PHOTODYNAMIC EFFECTS ON THE MICROSOMAL ELECTRON TRANSPORT SYSTEM

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ABSTRACT — We studied the photodynamic action of singlet oxygen, generated by Rose Bengal bound to polystyrene beads, on the electron transport components of rat liver microsomes. The decrease in the levels of cytochrome P_{450} and in the activities of NADPH-cytochrome P_{450} reductase, NADH-cytochrome b_5 reductase and cytochrome P_{450} peroxidase during illumination ($\lambda > 400$ nm) at 10°C, was significantly greater when in the presence of Rose Bengal beads. The inactivation was enhanced when microsomes were suspended in D_2O, whereas dark controls showed no inactivation. Levels of cytochrome b_5 were not affected under any of the experimental conditions.

Sodium azide, a well known singlet oxygen scavenger, protected to varying extents against loss of activity of NADPH- and NADH-reductases, but had no significant effect on cytochrome P_{450} destruction, cytochrome P_{450} peroxidase inactivation and lipid peroxidation. Aminopyrene, a type II substrate for cytochrome P_{450}, when coupled with dithiothrietol, successfully prevented the destruction of cytochrome P_{450} and the process of lipid peroxidation, but only partially protected the reductases. Using the Nash reagent to assay for formaldehyde production, we also found evidence for singlet-oxygen, cytochrome P_{450} — mediated demethylation of aminopyrine. The antioxidant butylated hydroxytoluene successfully prevented lipid peroxidation but had no effect on the photoinactivation of cytochrome P_{450}.

1 — INTRODUCTION

The absorption of light in living organisms by endogenous or exogenous photosensitizers in the presence of oxygen causes oxidation which leads to many chemical and biological effects,

most of which are detrimental [1]. Recently, the use of photosensitizers for the destruction of cancerous tissue has received increasing attention from clinicians and photobiologists. The dye Rose Bengal is a powerful photosensitizer well known [2], [3] for its visible-light-induced generation of singlet oxygen ($^1O_2$). The short lifetime of $^1O_2$ in water ($\sim 2\mu s$) does not prevent it from destroying membranes and cells. In an aqueous suspension of membranes, Rose Bengal is usually found associated with the membrane component of the system. Since the membrane structure of microsomes is still unclear, the localization of the dye within such a system would be hard to determine. However, Rose Bengal covalently bound to polystyrene beads [4] still retains its photosensitizing properties and has already been used to photokill water-borne bacteria [5].

We have used Rose Bengal beads, as an exogenous photosensitizer, to study the photodynamic effects of visible light ($\lambda > 400$ nm) on the electron-carrying components and lipids of microsomal membranes.

Unsaturated fatty-acids and several amino acids are known to be susceptible to sensitized photooxidation even though the chemistry of such processes is not well understood [2]. Previous work [6], [7] has shown that endogenous flavins in microsomal reductases are capable of acting as photosensitizers. We have minimized this effect by working at lower temperatures and never exceeding incubation periods of 6 hours.

2 — MATERIALS AND METHODS

Chemicals: NADPH, NADH, cytochrome *c*, sodium azide, tryptophan, aminopyrine, dithiothrietol (DTT), butylated hydroxytoluene (BHT), tetramethyl phenylenediamine (TMPD), biphenyl, trichloroacetic acid (TCA), thiobarbituric acid (TBA), acetic acid, sodium phenobarbital and Rose Bengal were purchased from Sigma Chemical Company. Sodium dithionite was from Aldrich Chemicals. Deuterium oxide (D_2O) and Chelex 100 were purchased from Bio-Rad Laboratories.

Liver microsome preparations from phenobarbital pretreated Long Evans male rats were obtained by a previously reported protocol [8]; they were suspended in a 50 mM potassium phosphate (pH = 7.5) chelexed buffer and all incubations, except where otherwise noted, were carried out in this buffer. Deuterium oxide was also buffered with 50 mM potassium phosphate at an equivalent pH ≈ 7.5 .

Protein concentration was measured by the biuret method using defatted bovine serum albumin as a standard [9]. Lipid peroxidation was assayed by formation of TBA reactive material [10].

The activity of each component of the microsomal electron transport chain was followed independently of the other components; NADPH-cytochrome P_{450} reductase activity was measured using cytochrome c as an electron acceptor [11]; NADH-cytochrome b_5 reductase was assayed using ferricyanide as an electron acceptor [12]; peroxidase activity was assayed using cumene hydroperoxide as a hydrogen donor and tetramethylphenylenediamine as an electron donor [13]; cytochrome b_5 and cytochrome P_{450} were measured by difference spectra in an Aminco DW-2 dual wavelength/split beam spectrophotometer [14], [15]. Aminopyrine demethylation was determined via the production of formaldehyde [16]. Tryptophan degradation was determined by changes in the absorption difference at 279 nm - 240 nm [17].

Incubation of dark and light samples was as previously described [18]; the light source was a battery of 300 W quartz iodide lamps covered by a 400 nm cut-off filter (corning glass n.° 3389). The maximum light intensity as measured by a LI-COR LI-185 Radiometer was 17 mW cm^{-2} s^{-1}. A variable rheostat was used for variable light intensities.

Anaerobic conditions were otbained by flushing humidified N_2 over the samples before (during 2 hrs in an ice bath with occasional stirring) and during illumination; aliquots were taken with a syringe for assays. Control experiments were performed in which (a) polystyrene beads with no Rose Bengal, and (b) polystyrene beads and unreacted Rose Bengal were added during the incubations. All 2 ml samples of microsomes at 2 mg protein/ml were incubated at 10°C in a shaking waterbath. When used, 10 mg of polystyrene beads and 0.15 μmoles of Rose Bengal (either bound or not bound) were added per ml of suspension.

3 — RESULTS

The photoinactivation of the NADPH - cytochrome P_{450} reductase is shown in Figure 1.

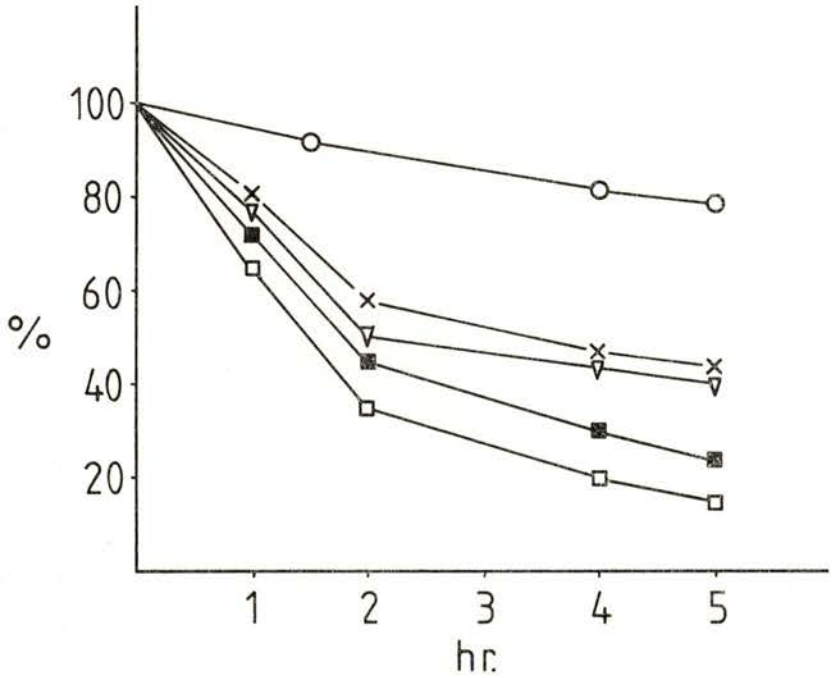

Fig. 1 — Photoinactivation of NADPH-cytochrome P_{450} reductase activity: 100 % corresponds to 164 ± 8 nmoles . min^{-1} . mg^{-1}; (○) in the absence of Rose Bengal beads. All others in the presence of Rose Bengal beads: (□) no further additions; (■) plus BHT (10 μM); (▽) plus azide (5mM); and (×) plus DTT (1mM).

Even in the absence of Rose Bengal beads there is some inactivation (~ 23 %) of this enzyme after 5 hrs of incubation at 10°C; in the presence of Rose Bengal beads, BHT (10 μM) affords no protection, while both DTT (1 mM) and sodium azide (5 mM) only partially protect the enzyme.

The effect of BHT, DTT and sodium azide on the photoinactivation of the NADH - cytochrome b_5 reductase is shown in Figure 2. In the case of this enzyme there is no inactivation in the absence of Rose Bengal beads and the extent of protection

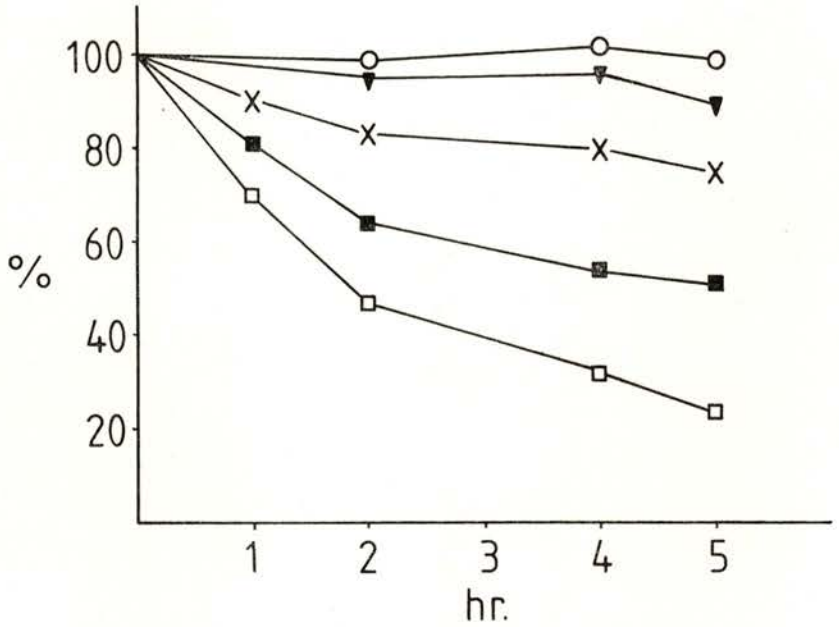

Fig. 2 — Photoinactivation of NADH-cytochrome b_5 reductase activity: 100 % corresponds to 2.7 ± 0.5 μmoles . min^{-1} . mg^{-1}; (○) in the absence of Rose Bengal beads. All others in the presence of Rose Bengal beads: (□) no further additions; (■) plus BHT (10μM); (▼) plus azide (5mM); and (×) plus DTT (1mM).

afforded by the compounds varies in the order BHT < DTT < azide with the latter protecting almost completely. Both reductases are therefore protected to varying degrees by azide and DTT while BHT only partially protects the NADH - cytochrome b_5 reductase.

The photoinactivation of cytochrome P_{450} is shown in Figure 3. Light by itself, in the absence of the photosensitizer destroys cytochrome P_{450} to some extent (~ 13 % in 5 hrs at 10°C) . In the presence of Rose Bengal beads the inactivation is far greater and is not protected by azide and/or BHT. DTT partially protects

and D_2O enhances the photoinactivation. The effects of two substrates of cytochrome P_{450}, biphenyl (1 mM) and aminopyrine (10 mM), on the photoinactivation of this enzyme were also

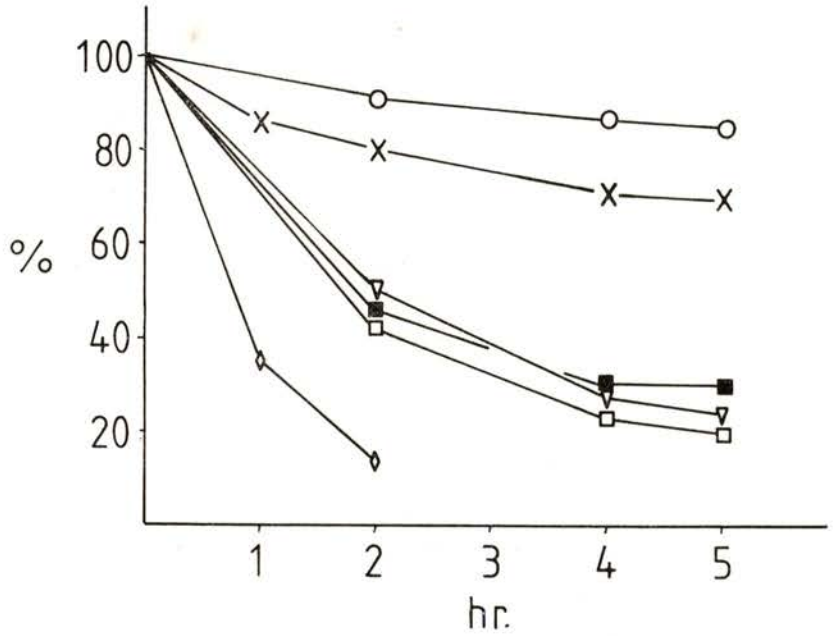

Fig. 3 — Photoinactivation of cytochrome P_{450}: 100 % corresponds to 1.4 ± 0.2 nmoles . mg^{-1}; (○) in the absence of Rose Bengal beads. All others in the presence of Rose Bengal beads: (□) no further additions; (■) plus BHT (10μM); (▽) plus azide (5mM); (×) plus DTT (1mM); and (◇) in D_2O buffer with no additions.

studied. Figure 4 shows that both aminopyrine and DTT are required for complete protection of cytochrome P_{450}. By itself, aminopyrine does to some extent, whereas biphenyl does not prevent the photoinactivation of cytochrome P_{450}. Aminopyrine does not protect the photoinactivation of the reductases (data not shown).

The peroxidase activity of cytochrome P_{450} was also investigated; in this case, aminopyrine also partially protected but azide did not its photosensitized inactivation (Figure 5).

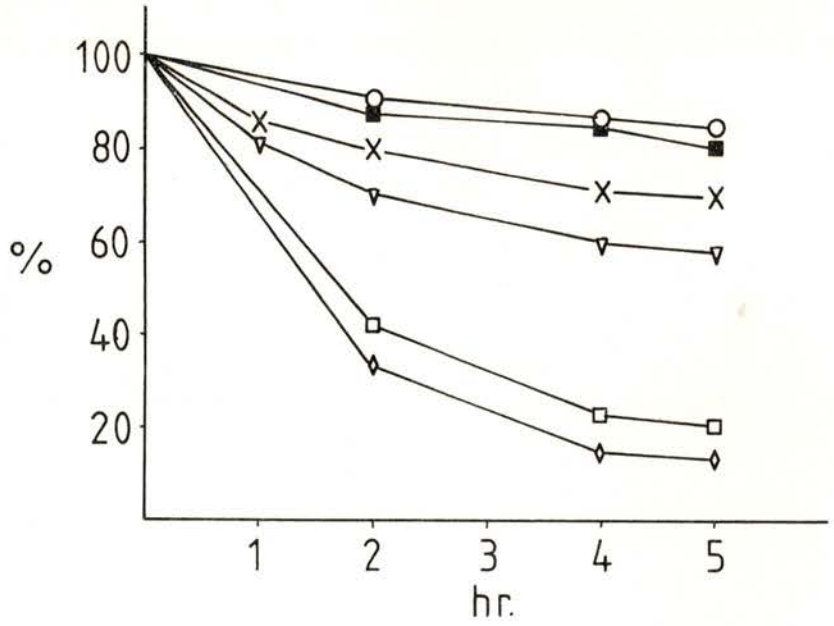

Fig 4 — Photoinactivation of cytochrome P_{450}: 100 % corresponds to 1.4 ± 0.2 nmoles . mg^{-1}; (○) in the absence of Rose Bengal beads. All others in the presence of Rose Bengal beads: (□) no further additions; (▽) plus aminopyrine (10mM); (×) plus DTT (1mM); (■) plus aminopyrine (10mM) and DTT (1mM); and (◇) plus biphenyl (1mM).

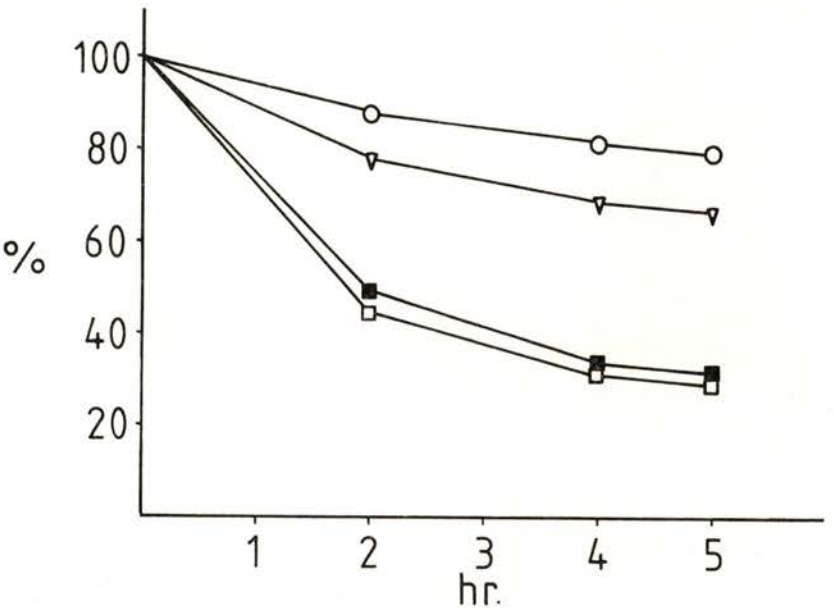

Fig. 5 — Photoinactivation of cytochrome P_{450} peroxidase activity: 100 % corresponds to 35 ± 3 nmoles . min^{-1} . mg^{-1}; (○) in the absence of Rose Bengal beads. All others in the presence of Rose Bengal beads: (□) no further additions; (▽) plus aminopyrine (10mM); (■) plus azide (5mM).

Figure 6 shows the production of formaldehyde (monitored using the Nash reagent) during the illumination of microsomes in the presence of aminopyrine. Sodium azide or DTT had no effect on this mechanism. In addition, the NADPH rate of generation of formaldehyde in the dark was the same before and after the illumination of microsomes in the presence of Rose Bengal beads, DTT and aminopyrine (M. P. G. Nova, unpublished results). Formaldehyde could also be photogenerated in the presence of 10 μM haemoglobin and 10 mM aminopyrine showed an absolute requirement for Rose Bengal beads. Microsomal lipid peroxidation is

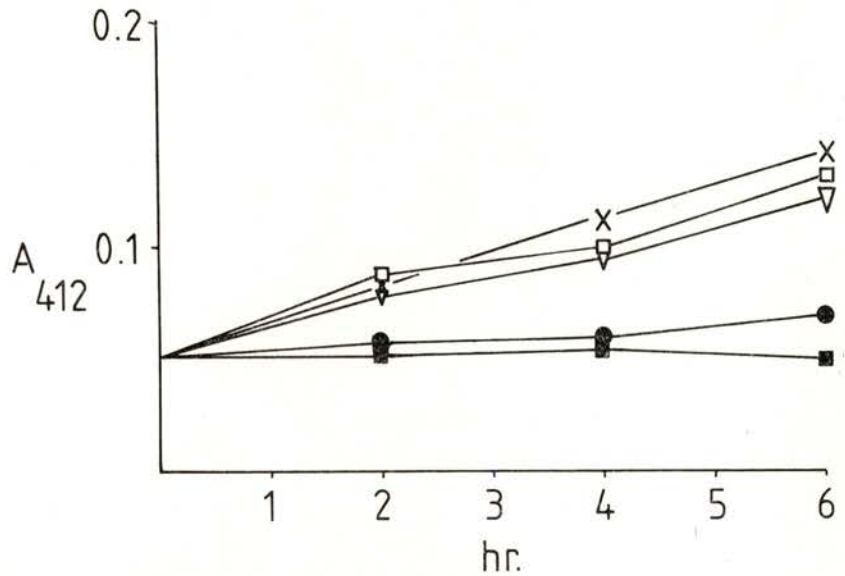

Fig. 6 — Generation of formaldehyde determined using the Nash reagent as described in Materials and Methods. Maximum rates correspond to 3.1 ± 0.5 nmoles . mg^{-1} . hr^{-1}; (■) in the absence of Rose Bengal beads and in the dark, (●) in the absence of Rose Bengal beads but in the light. All others in the presence of Rose Bengal beads: (▽) no further additions; (□) plus azide (5mM); (×) plus DTT (1mM).

shown in Figure 7. Sodium azide prevents only very slightly, while DTT and BHT prevent almost completely the generation of TBA reactive material.

In the presence of unreacted Rose Bengal (0.15 mM) and polystyrene beads (10 mg/ml), at 1/3 of the light intensity (also at 10°C) the NADPH - cytochrome P_{450} reductase and the cytochrome P_{450} were inactivated by 70 % and 50 % respectively in just 1 hr, while cytochrome b_5 remained unaffected. Polystyrene beads alone did not enhance the photoinactivation.

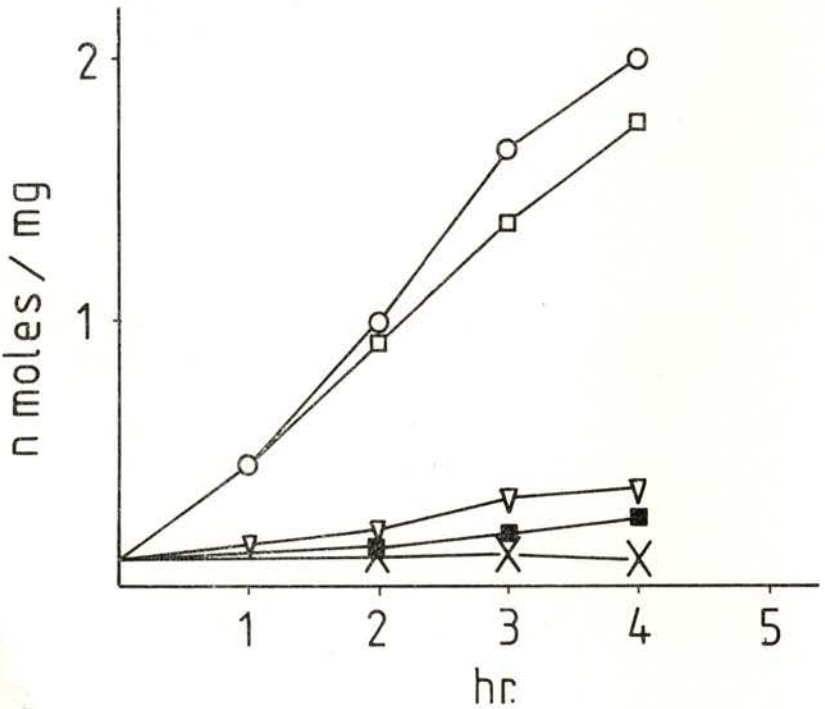

Fig. 7 — Photogeneration of TBA-reactants; (▽) in the absence of Rose Bengal beads. All others in the presence of Rose Bengal beads: (○) no further additions; (□) plus azide (5mM); (■) plus DTT (1mM); (×) plus BHT (10μM).

In the absence of oxygen, the photomediated effects on the electron transport and lipid components of microsomal membranes are abolished. Photooxidative effects are always enhanced in D_2O.

Table I shows changes in the difference between the absorbance at 279 nm and 240 nm for a 2 ml suspension of tryptophan (0.1 mM) after a 4 hr incubation at 10°C (Molar absorbance values used were 5580 at 279 nm and 1760 at 240 nm). Rose bengal beads are clearly required, and D_2O enhances the degradation of tryptophan during illumination.

TABLE I — Difference in absorption between 279 nm and 240 nm after a 4 hr incubation of tryptophan (0.1 mM) at 10°C (expressed as % of zero time value).

Dark	Light			
	H_2O (a)	D_2O (b)	H_2O+RB (c)	D_2O+RB
100 (d)	96	97	35	5

(a) H_2O — incubation in H_2O buffer

(b) D_2O — incubation in D_2O buffer

(c) RB — Rose Bengal beads (18 mg beads and 4 μmoles Rose Bengal per ml)

(d) 100 % corresponds to a value of 0.37 absorption units.

4 — DISCUSSION

The most interesting findings of the present investigation are:

a) That sodium azide, a well known scavenger of $^1O_2$, does not prevent the photodynamic action on cytochrome P_{450}, which in fact can only be fully protected by the combined presence of aminopyrine and dithiothrietol;

b) The photodynamic generation of formaldehyde when aminopyrine is added to an illuminated suspension of Rose Bengal beads and microsomes (or haemoglobin).

It is now well established that there is an important sulphydryl group near the haem of cytochrome P_{450} which contributes to the spectral characteristics of this cytochrome [19]. Dithiothrietol, a

strong sulphydryl reductant, is used as a protective agent during the isolation of cytochrome P_{450} [20] and will also, in some cases, regenerate P_{450} from its denatured P_{420} form [19]. It is not surprising therefore that dithiothrietol does afford some protection against the photodynamic inactivation of cytochrome P_{450} simply by maintaining the sulphydryl groups of the protein in the reduced state.

Substrates are also known to protect specific enzymes from denaturation and damage [21]. The reason why aminopyrine protects cytochrome P_{450} but biphenyl does not, may be due to the fact that biphenyl can only be added in a detergent solution, and detergents are well known for their denaturing action on this cytochrome [19]. Higher concentrations of biphenyl would also mean higher concentrations of detergent which would probably solubilize the membranes. Azide may not afford any protection against the photodynamic destruction of cytochrome P_{450} or the inactivation of its peroxidase activity simply because $^1O_2$ may have a higher reaction rate with cytochrome; β-carotene, one of the best $^1O_2$ quenchers [2], was not used since it also absorbs light in the visible range and would interfere with light absorption and with measurements of lipid peroxidation and cytochrome concentration. Azide does afford almost total protection to NADPH-cytochrome b_5 reductase, some protection to NADPH-cytochrome P_{450} reductase and negligible protection against lipid peroxidation. A strong correlation between lipid peroxidation and cytochrome P_{450} destruction has often been reported [22]. The fact that BHT prevents lipid peroxidation, but does not prevent cytochrome P_{450} destruction indicates that the photodynamic effect on this cytochrome is not mediated via lipid peroxidation. The varying levels of protection by azide to the different components in the microsomal system can always be explained in terms of varying reaction rates, even though we have no experimental data on such rates.

The results obtained with unreacted Rose Bengal and polystyrene beads show that the pattern of photodynamic inactivation of microsomal electron transport proteins depends largely on where the reactive oxygen species is generated. Unreacted Rose Bengal is found associated with microsomal membranes, most likely in the hydrophobic environment where the oxygen concentration is known to be higher and the life time of $^1O_2$ is longer [2];

this, and the fact that Rose Bengal in the membrane is likely to be closer to the membrane proteins, may explain the faster inactivation of the electron transport proteins observed in this case. The lack of a photodynamic effect on cytochrome b_5 is surprising, but has already been observed [6], [7].

The photodynamic demethylation of aminopyrine in the presence of microsomes or haemoglobin is interesting in so far as $^1O_2$ has often been suggested to be involved in many enzyme mediated oxidative reactions [23]-[25]. Its physiological relevance is far from being established [2], but it seems that in the presence of $^1O_2$, haemes are capable of demethylating aminopyrine. Cytochrome P_{450} is capable of accepting oxygen from a variety of donors, including hydroperoxides, hydrogen peroxide, sodium periodate and iodobenzene [26]-[31]. It may also accept $^1O_2$. On the other hand the effect could be totally unspecific, such that any haem would generate hydroxylamine oxides (in the presence of $^1O_2$ and secondary or tertiary amines) which would undergo rapid dehydration; the resulting nitrone intermediates would then liberate formaldehyde upon nonenzymatic hydrolisis. Our finding is however of sufficient importance to be reported, although a full understanding of the mechanism is still lacking.

We have no doubt that $^1O_2$ is being generated in our system. The unaerobic repression and the D_2O enhancement of all the effects (data not always shown) and the varying levels of protection afforded by azide provide strong evidence for the role of oxygen, particularly $^1O_2$ in the photodynamic damage to microsomal membrane components. Several enzymes have been inactivated by mechanisms involving $^1O_2$ [32]-[36]; unsaturated fatty acids have been photooxidized [37]-[39] to hydroperoxides [4] by mechanisms involving $^1O_2$. The possibility of generating other oxygen radicals from triplet sensitizers, such as Rose Bengal, cannot be ruled out, but electron transfer from sensitizer to oxygen leading to the superoxide ion is very inefficient [2]. In conclusion, it seems likely that the differential photodynamic effects on the components of the microsomal electron transport system are primarily singlet oxygen mediated and that further studies are required to determine the reaction rates of $^1O_2$ with the membrane components and to determine the involvement of other oxygen intermediates in the damage mechanisms.

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