Biochemical Pharmacology, Vol. 27, pp. 1805–1813.

0006-2952/78/0715-1805$02.00/0

EXCITED STATES OF OXYGEN IN BIOLOGY: THEIR POSSIBLE INVOLVEMENT IN CYTOCHROME P450 LINKED OXIDATIONS AS WELL AS IN THE INDUCTION OF THE P450 SYSTEM BY MANY DIVERSE COMPOUNDS

ALAN J. PAINE

M.R.C. Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.

Excited states of oxygen such as singlet oxygen and the superoxide ion are topics of the liveliest current research interest. Indeed they have been implicated in many processes as diverse as the fundamental mechanism of catalysis by oxygenase enzymes such as xanthine oxidase [1] and tryptophan pyrrolase [2] to their involvement in environmental pollution by industrial smog [3]. Although many excellent reviews on the superoxide ion and singlet oxygen have been presented these do tend to consider each excited state of oxygen independently of the other [4–9]. Accordingly, the purpose of this commentary is not to exhaustively review the chemistry and biology of the superoxide ion and singlet oxygen but, rather, is an attempt to integrate the work on the participation of both these species in biological processes; especially those of relevance to Biochemical Pharmacology. In order to account for the properties of these excited states of oxygen, however, it is useful to briefly consider the electronic structure of the oxygen molecule in the ground state.

The nature of excited states of oxygen. In the ground state the oxygen molecule has two unpaired electrons in its two outermost molecular orbitals. The excited states of oxygen can be very simply explained in terms of these two unpaired electrons. Figure 1 shows that these two outermost molecular orbitals, are of equal energy i.e. degenerate. Now chemical rules state that the two electrons present in these two orbitals will be in their lowest energy state if they are in different orbitals and have their spins parallel i.e. the electrons are unpaired and have their spins in the same direction. Accordingly, the ground state oxygen molecule (which is also called the triplet state of oxygen) can be conveniently designated as $^3O_2$. This is represented in Fig. 1 as having its two outermost electrons in different molecular orbitals and these two electrons have their spins parallel. However Fig. 1 also shows that two other configurations are possible in which the electrons have anti parallel spins and are either in the same or different orbitals. These configurations are excited states of oxygen, being of higher energy than the ground state (Fig. 1), and are called singlet states of oxygen. A convenient shorthand for singlet oxygen being $^1O_2$. Although two singlet states of oxygen exist, it is beyond the scope of this commentary to distinguish between them in greater detail, except to say that the lower energy state ($^1\Delta g$) is believed to be the only singlet oxygen species that leads to reaction in solution [9]. For this distinction as well as for a more precise and detailed description of the electronic structure of oxygen, cf. [7] and [9].

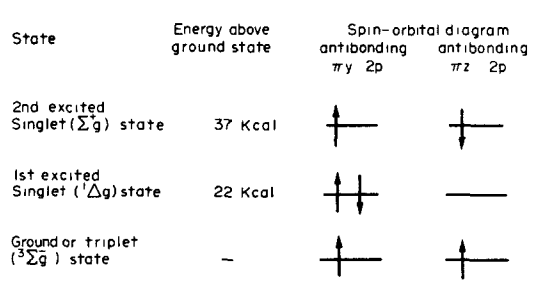

Fig. 1. Spin-orbital diagram of the electronic configuration of the outermost (π 2p) molecular orbitals for different states of molecular oxygen [8].

In summary, three states of molecular oxygen exist, (1) the ground state, and 2 excited states of oxygen, called singlet oxygen, which are produced by a re-arrangement of electrons in the oxygen molecule.

Figure 1 shows, that the oxygen molecule has two unpaired electrons in its two outermost molecular orbitals. However, these two orbitals can accommodate a further two electrons, that is one electron in each orbital, before they are full. Consequently the oxygen molecule can act as an electron sink, receiving electrons in a stepwise fashion. The addition of one electron produces the superoxide ion (O_2^-) as shown in Equation 1, whilst the addition of a second electron, which will fill the outermost molecular orbitals of the oxygen molecule, produces the peroxy anion (O_2^{2-}) as shown in Equation 2.

Equation 1. $O_2 + 1\,\acute{e} \rightleftharpoons O_2^-$

Equation 2. $O_2^- + 1\,\acute{e} \rightleftharpoons O_2^{2-}$

In conclusion, singlet oxygen is produced by a rearrangement of electrons in the oxygen molecule whilst the superoxide ion is an oxygen molecule with an extra electron. The superoxide ion in its protonated from (i.e. $HO_2\cdot$) is also called the perhydroxyl radical. The foregoing may seem a little removed from biological systems but it is now well established that these excited states of oxygen are involved in both non-enzymic as well as enzymic oxidations of biochemical significance (some of which are summarised in Table 1.) It is, therefore,

Table 1. Biological sources of O_2^- and $^1O_2$

Source	Excited states of oxygen involved O_2^-	$^1O_2$
Enzymes	Reference	
Xanthine oxidase	[1]	[10]
Aldehyde oxidase	[11]	
Dihydro-orotate dehydrogenase	[12]	—
Flavoprotein dehydrogenase	[13]	—
Tryptophan pyrrolase	[2]	
Galactose oxidase	[14]	
* Prostaglandin synthetase *in vitro*	[15]	[15], [123]
* Prostaglandin synthetase *in vivo*	—	[124]
* Liver microsomal lipid peroxidation and cytochrome P450 mono-oxygenase systems	[16], [17]	[17], [18], [126], [127]
Auto-oxidations		
Catecholamines	[19]	
Ferridoxins	[20]	
Haemoproteins	[21]	
Intact cells		
Phagocytosing granulocytes	[22], [23]	[52]
Illuminated chloroplasts (*i.e.* photosynthesis)	[25]	

* Footnote to Table 1. It is of interest to note, here, the possible involvement of cytochrome P450 and singlet oxygen mediated lipid peroxidation in prostaglandin synthesis by sheep vesicular gland microsomes [125].

energetically possible to produce these excited states of oxygen in biological systems.

The superoxide ion (O_2^-) can be produced *in vitro* by a variety of methods such as the dissolution of potassium superoxide (KO_2) in water, pulse radiolysis produced by ionising radiation such as X-rays, photochemically, by the illumination of photosensitizing dyes such as riboflavin or more commonly by the action of xanthine oxidase on xanthine [26–29]. This latter system is commonly used as it generates a low steady state concentration of O_2^- without the need for complex apparatus. Similarly singlet oxygen is said, although not unequivocably, to be produced, enzymatically by the action of lipoxidase on linoleic acid, or by the illumination of sensitizing dyes such as riboflavin, rosebengal or Methylene Blue [7, 30–32]. Physical methods for producing singlet oxygen include microwave discharge and laser photolysis [33–35].

Excited states of oxygen in biology. Because the superoxide ion and singlet oxygen have an electronic structure different from that of ground state oxygen their participation in biological processes can be determined by sophisticated physical techniques such as electron paramagnetic resonance. Indeed this was how the involvement of O_2^- in a biological process, i.e. the action of xanthine oxidase on xanthine, was first unequivocably established [1]. However it is much more common to infer the participation of these excited states of oxygen in biological processes by studying the effect of molecules, which are known to specifically scavenge a particular excited state of oxygen, on the reaction under study. Accordingly, if the addition of these scavenging molecules inhibits a given reaction, then it is inferred that a particular excited state of oxygen is involved. In this way two main analytical tools have made it possible to investigate the participation of the superoxide ion in biological processes. The first is an assay based on the oxidation of adrenalin to adrenochrome by the superoxide ion [19]. Since adrenalin is colourless and adrenochrome is a red-brown colour, the colour change associated with adrenochrome formation has formed the basis for a spectrophotometric assay of the superoxide ion [19]. Other spectrophotometric assays of O_2^- rely on its ability to reduce electron acceptors such as cytochrome *c* or nitroblue tetrazolium to formazan [36, 37]. The precise stochiometry of these reactions is given, cf. [38].

The second analytical tool is the discovery of the superoxide dismutase activity of the red cell protein erythrocuprein by McCord and Fridovich in 1969 [36] Figure 2 shows that by combining these techniques the co-oxidation of adrenalin to adrenochrome by xanthine oxidase has been attributed to the superoxide ion. In this way superoxide dismutase which reacts with free O_2^- ions in solution, produced by xanthine oxidase, prevents the formation of adrenochrome from adrenalin but has no effect on urate production from xanthine indicating that xanthine oxidase generates O_2^- during xanthine oxidation [4, 19, 36]. Similar techniques based on absorbance changes produced by the oxidation of

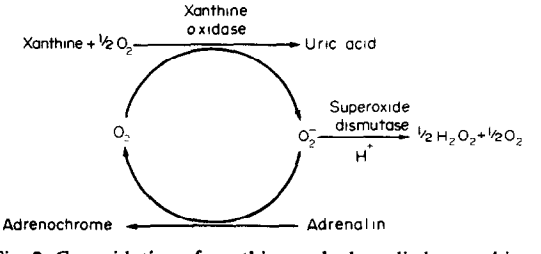

Fig. 2. Co-oxidation of xanthine and adrenalin by xanthine oxidase.

diphenylfuran [18], or tetracyclone by singlet oxygen and their inhibition by molecules which are known to specifically scavenge $^1O_2$ such as triethylenediamine (diazabicyclo 2,2,2 octane) [39, 40] or *B*-carotene [40], have implicated $^1O_2$ in biological oxidations, for examples of their use, cf. [10, 18, 41].

Singlet oxygen is said to produce chemiluminescence which can be measured on standard laboratory liquid scintillation counters, usually employed for the quantitation of radioactivity of β emitters such as [^{3}H] or ^{14}C [7, 30, 42]. Although chemiluminescence seems to be a valid indicator of $^1O_2$ when using highly purified enzymes it should be used with caution with tissue homogenates or semi-pure systems as the inhibition of chemiluminescence need not necessarily imply the scavenging of singlet oxygen and indeed chemiluminescence itself is not always due to singlet oxygen [43, 31, 44].

Oxygen toxicity and superoxide dismutase. The need for an enzyme to catalyse the dismutation of O_2^-, as shown in Fig. 2, which can proceed, very rapidly non-enzymically has been ascribed to the great biological reactivity of O_2^- [4]. Thus the biological significance of superoxide dismutase is based on the premise that respiration leads to some univalent reduction of oxygen and that superoxide dismutase exists to protect cells against the deleterious action of low levels of this radical. This hypothesis was originally strengthened by the finding that aerobic mico-organisms have high levels of superoxide dismutase whereas aerotolerant anaerobes contained less and obligate anaerobes contained no superoxide dismutase at all [45]. Even though superoxide dismutase has now been found in some obligate anaerobes [46], the initial conclusion that superoxide seems to protect cells against oxygen toxicity may still hold, especially as the fundamental basis of obligate anaerobiosis and microaerotolerance is not known. Oxygen is toxic to all organisms, but microaerotolerant organisms and anaerobes exhibit the effects of this toxicity at concentrations of oxygen below that found in the atmosphere [47]. The biochemical mechanisms of oxygen toxicity have been the subject of many recent reviews [48–50]. There is evidence that O_2^-, as well as $^1O_2$, will kill bacterial cells, and it is now clear that the bacterial effect of the antibiotic streptonigrin is due to the intracellular production of O_2^- followed by the cleavage of bacterial DNA by this radical [51, 52, 72]. The knowledge of this mechanism coupled with the finding that mammalian tumour cells, unlike normal cells, contain low levels of superoxide dismutase in addition to the H_2O_2 removing enzymes such as catalase and glutathione peroxidase has led to the suggestion that streptonigrin could be used in cancer chemotherapy [55, 56]. On the face of it, however, this would seem unlikely as the herbicide paraquat, like streptonigrin, also undergoes a cyclic oxidation-reduction process to generate O_2^- and is highly lethal to normal cells as will be discussed later. An alternative approach to the use of excited states of oxygen in cancer chemotherapy could be based on the finding that porphyrins, known to be powerful photosensitisers and to produce excited states of oxygen upon illumination [53], are selectively retained by tumour cells suggesting that porphyrins + light could be used therapeutically to destroy tumours, such as human bladder carcinoma which are accessible to a light source [66]. Even though the efficacy of excited states of oxygen such as O_2^- against tumour cells *in vivo* has yet to be firmly established, O_2^- will certainly damage cellular constituents such as membrane lipids [57]. In addition to O_2^- and $^1O_2$ other mechanisms of oxygen toxicity could include oxidative damage to vital cell constituents by the product of its dismutation H_2O_2, which is known to be cytotoxic to bacterial [51] as well as mammalian cells, [58] or by free radicals such as the hydroxyl radical (OH·) which can be produced by the interaction of O_2^- with H_2O_2 [59] as shown in Equation 3 below:*

Equation 3. $H_2O_2 + O_2^- \rightleftharpoons OH\cdot + OH^- + O_2$.

This reaction, in Equation 3, above can under certain conditions also give rise to singlet oxygen in addition to the hydroxyl radical [10]. Thus O_2^- may exert its destructive effects either directly or through the production of H_2O_2, OH· or singlet molecular oxygen. It seems that the production of any of these species could account for the bactericidal properties of phagocytosing leukocytes, as shown in Fig. 3. These cells produce the superoxide ion enzymatically via an NAD(P)H oxidase system which is located on the leukocyte plasma membrane [60, 61]. A similar NAD(P)H oxidase system has been found in Kupffer cells, the phagocytes of liver [62]. The superoxide ion is not produced in any large quantity when leukocytes are "resting". However contact with a bacterial cell or for that matter any particle such as zymosan or latex results in an increase in oxygen uptake and a burst of O_2^- production [63, 64]. Preliminary evidence, however, suggests that O_2^- production and internalisation (i.e. phagocytosis) of the bacteria are not tightly coupled as cytocholasin B, an inhibitor of phagocytosis, inhibits internalisation but not O_2^- production [65]. Indeed leukocytes seem to produce a whole battery

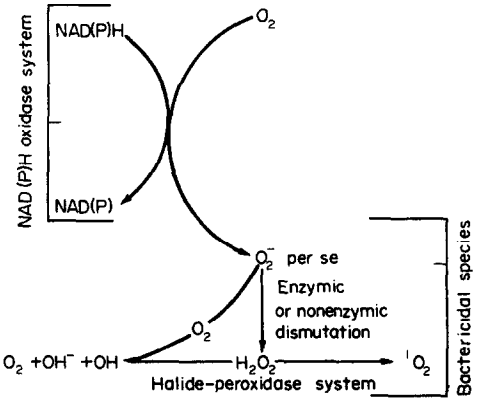

Fig. 3. Possible central role of O_2^-, produced by the NAD(P)H oxidase system, in the bactericidal action of leukocytes.

* It should be noted, however, that various experimental approaches have been unable to demonstrate the occurrence of the reaction in Equation 3 under what is considered to be most biological conditions. For examples cf. [128].

of destructive species such as $^1O_2$, OH and H_2O_2 all of which could be a consequence of O_2^- production by the NAD(P)H oxidase system as shown in Fig. 3. In this way either O_2^- *per se* or the H_2O_2 produced from it could be the major bactericidal agent of leukocytes; in addition H_2O_2 could also serve as a substrate for a halideperoxidase system which produces $^1O_2$ [43]. Additionally H_2O_2 could interact with O_2^- to produce the hydroxyl radical (OH). It therefore seems that whatever defence a bacterium has against a particular excited state of oxygen (such as high levels of superoxide dismutase or catalase or a high concentration of carotenoids, which are good scavengers of $^1O_2$ and are known to protect bacteria against the lethal effects of $^1O_2$ [40, 52]) it seems that leukocytes are sufficiently equipped to produce at least one reactive species to which the bacterial cell may have no defence. This central role of O_2^- in killing bacterial cells is further supported by the finding that leukocytes from patients suffering from "chronic granulomatous disease", which is characterised by a failure to destroy phagocytosed micro-organisms, do not form O_2^-. This is not the result of the rapid removal of O_2^- by increased levels of superoxide dismutase in leukocytes from "chronic granulomatous patients", especially as they do not form H_2O_2 either, but rather of a decreased production of O_2^- [67]. However in Down's syndrome, which results from an abnormality of chromosome number (i.e. Trisomy 21) increased levels of superoxide dismutase (the gene for which has been assigned to chromosome 21) have been proposed to explain the increased susceptibility of these individuals to bacterial and viral infections [68]. Although O_2^- formation by phagocytosing leukocytes seems to be beneficial by killing potentially infective bacterial cells it may also be involved in acute inflammation. O_2^- by reacting with hydrogen peroxide to produce the hydroxyl radical depolymerises hyaluronic acid as well as bovine synovial fluid. Since leukocytes produce O_2^- this reaction has been proposed as the mechanism of synovial fluid degradation *in vivo* in an inflamed joint [69]. Indeed superoxide dismutase injected into the knee and hip joints of osteoarthritic patients is said to make them ambulatory for more than 90 days [70]. Thus it seems that the pathological processes mediating both inflammation and cell mediated immune reactions resulting in the destruction of cells and tissues may share a common biochemical mechanism.

Even though superoxide dismutase appears to protect against the cytotoxicity of O_2^-, high levels of this enzyme in bacteria does not necessarily imply resistance to exogenously generated O_2^- as superoxide dismutase seems to be compartmentalised in the bacterial cell. Thus, *E. coli B* contains two types of superoxide dismutase, a manganese containing enzyme in the cell matrix and an iron containing enzyme in the periplasmic space. It is the concentration of the periplasmic space enzyme that will presumably determine the cells resistance to O_2^- generated exogenously i.e. by leukocytes, whilst the matrix enzyme probably serves to protect the cell against O_2^- produced from its cytochromes during oxidative metabolism [71]. Oxygen induces the manganо enzyme of *E. coli* [71] and this in turn makes these organisms more resistant to the antibiotic streptonigrin which generates O_2^- during oxidative metabolism in the cell [72]. On the other hand cultivation of *E. coli* in an iron-rich medium induces the ferri (periplasmic space) enzyme and accordingly increases the organisms resistance to O_2^- produced in the medium by phagocytes [73].

Eukaryotic cells also contain two types of superoxide dismutase, a copper–zinc enzyme in the cytosol and a manganese containing enzyme in the mitochondria [74]. The finding that the mitochondrial and bacterial enzymes are so similar, but differ from the cuprozinc enzyme found in the cytosol of many eukaryotes seems to add further support to the suggestion that bacteria and mitochondria share a common ancestor [4].

By analogy with the bacterial system the function of the mitochondrial enzyme is presumably to protect cells against production of O_2^- by mitochondrial cytochromes whilst the cytosol enzyme protects cytosolic constituents against oxidative damage. Certainly high oxygen concentrations, induce superoxide dismutase in the lungs of rats, and rats exposed to 85% oxygen become resistant to the lethal effects of 100% oxygen [75]. However the role of superoxide dismutase in protecting the cell solely against the toxicity of O_2^- now seems to be controversial question for the following reasons. Equation 4 shows that on thermodynamic considerations the non-enzymatic dismutation of O_2^- can produce singlet oxygen, although this has not yet been found experimentally [76, 77].

Equation 4. $2O_2^- + 2H^+ \rightleftharpoons H_2O_2 + {}^1O_2$

The half-life of $^1O_2$ is very dependent on its environment and can be as long as 45 min under limiting conditions or as short as 2 μ sec in water, although it is seven times longer in heavy water [7]. This deuterium effect on the lifetime of singlet oxygen in solution has been used as a test of the reactions involving singlet oxygen [54]. A half-life of 2 μ sec may not seem very long but it is sufficient for singlet oxygen to act as a characteristic and uniquely reactive species. Singlet oxygen is then said to pass to the ground state as shown in Equation 5 [7].

Equation 5. $2{}^1O_2 \rightarrow 2{}^3O_2 + h\nu$

Superoxide dismutase catalyses the reaction in Equation 4, however the resultant molecular oxygen produced has been shown to be the triplet or ground state (Equation 6) rather than the singlet state as shown in Equation 4 [78].

Equation 6. $2O_2^- + 2H^+ \overset{SOD}{\rightleftharpoons} H_2O_2 + {}^3O_2$

On thermodynamic considerations it is not possible for $^1O_2$ to be formed in the enzyme catalysed dismutation of O_2^- [76]. However the finding that superoxide dismutase produces ground state oxygen rather than singlet oxygen, has added to the suggestion that the function of superoxide dismutase is to catalytically scavenge singlet oxygen. Indeed superoxide dismutase (SOD) has even been given the acronymn "singlet oxygen decontaminase" [6]. Such an interaction between superoxide dismutase

and singlet oxygen is not possible on thermodynamic grounds [76] and has been eloquently argued against by Fridovich [6]. However the possibility that the interaction of superoxide dismutase with O_2^- consequently prevents the formation of singlet oxygen which could otherwise be formed in its absence in the non enzymic dismutation, still seems to hold. Thus a function of superoxide dismutase could be to prevent the formation of singlet oxygen not by catalytically interacting with singlet oxygen but, rather, as a consequence of its reaction with O_2^-. Whatever the precise function of superoxide dismutase, however, it is clear from the foregoing examples that the formation of $^1O_2$ and O_2^- during oxidative metabolism as well as the products of the latter's interaction with H_2O_2, i.e. the hydroxyl radical, are detrimental to normal physiological function. Superoxide dismutase can therefore serve to protect against the cytotoxicity of these species.

Superoxide dismutase appears to be present in every mammalian tissue so far examined, particularly high levels being found in organs with high rates of oxidative metabolism such as liver [79]. Indeed hepatic constituents (*in vitro*), such as microsomes seem to be very active in producing excited states of oxygen during NADPH oxidation by the microsomal cytochrome P450 linked drug metabolising system.

Excited states of oxygen and the hepatic microsomal cytochrome P450 system. Liver, as well as other mammalian tissues, contains a microsomal mono-oxygenase system which metabolises, mainly by hydroxylation, a great variety of lipophilic drugs to polar derivatives, which can then be excreted via the urine and bile. The activity of this system depends on the presence of NADPH, molecular oxygen and an enzyme complex consisting of cytochrome P450, a lipid factor and the flavoprotein NADPH cytochrome *c* reductase which serves to transfer electrons from NADPH to cytochrome P450 [80]. Cytochrome P450 is the site where the drug substrate, electrons from NADPH, and molecular oxygen all interact with resultant activation and insertion of an oxygen atom into the substrate as shown in Fig. 4.

The scheme in Fig. 4, cf. [80, 82, 83] shows the interaction of the ferric form of cytochrome P450 with the drug substrate (SH) followed by electron transfer from NADPH via the cytochrome *c* reductase to produce the ferrous cytochrome P450 substrate complex (step a, Fig. 4). The next step (b) is interaction of this complex with molecular oxygen to produce the oxy-ferrocytochrome-substrate complex. This is followed (step c in Fig. 4) by oxidation of the haem iron of the cytochrome from the ferrous to the ferric form with resultant electron transfer from the haem iron to the oxygen molecule to produce the activated form of oxygen that will be inserted into the substrate. The next step (d) is the transfer of the second electron from NADPH via the reductase which results in the oxygenation of the substrate and the regeneration of the ferric form of the substrate-free cytochrome P450 which can then re-enter the catalytic cycle. The precise nature of the activated form of oxygen produced by cytochrome P450 is still unknown. However, studies with purified cytochrome P450 suggest the possibility that it is O_2^-, especially as the generation of this species by the xanthine–xanthine oxidase system will support drug metabolism in the absence of NADPH and the cytochrome *c* (P450) reductase [82]. Furthermore studies with copper–tyrosine chelates, which are known to have a superoxide dismutase activity and are presumably small enough in molecular size to reach the active site of cytochrome P450, [96] inhibit drug metabolism but do not affect the rate of P450 reduction by NADPH [84]. This indicates that its mechanism of inhibition of drug hydroxylation is not a result of preventing electron transfer from NADPH to cytochrome P450, but is more probably a result of the dismutation of the activated form of oxygen produced at the active site of cytochrome P450. Since microsomal suspensions generate O_2^- as well as singlet oxygen during NADPH oxidation, it seems that the active form of oxygen envisaged in cytochrome P450 catalysed oxidations can easily dissociate from the system [85, 86, 18]. Alternatively, or in addition to a leak of the active species from the cytochrome, it seems that both O_2^- and $^1O_2$ can be produced by the interaction of the reduced form of the flavoprotein NADPH cytochrome *c* reductase with molecular oxygen as shown in Fig. 4. Indeed purified NADPH cytochrome *c* reductase has been shown to produce O_2^- during NADPH oxidation [16, 17, 86], and the dismutation of O_2^- to H_2O_2 followed by the interaction of H_2O_2 formed with another superoxide radical has been shown to produce both the hydroxyl radical and singlet oxygen both of which have been implicated in the peroxidation of membrane lipids by the P450 system [10, 18, 87]. In this way superoxide dismutase which scavenges the O_2^- produced by the P450 reductase prevents lipid peroxidation. However lipid peroxidation is not due solely to O_2^- in that the addition of H_2O_2 destroying enzyme catalase to the system also prevented membrane peroxidation [87]. The combined inhibition produced by scavenging O_2^- (by SOD) as well as H_2O_2 (by catalase) suggests that both $H_2O_2 + O_2^-$ are involved in peroxidative membrane damage, probably by producing the hydroxyl radical. Thus whilst SOD would not be expected to alter the concentration of

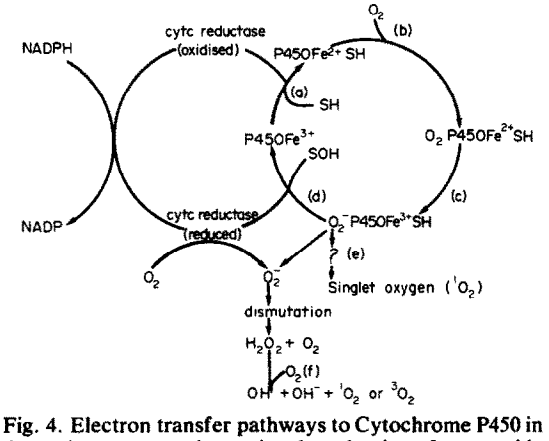

Fig. 4. Electron transfer pathways to Cytochrome P450 in liver microsomes and associated production of superoxide (O_2^-), hydroxyl radicals (OH), and singlet oxygen ($^1O_2$)
SH = substrate.

H_2O_2 formed from O_2^-, (since both the non enzymic and enzymic dismutation of O_2^- give rise to H_2O_2) SOD would by scavenging O_2^-, prevent the interaction of O_2^- with the hydrogen peroxide produced by either reaction. Thus in turn, this prevents the formation of the hydroxyl radical (OH·). Infact the production of OH· from the interaction of O_2^- and H_2O_2 has been found to be responsible for membrane peroxidation *in vitro* and this can be prevented as described above by the addition of superoxide dismutase, catalase as well as hydroxyl radical scavengers such as ethanol, mannitol, and sodium benzoate [87]. In addition to scavengers of the hydroxyl radicals, molecules which are known to specifically scavenge singlet oxygen also prevent lipid peroxidation in microsomal suspensions [18]. Whether singlet oxygen arises by the interaction of O_2^- with H_2O_2 (reaction f in Fig. 4), i.e. as it does with xanthine oxidase, in which case catalase and superoxide dismutase should also prevent its formation, or whether singlet oxygen is produced directly from the dissociation of the active oxygen cytochrome P450 complex (reaction e in fig 4) is unclear. Either way reactive oxygen species such as O_2^- and $^1O_2$ seem to be produced by the cytochrome P450 system both *in vivo* as well as *in vitro* as will be considered below.

Many studies have been made on the cytochrome P450 system, as its activity frequently parallels the toxicity and carcinogenicity of many natural and synthetic chemicals [88, 89]. These properties seem in the majority of cases to be due to the chemical nature of the metabolites produced by the P450 system, such as reactive epoxides etc rather than to the production of excited states of oxygen inside the cell [88, 89]. However it seems possible that some synergism between the production of active oxygen species by photosensitization of polycyclic hydrocarbon carcinogens on the skin as well as their metabolism by skin could occur [32]. Even though it seems that the majority of toxic compounds exert their toxicity either directly or through their metabolites there are now examples that the production of excited states of oxygen during metabolism could be responsible for the toxicity of some drugs, in particular paraquat.

Paraquat, a commercially available herbicide, is highly lethal to man and animals. Its toxicity is characterised by lung oedema which progresses to interstitial fibrosis. Paraquat is selectively retained in the lung, the most aerobic of all tissues, irrespective of its route of administration [90], which may explain the development of pulmonary lesions. The mechanism of paraquat toxicity has been proposed to result from the reduction of paraquat by the NADPH cytochrome *c* reductase of lung microsomes followed by a cyclic oxidation–reduction process which in the presence of oxygen generates the superoxide ion. Since singlet oxygen is known to peroxidase lipids [18, 91], and lipid peroxidation occurs in paraquat poisoning it has been proposed that singlet oxygen is produced by the non-enzymic dismutation of O_2^-; although the possibility that the hydroxyl radical produced from O_2^- could be responsible for lipid peroxidation does not appear to have been experimentally tested nor for that matter has the claimed role for singlet oxygen [92, 93]. Certainly O_2^- seems to be involved in the toxicity of paraquat and the injection of superoxide dismutase seems to confer a small protective effect in rats [94], and could have been one of the treatment's which may have aided recovery in a human case of paraquat poisoning [95, 24]. However, whether it is possible for a relatively large enzyme such as superoxide dismutase (MW 34,000) to penetrate a lung cell is unknown. It seems more likely that the much smaller copper tyrosine or copper lysine chelates, (which are known to have a pronounced superoxide dismutase activity [96]) would enter cells the possible protective effect of these compounds do not seem to have been evaluated.

The production of the superoxide ion by the cytochrome P450 system also seems to be responsible for the irreversible binding of estrogens, some of which are present in oral contraceptives, to liver proteins [97]. Whether this binding is harmful remains to be established. However the production of O_2^- from cytochrome P450 has been proposed as a possible mechanism for methyldopa hepatitis [98]. So it seems that the superoxide ion i.e. the active oxygen species envisaged in the mechanism of oxygen activation by cytochrome P450, can under certain conditions dissociate from the active oxygen–cytochrome complex and possibly give rise to both singlet oxygen and the hydroxyl radical, all of which are known to be produced by microsomes and all of which are capable of oxidising cellular constituents [18, 87]. This leak of active oxygen species from cytochrome P450 seems to be a result of an "uncoupling" of electron flow from NADPH and substrate oxidation which can be due to a failure to transfer the second electron to the active oxygen P450 substrate complex [99]. Alternatively it may be due to particular substrates being poor acceptors of the active oxygen species produced by cytochrome P450. In this respect it would be interesting to know if perfluoro-*n*-hexane a powerful uncoupler of microsomal electron transport due to the strength of the carbon–fluorine bond results in a leak of active oxygen species from cytochrome P450 [100]. This concept of uncoupling together with other findings considered below could provide a possible mechanism for the regulation of the cytochrome P450 enzymes.

Excited states of oxygen: a common factor in the induction of cytochrome P450 by many diverse compounds? The induction of the hepatic microsomal P450 system, which includes benzo(a)pyrene mono-oxygenase, by many pharmacological and structurally unrelated compounds in the whole animal and in liver cell culture involves DNA dependent RNA synthesis and requires protein synthesis [101, 102]. However, the intracellular site of action as well as the precise properties that allow over two hundred diverse compounds to induce the enzyme system are unknown [101].

It seems improbable that the many diverse inducers produce essentially the same response by acting on a DNA repressor site in the classical fashion as discussed by Venkatesan *et al.* [103]. It seems more likely that some common denominator exists between the many diverse inducers. An

attractive hypothesis is that induction by many diverse compounds is mediated through a common endogenous inducer [104].

I believe that excited states of oxygen such as singlet oxygen are possible candidates for a common denominator in induction as their generation by various processes in rat liver cell culture results in a typical induction of benzo(a)pyrene mono-oxygenase activity [42, 105–107]. This finding may be of special significance as excited states of oxygen are envisaged in the mechanism of oxygen activation by cytochrome P450 and can probably be produced inside cells during electron flow in the endoplasmic reticulum as microsomal suspensions generate singlet oxygen during NADPH oxidation [82]. It therefore seems theoretically possible that inducers could produce their effects by generating singlet oxygen inside cells, by binding to cytochrome P450 and stimulating electron flow from NADPH. As chemically unrelated inducers are known to bid to cytochrome P450, such a working hypothesis (Fig. 5) could account for the great diversity of inducers by utilising the well established versatility of cytochrome P450 [81]. Although the experimental findings used in the development of the model are indirect and cannot be taken as conclusive, it is hoped that the model will provide an experimental basis to test the many points about the induction of cytochrome P450 which are still obscure.

The scheme in Fig. 5 proposes that inducing compounds bind to cytochrome P450 and stimulate electron flow from NADPH resulting in the formation of an active oxygen–cytochrome–inducer complex in a manner similar to that envisaged for substrates [82]. However, unlike non-inducing substrates, the model implies that inducing compounds are poor acceptors of the active oxygen species and may effectively 'uncouple' electron flow from substrate oxidation permitting a leak of singlet oxygen from cytochrome P450 [99, 100]. The implication that inducers may be poor substrates of the P450 system seems to be supported by the knowledge that the ability of barbiturates to induce drug metabolism is related to their biological half life [108]. In addition, Dioxin, the most effective inducer known is very

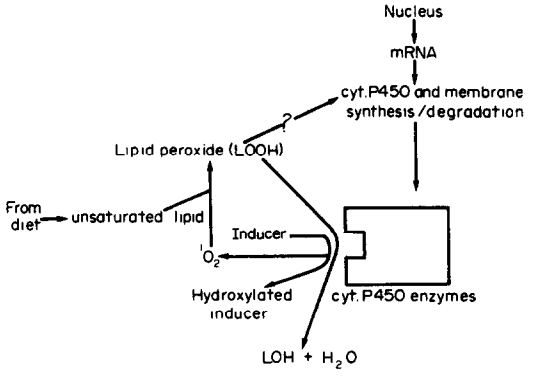

Fig. 5. Proposed model for the induction of the microsomal cytochrome P450 linked enzymes by a common inducing intermediate produced by a singlet oxygen ($^1O_2$) leak from the active oxygen–cytochrome–inducer complex.

slowly metabolised [109, 110]. Furthermore although inducers have not been tested as 'known, uncouplers' such as cyclohexane are inducers of the P450 system [100, 111, 112]. The proposed leak of singlet oxygen, which is 1500 times more effective in initiating lipid peroxidation than ground state oxygen [91], is envisaged as increasing the hepatic concentration of lipid peroxides by peroxidising free (i.e. non-membrane bound) unsaturated fatty acids. The peroxidation of the lipids which constitute the microsomal membrane, with which cytochrome P450 is intimately associated [80], seems to result in destruction of cytochrome P450 [113]. It is the concentration of free (non-membrane bound) lipid peroxides that is proposed to control the activity of the P450 system. Lipid peroxides are envisaged as playing a central role in cytochrome P450 enzyme induction because nutritional studies have shown that certain readily peroxidisable lipids have a very weak inducing activity on their own but are necessary to permit the more powerful induction by the classical inducers, [104] (unpublished work). In addition, as cytochrome P450 has been shown to function as a lipid peroxidase lipid peroxides can be envisaged as exerting a feedback control on the activity of the P450 system [114]. In this way lipid peroxides are themselves considered to be substrates of the enzyme system of which they regulate the synthesis. As lipid peroxides are known to exert an inhibitory effect on the P450 system and the mono-oxygenase and peroxidase activities of cytochrome P450 appear to be inversely related it seems possible that inducing compounds may compete with lipid peroxides for the P450 site [115]. Thus when an inducer occupies the site the lipid peroxide concentration rises, due to the combination of the singlet oxygen leak as well as the inhibition of lipid peroxide inactivation.

Then as the inducer is cleared from the liver and lipid peroxide concentration falls as a result of the increased peroxidase activity, due to the increase in P450 molecules, decreased singlet oxygen generation, as well as decreased competition by the inducing substrate for the peroxidase site. This fall in the hepatic lipid peroxide concentration should precede the return of the induced activity to control levels. Preliminary experiments by Marshall and Naughton [116] have shown that these changes in the hepatic lipid peroxide concentration do indeed occur after the administration of a single dose of the inducer phenobarbitone, and are compatible with the hypothesis that lipid peroxides may play a central role in induction. Lipid peroxides are also likely candidates for controlling cytochrome P450 levels from a teleological point of view, as it is most likely that the P450 system evolved to protect organisms from the toxic effects of these as well as other naturally occurring dietary anutrients rather than to detoxify synthetic organic chemicals.

The model implies that in order for a compound to be an inducer it must occupy its site on cytochrome P450 for long enough so that the lipid peroxide concentration can reach the level that results in induction. This pre-requisite could account for certain rather general, although well established physiochemical characteristics that render a com-

pound particularly potent as an inducer. These include tight binding to cytochrome P450 and a long biological half life both of which could imply a poor rate of oxygenation by cytochrome P450 [81, 101, 117]. Furthermore the model could also account for the finding that inducers are usually, if not always, initially inhibitors of the metabolism of other substrates [118, 119].

A limitation of the model is that it is difficult to account for the differences in cytochrome P450 linked activities obtained in response to the two main classes of inducers i.e. the phenobarbitone type and those typified by benzo(a)pyrene [101]. This limitation may be overcome by speculating that the site of the singlet oxygen leak for each class of inducer occurs in different cells. Multiple forms of cytochrome P450, having different substrate specificities are known [120]. Similarly the type of induction observed could be determined by the specificity of a cell's P450 type for a particular inducer since the scheme in Fig. 5 proposes that inducers must dissociate slowly from the active oxygen–cytochrome–inducer complex in order for induction to occur. Although differences between individual liver parenchymal cells with regard to their microsomal hydroxylation enzymes and response to phenobarbitone have been shown it is still unclear whether or not all cells express the same drug metabolising capacity [121, 122]. Attempts to isolate liver cells expressing different drug metabolising enzymes should help solve this question and hopefully give some insight as to whether this is the reason why toxins requiring activation by P450 can produce necrosis in different regions of the liver.

Acknowledgements—I am grateful to P. J. Colley for many discussions on excited states of oxygen and to Drs. F. De Matteis and A. E. M. McLean for many ideas which have led to the development of the proposed model for the induction of the cytochrome P450 linked enzymes.

REFERENCES

1. P. F. Knowles, J. F. Gibson, F. M. Pick and R. C. Bray, *Biochem. J.* **111**, 53 (1969).
2. F. Hirata and O. Hayaishi, *J. biol. Chem.* **246**, 7825 (1971).
3. J. N. Pitts, *Ann N.Y. Acad. Sci.* **171**, 239 (1970).
4. I. Fridovich, *Acc. Chem. Res.* **5**, 321 (1972).
5. I. Fridovich, *Adv. Enzymol.* **41**, 35 (1974).
6. I. Fridovich, *Ann. Rev. Biochem.* **44**, 147 (1975).
7. M. Kasha and A. U. Khan, *Ann. N.Y. Acad. Sci.* **171**, 5 (1970).
8. I. R. Politzer, G. W. Griffin and J. L. Laseter, *Chem-Biol. Interact.* **3**, 73 (1971).
9. C. S. Foote, in *Free Radicals in Biology* (Ed. W. A. Pryor) Vol. 2. Academic Press, London (1976).
10. E. W. Kellogg (3rd) and I. Fridovich, *J. biol. Chem.* **250**, 8812 (1975).
11. K. V. Rajagopalan, I. Fridovich and P. Handler, *J. biol. Chem.* **237** 922 (1962).
12. V. Aleman and P. Handler, *J. biol. Chem.* **242**, 4087 (1967).
13. V. Massey, S. Strickland, S. G. Mayheny, L. G. Howell, R. G. Engel, M. Mathews, M. Schuman and P. A. Sullivan, *Biochem biophys. Res. Commun.* **36**, 891 (1969).
14. G. A. Hamilton and R. D. Libby. *Biochem. biophys. Res. Commun.* **55**, 333 (1973).
15. L. J. Marnett, P. Wlodawer and B. Samuelsson, *Biochem. biophys. Res. Commun.* **60**, 1286 (1974).
16. T. C. Pederson and S. D. Aust, *Biochem. biophys. Res. Commun.* **48**, 789 (1972).
17. M. Nakano, T. Noguchi, Y. Tsutsumi, K. Sugioka, Y. Shimizu, Y. Tsuji and H. Inaba, *Proc. Soc. exp. Biol. Med.* **147**, 140 (1974).
18. M. M. King, E. K. Lai and P. B. McCay, *J. biol. Chem.* **250**, 6496 (1975).
19. H. P. Misra and I. Fridovich, *J. biol. Chem.* **247**, 3170 (1972).
20. H. P. Misra and I. Fridovich, *J. biol. Chem.* **246** 6886 (1971).
21. W. J. Wallace, J. C. Maxwell and W. S. Caughey, *Biochem. biophys. Res. Commun.* **57**, 1104 (1974).
22. B. M. Babior, R. S. Kipnes and J. T. Curnutte, *J. clin. Invest.* **52**, 741 (1973).
23. D. B. Drath and M. L. Karnovsky, *J. exp. Med.* **141**, 257 (1975).
24. D. S. Davis and D. L. Davis, *Fedn Proc.* **33**, 228 (1974).
25. K. Asada, K. Kiso, K. Yoshikawa, *J. biol. Chem.* **249**, 2175 (1974).
26. L. E. A. Henry, B. Halliwell, D. O. Hall, *FEBS Letters* **66**, 303 (1976).
27. G. Rotilio, R. C. Bray and E. M. Fielden, *Biochim. biophys. Acta* **268**, 605 (1972).
28. R. W. Miller, *Can. J. Biochem.* **48**, 935 (1970).
29. C. Beauchamp and I. Fridovich, *Analyt. Biochem.* **44**, 276 (1971).
30. A. Finazzi-Agro, P. De Sole, G. Rotilio, B. Mondovi, *Ital. J. Biochem.* **22**, 217 (1973).
31. A. H. Michelson, *FEBS Letters* **44**, 97 (1974).
32. A. U. Khan and M. Kasha, *Ann. N.Y. Acad. Sci.* **171**, 23 (1970).
33. E. J. Corey and W. C. Taylor, *J. Am. Chem. Soc.* **86**, 3881 (1964).
34. W. S. Gleason, A. D. Broadbent, E. Whittle and J. N. Pitts, *J. Am. Chem. Soc.* **92**, 2068 (1970).
35. A. Farmilo and F. Wilkinson, *Photochem. Photobiol.* **18**, 447 (1973).
36. J. M. McCord and I. Fridovich, *J. biol. Chem.* **244**, 6049 (1969).
37. B. Lippit and I. Fridovich, *Archs. Biochem. Biophys.* **159**, 738 (1973).
38. V. Mishin, A. Pokrovsky and V. Lyakhovich, *Biochem. J.* **154**, 307 (1976).
39. C. Ouannes and T. Wilson, *J. Am. Chem. Soc.* **90**, 6527 (1968).
40. C. S. Foote, R. W. Denny, L. Weaver, Y. Chang and J. Peters, *Ann. N.Y. Acad. Sci.* **171**, 139 (1970).
41. A. J. Paine, *Biochem. J.* **158**, 109 (1976).
42. R. C. Allen, S. J. Yevich, R. W. Orth and R. H. Steele, *Biochem. biophys. Res. Commun.* **60**, 909 (1974).
43. J. F. Piatt, A. S. Cheema and P. J. O'Brien, *FEBS Letters* **74**, 251 (1977).
44. E. Hodgson and I. Fridovich, *Archs Biochem. Biophys.* **172**, 202 (1976).
45. J. M. McCord, B. B. Keele and I. Fridovich, *Proc. natn. Acad. Sci. U.S.A.* **68**, 1024 (1971).
46. J. Hewitt, and J. G. Morris, *FEBS Letters* **50**, 315 (1975).
47. S. F. Gotleib, *Ann. Rev. Microbiol.* **25**, 111 (1971).
48. N. Haugaard, *Physiol. Rev.* **48**, 311 (1968).
49. J. G. Morris, *J. Gen. Microbiol.* **60**, 000 (1970).
50. D. B. Menzel, *Ann. Rev. Pharmac.* **10**, 379 (1970).
51. F. J. Yost and I. Fridovich, *Archs Biochem. Biophys.* **175**, 514 (1976).
52. N. I. Krinsky, *Science, N.Y.* **186**, 363 (1974).
53. H. R. Rawls and P. J. Van Santen, *J. Am. Oil Chem. Soc.* **47**, 121 (1970).
54. P. B. Merkel, R. Nilsson and D. R. Kearns, *J. Am. Chem. Soc.* **94**, 1030 (1972).

55. A. Bozzi, I. Mavelli, A. Finazzi-Agro, R. Strom, A. M. Wolf, B. Mondovi and G. Rotilio, *Molec. Cell. Biochem.* **10**, 11 (1976).
56. J. W. Lown and S. Sim, *Can. J. Biochem.* **54**, 446 (1976).
57. D. Tyler, *FEBS Letters* **51**, 180 (1975).
58. B. Peterkofsky and W. Prather, *J. Cell Physiol.* **90**, 61 (1977).
59. C. Beauchamp and I. Fridovich, *J. biol. Chem.* **245**, 4641 (1970).
60. R. T. Briggs, B. D. Drath, M. L. Karnovsky and M. J. Karnovsky, *J. Cell Biol.* **67**, 566 (1975).
61. I. M. Goldstein, M. Cerqueira, S. Lind and H. B. Kaplan, *J. clin. Invest.* **59**, 249 (1977).
62. T. J. C. Van Berkel and J. K. Kruijt, *Archs Biochem. Biophys.* **179**, 8 (1977).
63. D. B. Drath and M. L. Karnovsky, *J. exp. Med.* **141**, 257 (1975).
64. B. M Babior, R. S. Kipnes and J. T. Curnutte, *J. clin. Invest.* **52**, 741 (1973).
65. I. M. Goldstein, D. Roos, H. B. Kaplan and G. Wleissman, *J. clin. Invest.* **56**, 1155 (1975).
66. J. F. Kelly, M. E. Snell and M. C. Berenbaum, *Br. J. Cancer* **31**, 237 (1975).
67. R. B. Johnston, B. B. Keele, H. Misra, J. E. Lehmeyer, L. S. Webb, R. L. Baehner and K. V. Rajagopalan, *J. clin. Invest.* **55**, 1357 (1975).
68. R. Frants, A. W. Eriksson, P. H. Jongbloet and A. J. Hames, *Lancet* **2**, 43 (1975).
69. J. M. McCord, *Science, N.Y.* **185**, 529 (1974).
70. K. Lund Olesen and K. B. Menander, *Curr. Therapeut Res.* **16**, 706 (1974).
71. E. M. Gregory, F. J. Yost and I. Fridovich, *J. Bact.* **115**, 987 (1973).
72. E. M. Gregory and I. Fridovich, *J. Bact.* **114**, 1193 (1973).
73. F. J. Yost and I. Fridovich, *Archs Biochem. Biophys.* **161**, 395 (1974).
74. R. A. Weisiger and I. Fridovich, *J. biol. Chem.* **248**, 3582 (1973).
75. J. D. Crapo and D. F. Tierney, *Am. J. Physiol.* **226**, 1401 (1974).
76. W. H. Koppenol, *Nature, Lond.* **261**, 420 (1976).
77. R. Nilsson and D. R. Kearns, *J. Phys. Chem.* **78**, 1681 (1974).
78. K. Goda, T. Kimura, A. L. Thayer, K. Keens and A. P. Schaap, *Biochem. biophys. Res. Commun.* **58**, 660 (1974).
79. P. Van Hien, K. Kovacs and B. Matkovics, *Enzyme* **19**, 1 (1975).
80. A. Y. H. Lu and W. Levin, *Biochim. Biophys. Acta* **244**, 205 (1974).
81. H. Remmer, *Am. J. Med.* **49**, 617 (1970).
82. M. J. Coon, H. W. Strobel and R. F. Boyer, *Drug Metab. Disp.* **1**, 92 (1973).
83. R. W. Estabrook, T. Matsubara, J. I. Mason, J. Werringloer and J. Baron, *Drug Metab. Disp.* **1**, 98 (1973).
84. C. Richter, A. Azzi and A. Wendel, *FEBS Letters* **64**, 332 (1976).
85. P. Debry and C. Balny, *Biochimie* **55**, 329 (1973).
86. S. D. Aust, D. L. Roerig and T. C. Pederson, *Biochem. biophys. Res. Commun.* **47**, 1133 (1972).
87. K. L. Fong, P. B. McCay, J. L. Poyer, B. B. Keele and H. Misra, *J. biol. Chem.* **248**, 7792 (1973).
88. J. R. Gillette, *Biochem. Pharmac.* **23**, 2785 and 2927 (1974).
89. J. A. Miller, *Cancer Res.* **30**, 559 (1970).
90. C. W. Sharp, A. Ottolenghi and H. S. Posner, *Toxic App. Pharmac.* **22**, 241 (1972).
91. H. R. Rawls and P. J. Van Santen, *Ann. N.Y. Acad. Sci.* **171**, 135 (1970).
92. J. S. Bus, S. D. Aust and J. E. Gibson, *Res. Commun. Chem. Path. Pharm.* **11**, 31 (1975).
93. J. S. Bus, S. Z. Cagen, M. Olgarard and J. E. Gibson, *Toxic. Appl. Pharmac.* **35**, 501 (1976).
94. A. Autor, *Life Sci.* **14**, 1309 (1974).
95. Anonymous, *Nature, Lond.* **245**, 64 (1973).
96. R. Brigelius, R. Spottl, W. Bors, E. Lengfelder, M. Sauran and U. Weser, *FEBS Letters* **47**, 72 (1974).
97. S. D. Nelson, J. R. Mitchell, E. Dybing and H. A. Sasame, *Biochem. biophys. Res. Commun.* **70**, 1157 (1976).
98. E. Dybing, S. D. Nelson, J. R. Mitchell, H. A. Sasame, and J. R. Gillette, *Molec. Pharmac.* **12**, 911 (1976).
99. H. A. Sasame, J. R. Mitchell and J. R. Gillette, *Fedn Proc.* **34**, 729 (1975).
100. V. Ullrich and J. H. Diehl, *Eur. J. Biochem.* **20**, 509 (1971).
101. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
102. I. S. Owens and D. W. Nebert, *Molec. Pharmac.* **11**, 94 (1975).
103. M. Venkatesan, J. C. Arcos and M. F. Argus, *J. theor. Biol.* **33**, 517 (1971).
104. W. J. Marshall and A. E. M. McLean, *Biochem. J.* **122**, 569 (1971).
105. A. J. Paine and A. E. M. McLean, *Biochem. Pharmac.* **23**, 1910 (1974).
106. A. J. Paine and A. E. M. McLean, *Biochem. Soc. Trans.* **2**, 605 (1974).
107. A. J. Paine and A. E. M. McLean, *Biochem. biophys. Res. Commun.* **58**, 482 (1974).
108. O. Pelkonen and N. J. Karki, *Chem.-Biol. Interact.* **7**, 93 (1973).
109. J. B. Greig and F. De Matteis, *Environ. Hlth Perspect.* **5**, 211 (1973).
110. G. F. Fries and G. S. Marrow, *J. Agr. Food Chem.* **23**, 265 (1975).
111. G. Mohn and E. M. Philipp, *Hoppe-Seyler's Z Physiol. Chem.* **355**, 564 (1974).
112. G. Mohn, *Biochem. Soc. Trans.* **3**, 972 (1975).
113. F. De Matteis and R. G. Sparks, *FEBS Letters* **29**, 141 (1973).
114. E. G. Hrycay and P. J. O'Brien, *Archs Biochem. Biophys.* **147**, 14 (1971).
115. A. N. Kotake, L. B. Deloria, V. S. Abbot and G. J. Mannering, *Biochem. biophys. Res. Commun.* **63**, 209 (1975).
116. W. J. Marshall and C. Naughton, *Biochem. Soc. Trans.* **2**, 992 (1975).
117. J. R. Gillette, D. C. Davis and H. A. Sasame, *Ann. Rev. Pharmac.* **12**, 57 (1972).
118. R. Kato, E. Chiesara and P. Vassanelli, *Biochem. Pharmac.* **13**, 69 (1964).
119. E. S. Vessell, C. J. Lee, G. J. Passananti and C. A. Shirely, *Pharmacology* **8**, 217 (1972).
120. D. Ryan, A. Y. H. Lu, S. West and W. Levin, *J. biol. Chem.* **250**, 2157 (1975).
121. L. W. Wattenberg and J. L. Leong, *J. Histochem. Cytochem.* **10**, 412 (1962).
122. J. C. Wanson, P. Drochmans, C. May, W. Penasse and A. Popowski, *J. Cell Biol.* **66**, 23 (1975).
123. A. Rahimtula and P. J. O'Brien, *Biochem. biophys. Res. Commun.* **70**, 893 (1976).
124. C. Deby, Drugs *Exptl Clin. Res.* **2**, 105 (1977).
125. P. J. O'Brien and A. Rahimtula, *Biochem. biophys. Res. Commun.* **70**, 832 (1976).
126. M. Nakano, T. Noguchi, K. Sugioka, H. Fukuyamá, M. Sato, Y. Shimizu, Y. Tsuji and H. Inaba, *J. biol. Chem.* **250**, 2404 (1975).
127. M. Nakano, K. Takayama, Y. Shimizu, Y. Tsuji, H. Inaba and T. Migita, *J. Am. Chem. Soc.* **98**, 1974 (1976).
128. A. Rigo, R. Stevanato, A. Finazzi-Agro and G. Rotilio, *FEBS Letters* **80**, 130 (1977).