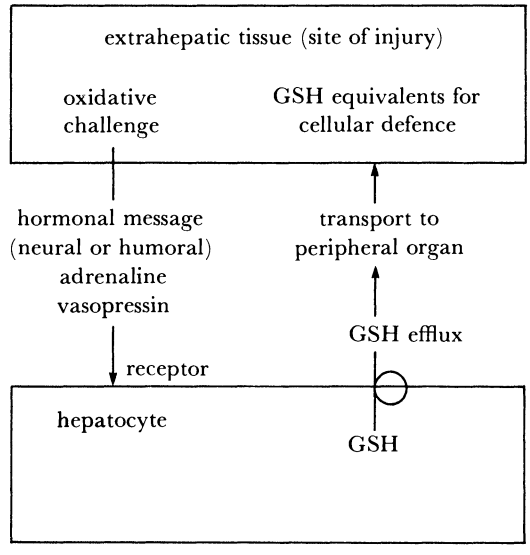

FIGURE 3. Simplified scheme or interorgan feedback system of hormone-stimulatable GSH efflux between extrahepatic tissue and liver. Interrelations of hormone receptor and GSH transport system are yet unknown. Uptake of GSH equivalents by peripheral (extrahepatic) cells may involve prior hydrolysis and subsequent *de novo* synthesis in cells (see figure 1).

balance. The peripheral site (for example, an inflammatory response, or tissue injury) would generate reactive oxygen species which, in turn, lead to a transportable signal of hormone nature. This can be of humoral or neural nature. The stimulation of GSH release form the liver then would counteract the loss of thiol groups in the periphery (see figure 3).

Metabolism of hydroperoxides

Glutathione-dependent hydroperoxide reduction

Reduction of model hydroperoxides (t-butyl hydroperoxide, hydrogen peroxide, cumene hydroperoxide) by perfused rat liver is accompanied by an intracellular decrease of GSH, an intracellular increase of GSSG, GSSG efflux into the bile (see Sies *et al.* 1983*a*). The glutathione content in heart is only about one-fifth of that in liver. The existence of a transport system for GSSG in rat heart were recently shown (Ishikawa & Sies 1984*a*), its capacity also being substantially lower than in liver. Thus, the sensitivity of heart to oxidative stress may be explained, in part, by a low capacity of GSSG export. It is suggested that this transport system takes part in detoxication in cooperation with cardiac glutathione S-transferase. The release by perfused heart of substantial amounts of glutathione S-conjugate (S(2,4-dinitrophenyl) glutathione) in response to infusion of 1-chloro-2,4-dinitrobenzene (Ishikawa & Sies 1984*b*) indicates the existence of cardiac glutathione S-transferases and of an export system for the glutathione S-conjugate formed. The isozyme pattern of glutathione S-transferases in heart differs from that found in liver (Ishikawa & Sies 1984*b*); the cardiac S-transferases consist predominantly of acidic proteins, many of which are absent in the liver (as also found in the testis (Guthenberg *et. al* 1983)). These acidic S-transferases may take a biologically significant share which differs from that for the basic enzymes in liver.

The oxidation of NADPH during either the glutathione-dependent organic hydroperoxide reduction or the cytochrome P_{450}-dependent drug oxidations is linked to calcium movements as shown perfused rat liver (Sies *et al.* 1981) and in isolated hepatocytes (Bellomo *et al.* 1982; Moore *et al.* 1983).

Glutathione peroxidase like and antioxidant activity of the seleno-organic compounds PZ-51

A new aspect on cellular hydroperoxide metabolism was introduced by the use of a seleno-organic compound that exhibits glutathione peroxidase activity, 2-phenyl-1,2-benzoisoselenazol 3(2H)-one, called PZ-51 or Ebselen. The function of PZ-51 can be described within the frame of a glutathione-peroxidase-like activity and a free-radical quenching activity. The former activity was demonstrated *in vitro* by the reduction of t-butyl hydroperoxide by PZ-51 in contrast to its sulphur analogue, PZ-25 (Müller *et al.* 1984; Wendel *et al.* 1984). The temporary protection of microsomes against ascorbate–Fe^{3+}-induced lipid peroxidation did not require GSH and therefore is thought to be a result of the antioxidant capacity of PZ-51. This effect is also exhibited by compounds that are relatively well soluble in lipids, such as diethyldithiocarbamate (Bartoli *et al.* 1983). In contrast, in isolated hepatocytes the protection of PZ-51 against lipid peroxidation requires GSH, therefore being attributed to the GSH Px activity of the compound (Müller *et al.* 1985).

Lipid peroxide formation and chemiluminescence

The occurrence of chemiluminescence will take place in intact hepatocytes or perfused liver when the glutathione-dependent enzymatic detoxication of hydroperoxides is saturated.

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Glutathione-depleted hepatocytes (after treatment with phorone (2,6-dimethyl(1)-2,5-heptadiene-4-one) yield low-level chemiluminescence with levels of t-butyl hydroperoxide much lower than those with intact hepatocytes (Sies *et al.* 1983*b*). Likewise, O_2-induced (lipid peroxidation mediated) chemiluminescence of glutathione-depleted hepatocytes is about three times higher in GSH-depleted hepatocytes than in controls (Cadenas *et al.* 1981). Chemiluminescence traces in different cellular glutathione states are shown in figure 4.

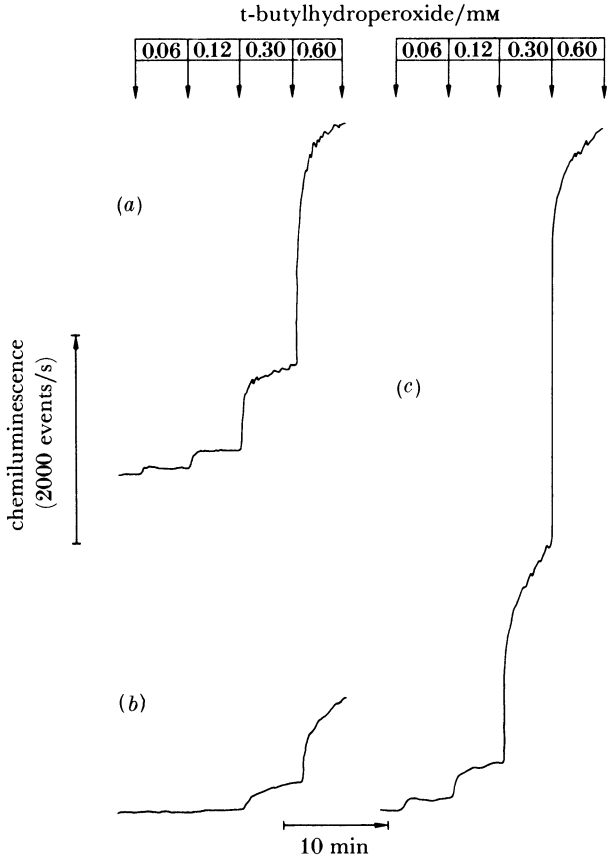

FIGURE 4. Low-level chemiluminescence recorded from surface of perfused rat liver in control (*b*), selenium deficiency (*a*), and selenium deficiency plus GSH-depleted(phorone)state (*c*), in response to increasing concentrations of t-butyl hydroperoxide. Three separate livers were exposed to the same sequence of hydroperoxide concentrations. Modified from Sies *et al.* (1983*b*).

Hydroperoxide metabolism in endoplasmic reticulum

In vitro studies with isolated cytochrome P_{450} and microsomal fractions led to proposed molecular mechanisms for the hydroperoxide-supported mono-oxygenations in terms of homolytic (White & Coon 1980; McCarthy & White 1983) or heterolytic (Ullrich 1977) scission. The homolytic cleavage of the O—O bond of the hydroperoxide is understood as a 'quasi-Fenton' pathway, whereas the heterolytic cleavage can be called an 'oxenoid' pathway. Figure 5 illustrates these types of mechanism for the generation of excited states and, hence, chemiluminescence during the metabolism of hydroperoxides by cytochrome P_{450}.

During the heterolytic cleavage the hydroperoxide coordinates to the haem iron followed by extrusion of alcohol to generate a transient $(FeO)^{3+}$ complex similar to compound I. This

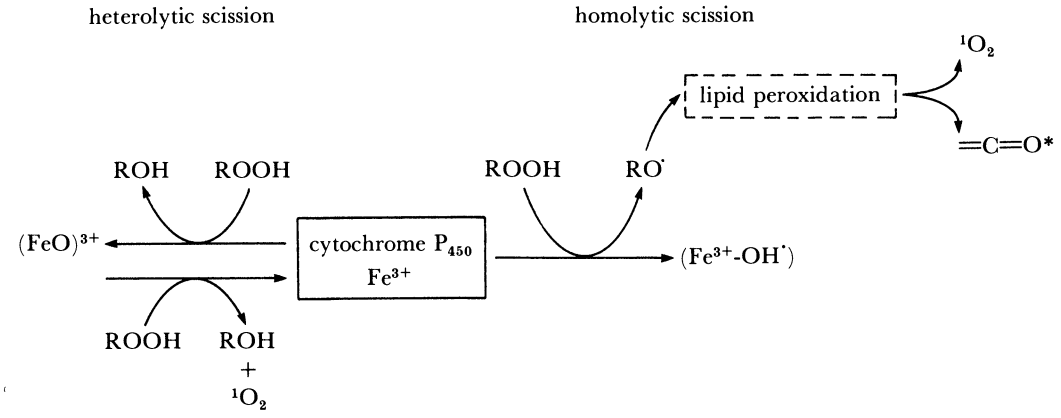

FIGURE 5. Scheme of heterolytic and homolytic scission of hydroperoxides by haemproteins. Iron protoporphyrin is denoted as Fe^{3+}.

$(FeO)^{3+}$ species could either hydroxylate or epoxidize a mono-oxygenation substrate or react with a second molecule of hydroperoxide and yield singlet oxygen (Cadenas *et al.* 1983*c*). During the heterolytic cleavage the generation of singlet oxygen can be viewed as a disproportionation of hydroperoxides (Cadenas *et al.* 1983*a*) (reaction (8)),

$$\text{ROOH} + \text{ROOH} \rightarrow 2\text{ROH} + {}^1\text{O}_2 \qquad (8)$$

which formally operates in a manner similar to that described for the generation of singlet oxygen during the enzymatic reduction of prostaglandin G_2 to prostaglandin H_2 (Cadenas *et al.* 1983*d*) and during the disproportionation of hydrogen peroxide catalysed by lacto-, myelo-, or chloroperoxidase–halide systems (Khan 1984; Kanofsky 1984). The products of the homolytic pathway are alkoxy radical ($RO^{\cdot}$) and a ferric hydroxide haem. The formation of $RO^{\cdot}$ in the presence of large amounts of hydroperoxide and absence of a hydroxylatable substrate can account for the destruction of the haem observed under these conditions and the accompanying lipid peroxidation. The formation of excited states by a homolytic mechanism results from the attack of such free radicals on microsomal unsaturated fatty acids; therefore, this photoemission is difficult to distinguish from the lipid peroxidation supported chemiluminescence (Cadenas & Sies 1982).

In addition to various reports suggesting the generation of singlet oxygen as a consequence of lipid peroxidation, it was recently found that excited triplet carbonyls are also formed (Cadenas *et al.* 1984*b*). The detection is based on energy transfer from a triplet carbonyl to a suitable acceptor such as chlorophyll *a* (see reactions 4*a*,*b*). This approach was based on previous reports of an efficient energy transfer from triplet carbonyls generated enzymatically to chlorophyll *a* as such, or incorporated in micelles or as constituents of chloroplasts (Nassi & Cilento 1984; Brunetti *et al.* 1983).

METABOLISM OF ALDEHYDES

The cytotoxic effects derived from aldehyde metabolism in isolated hepatocytes and perfused liver has been evaluated by detection of volatile hydrocarbons and chemiluminescence. Either exogenously added aldehydes, or enzymatically generated aldehydes, or lipid peroxidation

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derived aldehydes promote alkane release from the perfused liver (Müller & Sies 1982; 1983*a*, *b*). Aldehyde cytotoxicity might rely on (*a*) the ongoing production of radicals (as indicated by a protective effect of radical quenchers such as (+)-cyanidanol-3 and vitamin E (Müller & Sies 1982) and (*b*) the accompanying decreased cellular level of GSH, which might in part be a result of conjugation of the aldehyde with glutathione (Vina *et al.* 1981).

Enzymatically generated aldehydes

The hepatotoxicity elicited by ethanol requires its oxidation to acetaldehyde as indicated by a decreased rate of production of volatile hydrocarbons during alcohol dehydrogenase inhibition (by methyl- or propylpyrazole) and by a hundred-fold higher efficiency of acetaldehyde than ethanol in promoting alkane release by the perfused liver. Moreover, a decreased rate of ethane formation is observed during the inhibition of acetaldehyde oxidation by pargyline, disulphiram, or cyanamide. This indicates that in the reaction chain alcohol–aldehyde–acid, the last step is responsible for ethane production. Neither acetate nor NADH have an effect on ethane release (Müller & Sies 1983*a*, *b*).

The oxidation of monoamines via monoamine oxidase represents a metabolic source of aldehydes. The ethane release observed during the oxidation of benzylamine or phenylethylamine is inhibited in the presence of monoamine oxidase inhibitors, such as pargyline or the more specific tranylcypromine (Müller & Sies 1983*b*). Another aspect of monoamine oxidation cytotoxicity is brought forward by the increased GSSG efflux from liver caused during benzylamine infusion into the perfused liver (Akerboom *et al.* 1982*a*). This, along with the substantially increased cellular content of GSSG, is interpreted as an increased rate of hydrogen peroxide production formed during the oxidation of benzylamine or tryptamine. Reduction of hydrogen peroxide by the glutathione peroxidase system is indicated by an increased cellular content of GSSG (Akerboom *et al.* 1982*a*) at the expense of GSH. If 1-chloro-2,4-dinitrobenzene (CDNB) is given in addition to the oxidative conditions caused by benzylamine, a further increase in GSSG content is observed. This is partly explained by the action of glutathione S-transferase converting CDNB to a thioether of glutathione, which depresses GSSG release, as described above (Akerboom *et al.* 1982*a*).

Lipid peroxidation derived aldehydes

Aldehyde products from the free-radical peroxidative breakdown of polyunsaturated fatty acids include, among others, 4-hydroxyalkenals (Esterbauer *et al.* 1982). The aldehydes 4-hydroxynonenal, 4-hydroxyoctenal, and 4-hydroxyundecenal exert powerful cytotoxic effects, which have been evaluated by detection of low-level chemiluminescence and alkane formation. Cell damage elicited by 4-hydroxynonenal is reflected by an enhanced chemiluminescence intensity and alkane production, in addition to changes in the glutathione status of hepatocytes involving a rapid loss of GSH with formation of a glutathion S-conjugate with 4-hydroxynonenal (Cadenas *et al.* 1983*b*). Several aldehydes, when added to the perfused liver, elicit an extra ethane release that mimics the effect produced by endogenously generated aldehydes during ethanol- or monoamine oxidation. Examples of this are the ethane release promoted by acetaldehyde, benzaldehyde, crotonaldehyde, propionaldehyde, etc. (Müller *et al.* 1983*a*, *b*). As stated above for the endogenously generated aldehydes, the oxidation of the carbonyl is required to observe ethane release, a process which probably involves generation of free-radical intermediates leading to membrane lipid peroxidation.

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Redox cycling

The molecular mechanism for activation of certain xenobiotics (often of quinone structure) involves the univalent reduction of the compound with formation of the superoxide anion radical and, subsequently, other oxygen radical derived species (Kappus & Sies 1981; Doroshow & Hochstein 1982). This reduction is accomplished by different enzymatic activities present in the endoplasmic reticulum, mitochondria and nuclei of different tissues. O_2 is required for cytotoxicity; O_2^-, H_2O_2, $HO^\bullet$, and $^1O_2$ were thought to be the species responsible for cell damage, whereas the radical form of the xenobiotic appeared less likely to be responsible for cytotoxicity. The relative contribution by the quinone itself remains to be evaluated. The one-electron reduction of the redox cyclers that leads to O_2^- and subsequent hydrogen peroxide generation (via superoxide dismutase reaction) provides substrate for GSH peroxidase, hence an increase in cellular GSSG and (probably catalysed by thioltransferase) an increase in mixed disulphides. These observations suggest that substantial losses of GSH caused by intracellular redox cycling are linked to an increase in intracellular GSSG and, concomitantly, to an increase in mixed disulphides.

Changes in the cellular GSH status may have regulatory significance for several metabolic processes, including transport, by altering the membrane thiol status and the amount of protein mixed disulphides. Redox cycling of menadione affects the hepatic disposition of taurocholate (Akerboom *et al.* 1984). Biliary taurocholate efflux is almost completely inhibited, whereas the hepatic uptake of taurocholate remains unaltered. The intracellular GSSG content rises from 18 (controls) to 540 nmol g^{-1} liver. GSH decreased from 5.5 to 3.5 μmol g^{-1} liver because of the formation and biliary excretion of GSSG. In livers from Se-deficient rats the inhibition of taurocholate transport amounted to only 25% of that in Se-adequate controls, thus suggesting that taurocholate transport is related to the flux through Se-dependent glutathione peroxidase. Adriamycin, a known anticancer drug that was shown *in vitro* to be activated by a redox cycling mechanism (Doroshow & Hochstein 1982), promoted no GSSG efflux from either perfused rat liver or heart (T. Ishikawa, T. P. M. Akerboom & H. Sies, unpublished results).

In addition to these changes in the glutathione status and an intracellular increase of $NADP^+$, menadione redox cycling is associated with the production of low-level chemiluminescence when infused into the perfused liver or during its metabolism by isolated hepatocytes or microsomal fractions (Wefers & Sies 1983*a*). Light emission is increased during conditions which support univalent reduction of the quinone as indicated by (*a*) the enhanced emission during inhibition of the two-electron transfer by dicoumarol or (*b*) the inhibition of chemiluminescence during an enhanced NAD(P)H:quinone reductase activity promoted by pretreatment of the animals with butylated hydroxyanisole (Wefers *et al.* 1984). The cellular decrease of GSH is partly explained by the formation of a glutathione S-conjugate with menadione. This conjugation reaction is not in itself of protective nature and does not abolish semiquinone formation. The glutathione conjugate of menadione can also redox cycle with formation of excited states which lead to chemiluminescence. This could explain the weaker intensity of menadione-induced photo-emission in glutathione-depleted liver, at variance with the intensity observed under similar conditions during hydroperoxide-induced photoemission (Wefers & Sies 1983*a*).

It is interesting that, in a model system *in vitro* with the use of xanthine or acetaldehyde as substrate and xanthine oxidase for O_2^- generation, GSH was shown to increase low-level

chemiluminescence in the red spectral region (above 620 nm) and, as a stable end product of GSH, GSSG, and GSO_2^-, the glutathione sulphonate (Wefers & Sies 1983*b*). The mechanism proposed involves the formation of thiyl radical and, further, of the peroxysulphenyl radical (reactions (9) and (10)).

$$GSH + HO_2^{\bullet} \rightarrow GS^{\bullet} + H_2O_2, \quad (9)$$

$$GS^{\bullet} + GS^{\bullet} \rightarrow GSSG, \quad (10a)$$

$$GS^{\bullet} + O_2 \rightarrow GSOO^{\bullet}. \quad (10b)$$

The reactions of thiyl radicals in biological tissue are of interest not only in radiation biochemistry but also in metabolism. For example, the biological significance of the observation by Glatt *et al.* 1983 of a mutagenicity of glutathione and cysteine in the Ames test remains to be evaluated.

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Discussion

M. C. R. Symons (*Department of Chemistry, Leicester University*). I accept that it is conventional wisdom that the two visible emission bands associated with $^1\Delta$ oxygen are a result of emission from the dimer $^1\Delta O_2 \cdots {}^1\Delta O_2$, and that the near-i.r. band is simply due to $^1\Delta O_2$ itself. However, I find this hard to understand, and wonder if there might be some other explanation. This is because the intensity of the visible emission seems to be greater than the i.r. emission. Because $^1\Delta O_2$ has only a very limited lifetime, the probability of any sort of 'collision' involving orbital overlap must surely be small. If one argues that the i.r. band is weak because of thermal pathways, this will also serve to reduce the visible bands because the concentration of dimer will also be reduced. Has Professor Sies any explanation for the apparently extraordinary efficiency of dimer emission? Is it possible that one fails to 'see' much of the i.r. emission because of absorption by water, which has vibrational overtone features in this region? (If this were so, then the i.r. emission would be enhanced by using D_2O, but not the visible emission.)

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H. Sies. This problem has not yet been solved in simple physical–chemical systems. In our biological models, as in intact cells, we have a multi-phase system, so that there are differences in localized concentrations of $^1\Delta O_2$; this can contribute to the preference of dimol over monomol emission. In a purely aqueous phase with H_2O_2–NaOCl *in vitro*, we found that DABCO enhanced both monomol and dimol emission, but enhancement was more pronounced for the latter.

Reference

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M. C. R. Symons. In Professor Sies's scheme outlining the reactions of GSH, he included reaction (1) below as a route to GSSG. In my view, an important alternative mechanism is given in steps (2)–(4), which leaves open the route involving electron loss from $GSSG^-$.

$$2GS^{\cdot} \longrightarrow GS{-}SG, \tag{1}$$

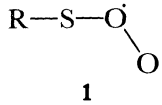
$$GS^{\cdot} + GSH \longrightarrow GS{-}\dot{S}\begin{matrix}H\\G\end{matrix}, \tag{2}$$

$$GS{-}\dot{S}\begin{matrix}H\\G\end{matrix} + H_2O \rightleftharpoons GS{-}\dot{S}G^- + H_3O^+, \tag{3}$$

$$GS{-}\dot{S}G^- \longrightarrow GSSG + [e^-]. \tag{4}$$

We think that we have e.s.r. evidence for intermediates of the type RS–$\dot{S}$HR, (Nelson *et al.* 1977, 1978) although this remains a matter of controversy (Hadley & Gordy 1974; Symons 1985). The anions RS–$\dot{S}R^-$ are, of course, relatively stable, and at neutral pH, proton loss from RS–$\dot{S}$HR should be favoured.

I would also like to support Professor Sies's suggestion that $GS^{\cdot}$ radicals can react with oxygen to give the 'peroxide' radical GS—$OO^{\cdot}$. We find that in the presence of oxygen at low temperatures, RS–$\dot{S}$HR–$RSSR^-$ is suppressed in favour of a species having e.s.r. properties commensurate with the formation of a bent species, **1**, which is the structure expected for this radical. Such a 'peroxide', however, may be much less reactive than alkyl peroxide radicals or $HO_2^{\cdot}$. Furthermore, at room temperature, the equilibrium may not favour $RSOO^{\cdot}$ formation as much as it does at low temperatures.



1

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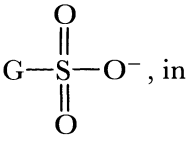
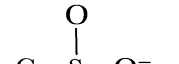
H. Sies. As I said, there are several potential reactions involving $GS^{\bullet}$, and reaction (2) is certainly one of them; alternatively, $GS^{\bullet}$ may react with GS^{-} also directly. Thus, I agree fully with your comment. One interesting electron acceptor for reaction (4) can be O_2, this way leading to formation of the superoxide anion radical.

G. Scott (*Department of Molecular Sciences, Aston University*). In his talk Professor Sies referred to the formation of glutathione sulphonate species, by which I understand him to mean that the contain two oxygens attached to sulphur. We know that *in vitro* these species are very effective antioxidants (Armstrong *et al.* 1979) by virtue of their ability to trap radicals and destroy hydroperoxides. Is there any evidence that the oxidized sulphur species he has observed are antioxidants *in vivo* or do you consider this a 'wasteful' reaction of thiyl radicals?

Reference

Armstrong, C., Husbands, M. & Scott, G. 1979 *Eur. Polym. J.* **15**, 241.

H. Sies. We have detected glutathione sulphonate,  , in our *in vitro* system. So far, this species has not yet been detected *in vivo*. Regarding the sulphinate,  , and the peroxy sulphenyl radical $GSOO^{\bullet}$, we have no information as to their potential pro-oxidant or antioxidant activity.

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