Induction of Benzo[a]pyrene Mono-oxygenase in Liver Cell Culture by the Photochemical Generation of Active Oxygen Species

EVIDENCE FOR THE INVOLVEMENT OF SINGLET OXYGEN AND THE FORMATION OF A STABLE INDUCING INTERMEDIATE

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1. The photochemical generation of excited states of oxygen in liver cell culture by the mild illumination of culture medium containing riboflavin, results in stimulation of benzo[a]pyrene 3-mono-oxygenase, a cytochrome *P*-450-linked mono-oxygenase.
2. The same large increase in mono-oxygenase activity was found when medium containing riboflavin was illuminated in the absence of cells and then stored in the dark for 24h before contact with the cells. From this it may be inferred that stimulation is due to the formation of a stable inducer in the culture medium. Further experiments indicate that the stable inducer is due to the photo-oxidation of an amino acid.
3. Evidence that singlet oxygen is responsible for initiating the stimulation of the mono-oxygenase is based on the use of molecules that scavenge particular active oxygen species. Of all the scavengers tested, only those that scavenge singlet oxygen inhibited the stimulation.
4. A hypothesis is developed to relate the stimulation of the mono-oxygenase by singlet oxygen in cultured cells to the regulation of the cytochrome *P*-450 enzyme system *in vivo*. It is suggested that singlet oxygen generation within cells may be a common factor linking the many structurally diverse inducers of the enzyme system.

The properties that allow numerous pharmacological and structurally unrelated compounds to induce the activity of the hepatic microsomal cytochrome *P*-450-linked mono-oxygenases are unknown (Conney, 1967; Kuntzman, 1969). An attractive hypothesis is that induction by many diverse compounds is mediated through a common endogenous inducer (Marshall & McLean, 1971). Excited states of oxygen such as the superoxide ion (O_2^-) are possible candidates for a common denominator in induction, as the photochemical generation of these species in liver cell culture, by the mild illumination of medium containing riboflavin, results in a typical induction of benzo[a]pyrene mono-oxygenase activity (EC 1.14.14.2), commonly referred to as aryl hydrocarbon hydroxylase (Paine & McLean, 1974*a,b*).

This proposal seems to be of special significance, as the superoxide ion (O_2^-) is envisaged in the mechanism of oxygen activation and substrate oxidation by cytochrome *P*-450 (Coon *et al.*, 1973; Estabrook *et al.*, 1973). In addition, both microsomal suspensions (Debry & Balny, 1973) and a

purified microsomal enzyme involved in cytochrome *P*-450 reduction (Aust *et al.*, 1972) generate O_2^- during electron transfer. These findings suggest that inducing compounds could permit a leak of O_2^- within cells, from their site of metabolism on cytochrome *P*-450, which results in induction.

However, it is difficult to assign a precise identity to the species responsible for induction in cell culture, as the riboflavin molecule sensitized by visible-light absorption can undergo either photoreduction, in the presence of electron donors, such as methionine in the cell culture medium, to generate the superoxide ion (Ballou *et al.*, 1969; Lippitt & Fridovich, 1973), or else intermolecular energy transfer to ground-state oxygen (3O_2) to generate singlet oxygen (1O_2), which is produced by a re-arrangement of electrons in the oxygen molecule (Kasha & Khan, 1970; Khan & Kasha, 1970). Since the illumination of culture medium containing riboflavin generates more than one excited state of oxygen, and the interactions between the products of illumination can give rise to free radicals such as the hydroxyl radical (Beauchamp & Fridovich, 1970), the work reported here was undertaken to ascertain if one of these species is responsible for induction in cell culture, and hence to determine the way in which excited states of

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oxygen may be involved in induction in the whole animal.

Materials and Methods

Materials

Cells of the rat liver epithelial cell line (Paine & McLean, 1974c) and adult-rat liver cells previously described by Montesano *et al.* (1973), given by Dr. K. Rees, Department of Biochemical Pathology, University College Hospital Medical School, London, were grown in Dulbecco's modification of Eagle's medium supplemented with 5% foetal calf serum, 100 i.u. of penicillin/ml and 100 µg of streptomycin/ml (Flow Laboratories, Irvine, Scotland, U.K.). The cells, when just confluent, were illuminated in the above medium (final volume 6ml) with or without riboflavin (British Drug Houses, Poole, Dorset, U.K.) by a 60W household tungsten lamp, 17cm from the culture flasks (25cm² Falcon bottles: Gibco-Biocult Ltd., Paisley, Scotland, U.K.). The cells were maintained at 37°C both during and after exposure to light. Medium containing benz[a]-anthracene was prepared as previously described (Paine & McLean, 1974c).

Special media of different composition were made from Earle's balanced salt solution without glucose (10×normal strength), with added 10% (w/v) glucose, 'Dulbecco's modification of Eagle's medium amino acids×25 strength', 'non-essential amino acids×100 strength' and 'Dulbecco's modification of Eagle's medium' vitamins×25 strength' (Flow Laboratories).

Triethylenediamine (1,4-diazabicyclo[2,2,2]octane), Tiron (1,2-dihydroxybenzene-3,5-disulphonic acid disodium salt), Triton X-100, ethanol, mannitol and sodium benzoate were obtained from British Drug Houses. Benz[a]anthracene, β -carotene type 1 and Nitro Blue Tetrazolium grade III were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. A crystalline suspension of catalase (EC 1.11.1.6) in water, saturated with thymol (specific activity 39000 µmol of H₂O₂ decomposed/min per mg) was purchased from Boehringer Corp., London W5 2TZ, U.K. Superoxide dismutase (EC 1.15.1.1) was prepared from human erythrocytes, from out-of-date blood as described by McCord & Fridovich (1969). The specific activity of purified enzyme was 3500 units/mg of enzyme. Crystalline bovine serum albumin was purchased from Armour Pharmaceuticals, Eastbourne, Sussex, U.K.

Methods

Enzyme assays. Benzo[a]pyrene 3-mono-oxygenase (EC 1.14.14.2) activity was assayed in cell homogenates as previously described (Paine & McLean,

1974c). The mono-oxygenase activity is expressed as pmol of 3-hydroxybenzopyrene formed/30min per mg of cell protein.

Superoxide dismutase was assayed by the cytochrome *c* reduction procedure as described by McCord & Fridovich (1969).

Catalase was assayed by the method described by Luck (1963).

Other determinations. Cell protein was assayed by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as the standard.

H₂O₂ was determined as described by Egerton *et al.* (1954). β -Carotene was measured by centrifuging culture medium to which β -carotene had been added, at 2500 rev./min for 10min in an MSE bench centrifuge to remove any undissolved β -carotene. Then a 2ml portion of the medium was made up to 3ml with 0.15M-NaCl. To this solution 3ml of ethanol was slowly added with shaking, to precipitate the calf serum proteins; this was followed by the addition of 2ml of hexane and the mixture was vigorously shaken in stoppered tubes for 10min to extract the β -carotene into the hexane phase. The mixture was then centrifuged at 1500 rev./min for 10min to clarify the phases, and the upper (hexane) phase was removed and the E_{440} measured and compared with that of culture medium without added β -carotene, which had been treated in the same way, and to a standard curve obtained from solutions containing up to 10 µg of β -carotene/ml of hexane.

Nitro Blue Tetrazolium reduction by the superoxide ion produced during the photoreduction of riboflavin was measured by determination of the formazan production measured at 568nm essentially as described by Lippit & Fridovich (1973). The rate of formazan production in the absence of cells was measured under the same conditions used for illuminating cultures of cells. The reaction mixture (final volume 6ml) in a 25cm² Falcon culture flask contained 0.17mM-Nitro Blue Tetrazolium and 15 µM-riboflavin in Dulbecco's modification of Eagle's medium containing 5% foetal calf serum but without the Phenol Red pH indicator. It is necessary to omit the Phenol Red pH indicator, as in the bicarbonate-buffered culture medium the pH of the sample and reference cuvettes can vary slightly, causing the pH indicator, which absorbs at the same wavelength as formazan, to interfere with the determination.

Results

Table 1 shows that when cultured rat liver cells were illuminated in a medium containing 15 µM-riboflavin there was a marked increase in the activity of benzo[a]pyrene mono-oxygenase. Treatment of cells with riboflavin but without

Table 1. Effect of illumination time on the activity of benzo[a]pyrene mono-oxygenase in rat liver cells cultured with and without 15 μ M-riboflavin

Duplicate flasks (25cm² Falcon bottles) of rat liver epithelial cells cultured in Dulbecco's modification of Eagle's medium, with or without 15 μ M-riboflavin (final volume 6ml), were illuminated by a 60 W household tungsten lamp placed 17cm from the culture flasks for 1 or 24h. The cells were maintained at 37°C throughout the experiment and 24h after illumination commenced the cells were harvested and the mono-oxygenase activity (expressed as pmol of 3-hydroxybenzopyrene formed/30min per mg of cell protein) were assayed. The illumination of medium containing riboflavin concentrations greater than 20 μ M produced cytotoxic effects. A 24h exposure, in the dark, to medium containing 17.5 μ M-benz[a]anthracene induced the mono-oxygenase to a specific activity of 186pmol/30min per mg of protein. Variation in mono-oxygenase activity between duplicate flasks of cells from the same subculture rarely exceeded 10%.

Illumination time (h)	Mono-oxygenase activity (pmol/30min per mg of protein)	
	No riboflavin	+ 15 μ M-riboflavin
0	30	35
1	29	187
24	23	198

illumination, or exposure to light without riboflavin, gave no increase in the mono-oxygenase activity, whereas the mild illumination of a culture medium containing 15 μ M-riboflavin was as effective as medium containing a maximal inducing concentration of the classical inducer benz[a]anthracene. These same large increases in mono-oxygenase activity were found whether illumination with riboflavin was for 1h followed by 23h in a dark incubator, or continuously for 24h (Table 1). Since the generation of active oxygen species by the riboflavin system is dependent on visible-light absorption, the maximal stimulation of the mono-oxygenase 23h after 1h illumination cannot be due to their continued production after illumination ceases. Indeed the generation of the superoxide ion, as detected by its ability to reduce Nitro Blue Tetrazolium to formazan (Lippitt & Fridovich, 1973), ceases immediately when illumination stops. In addition, culture medium containing riboflavin that has been illuminated in the absence of cells and then stored in the dark at 4°C or 37°C for 24h before contact with the cells will still enhance the mono-oxygenase activity. This result suggests that stimulation of the mono-oxygenase by the riboflavin-and-light system is due to the formation of a stable inducer in the culture medium rather than to an excited oxygen species directly activating the inducing mechanism inside the cell. From this consideration

the following experiments to determine the nature of the stable inducer were carried out by illuminating culture medium on its own, before contact with the cells.

Nature of the stable inducer

The stable inducer is not formed as a result of peroxidative attack on the plastic culture flask, as the illumination of culture medium containing 15 μ M-riboflavin in a glass vessel still induced the mono-oxygenase when placed in contact with the cells.

Culture medium containing 15 μ M-riboflavin that had been illuminated for 1h in the absence of cells had a H₂O₂ concentration of 13 μ M. H₂O₂ is probably produced by the non-enzymic dismutation of O₂⁻, as shown in eqn. (1) (Khan, 1970; Goda *et al.*, 1974).

However, the incorporation of 0.13 μ M-catalase (7800units/6ml) sufficient to remove 10⁵ times this concentration of H₂O₂ was without effect on the stimulation of the mono-oxygenase. Although a 50% loss in catalase activity was found after a 1h illumination of medium containing 15 μ M-riboflavin, H₂O₂ was not detectable. Similarly, the stable inducer cannot be due to a riboflavin-breakdown product, as riboflavin photolysis is practically eliminated in the presence of certain amino acids, such as methionine, which are present in the culture medium. These amino acids protect riboflavin from photolysis by acting as electron donors instead of the ribityl side chain (Knobloch, 1971). In addition, when riboflavin photolysis was allowed to proceed by illuminating Earle's balanced salt solution containing 15 μ M-riboflavin, that is in the absence of added electron donors, induction of the mono-oxygenase did not occur.

The Dulbecco's modification of Eagle's medium used to culture cells of the rat liver epithelial line consists of six components, namely a mixture of inorganic salts to maintain iso-osmoticity and buffering capacity called Earle's balanced salt solution, 25mM-glucose, a mixture of vitamins, 5% (v/v) foetal calf serum, a mixture of non-essential amino acids and an essential amino acid mixture (Dulbecco & Freeman, 1959; *Flow Manual*, Flow Laboratories). To determine which of these components gives rise by photo-oxidation, to the stable inducer, Earle's balanced salt solution containing 15 μ M-riboflavin and 0.2mM-methionine, as an electron donor, which is referred to as 'basal medium' for brevity in Table 2, was illuminated with each individual component of the culture medium for 1h. The medium was then completed by adding the missing components and placed in contact with the

Table 2. *Effect of illuminating Earle's balanced salt solution containing 15 μ M-riboflavin and 0.2 mM-methionine (basal medium) with each component of Dulbecco's modification of Eagle's medium (DMEM) on stimulation of the mono-oxygenase in adult-rat liver cells*

Duplicate 25 cm² Falcon flasks containing Earle's balanced salt solution, without glucose and the Phenol Red pH indicator but containing 15 μ M-riboflavin and 0.2 mM-methionine (referred to in the Table as basal medium for brevity), were illuminated with the individual medium components, for 1 h, in the absence of cells as described in Table 1. After illumination ceased these special media were supplemented with the components omitted during illumination, so that all media had the same formulation as Dulbecco's modification of Earle's medium (DMEM) when placed in contact with the cells. This was done by adding the required volume of the respective concentrates of the medium components which were 'DMEM Vitamins $\times$ 25 strength', 10% (w/v) glucose, foetal calf serum, 'non-essential amino acids $\times$ 100 strength' and 'DMEM amino acids $\times$ 25 strength', all of which were purchased from Flow Laboratories. At 23 h after contact with these media the cells were harvested and the mono-oxygenase activity (expressed as pmol of 3-hydroxybenzopyrene formed/30 min per mg of cell protein) was assayed.

Addition to basal medium	Benzo[a]pyrene mono-oxygenase activity (pmol/30 min per mg of protein)
None	23
'DMEM' mixed vitamins	24
25 mM-Glucose	22
5% (w/v) calf serum	39
'DMEM' mixed non-essential amino acids	39
'DMEM' mixed essential amino acids	180
Complete medium (i.e. DMEM)	185

cells. Table 2 shows that the oxidation of methionine, which is evidenced by a characteristic odour of decaying cabbage, is not responsible for inducing the mono-oxygenase, as the illumination of basal medium containing only riboflavin and methionine did not stimulate the mono-oxygenase. The only component of the culture medium which was effective in stimulating the mono-oxygenase after illumination with riboflavin was the mixture of essential amino acids. Exactly the same result was obtained when the disodium dicalcium salt of EDTA (0.1 mM) was used as the electron donor instead of methionine.

Of the 13 amino acids (arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine, present in the essential-amino acid mixture only histidine, methionine, tryptophan and tyrosine are photo-oxidized at appreciable rates on illumination in the presence of sensitizing dyes, such as

riboflavin (Spikes & MacKnight, 1970). However, preliminary experiments in which each of these amino acids was individually illuminated with 15 μ M-riboflavin in Earle's balanced salt solution containing 0.1 mM-EDTA failed to stimulate the mono-oxygenase activity.

It is probable that in a mixture of amino acids, competition between amino acids for the oxidizing species occurs. Since many products can be formed from the photo-oxidation of a single amino acid (Spikes & MacKnight, 1970), it seems likely that competition between amino acids in a mixture results in qualitative as well as quantitative differences in the oxidation products produced from an individual amino acid. Thus the illumination of a single amino acid with riboflavin would not produce the same products as when photo-oxidized in a mixture and hence would not induce the mono-oxygenase. It seems that the more complex experiment in which each individual amino acid is removed from the medium is necessary to determine the identity of the stable inducer. Whatever its nature, it is noteworthy that the mild illumination of culture medium containing riboflavin has converted a physiological component of the medium, which is not normally an inducer, into a compound which is as effective a stimulator of the mono-oxygenase as the classical inducer benz[a]anthracene (Table 1).

Effect of superoxide dismutase

If the superoxide ion (O_2^-) is the effective molecule in initiating the stimulation of the mono-oxygenase by the riboflavin-and-light system, then superoxide dismutase, which scavenges this species by catalysing the dismutation reaction in eqn. (2) (McCord & Fridovich, 1969; Goda *et al.*, 1974), should prevent the stimulation.

Fig. 1 shows the reduction of Nitro Blue Tetrazolium to formazan by O_2^- produced during the continuous illumination of a culture medium containing 15 μ M-riboflavin. The incorporation of 0.3 μ M-0.6 μ M- and 1.2 μ M-superoxide dismutase, assuming a mol.wt. of 34000 (Hartz & Deutsch, 1969) into the culture medium greatly decreased the rate of formazan production, during a 1 h illumination under the same conditions as when the cells were illuminated (Fig. 1). It is noteworthy that formazan production by the riboflavin-and-light systems is proportional to the riboflavin concentration, and the formazan produced in the presence of 1.2 μ M-dismutase and 15 μ M-riboflavin is equivalent to a 1 h illumination of culture medium containing only 1 μ M-riboflavin, a treatment that is not enough to stimulate the mono-oxygenase. Even though Fig. 1 shows that

Fig. 1. Reduction of Nitro Blue Tetrazolium to formazan by O_2^- in Dulbecco's modification of Eagle's medium containing $15 \mu\text{M}$ -riboflavin + superoxide dismutase

Dulbecco's modification of Eagle's medium containing $15 \mu\text{M}$ -riboflavin and 0.17 mM -Nitro Blue Tetrazolium plus 5% calf serum, but without the Phenol Red pH-indicator (final volume 6ml), was illuminated in the absence of cells in a 25 cm^2 Falcon bottle as described in the Materials and Methods section. Line (a) (○) shows formazan production during continuous illumination in the absence of superoxide dismutase. Lines (b), (c) and (d) show the formazan produced during the continuous illumination of medium containing $15 \mu\text{M}$ -riboflavin and $0.3 \mu\text{M}$ - (●), $0.6 \mu\text{M}$ - (□) and $1.2 \mu\text{M}$ - (■) superoxide dismutase respectively. Line (e) shows the absorbance due to continuous illumination in the absence of the dismutase, of a medium containing Nitro Blue Tetrazolium but without riboflavin, or medium without Nitro Blue Tetrazolium but with $15 \mu\text{M}$ -riboflavin. Such blanks had the same absorption at 568 nm as non-illuminated medium containing both $15 \mu\text{M}$ -riboflavin and Nitro Blue Tetrazolium.

there was sufficient dismutase present to scavenge all the superoxide ions produced, culture medium containing $0.3 \mu\text{M}$ -, $0.6 \mu\text{M}$ - or $1.2 \mu\text{M}$ -dismutase was without effect on the stimulated mono-oxygenase activity found 23h after a 1h illumination of rat liver cells cultured in a medium containing $15 \mu\text{M}$ -riboflavin (Table 3).

The variation in the final induced activity of the mono-oxygenase between experiments is due to the number of times the cells have been passaged (Paine,

1976). Such variation makes a statistical analysis of the results from different subcultures difficult. However, it is noteworthy that in all the experiments, the illumination of culture medium containing riboflavin is always as effective a stimulator of the mono-oxygenase as the classical inducer benz[a]-anthracene. Since the variation in mono-oxygenase activity between duplicate flasks of cells from the same subculture rarely exceeds 10%, the results in Table 3 clearly demonstrate that the dismutase is without effect on the increased mono-oxygenase activity found 23h after a 1h illumination of rat liver cells cultured in a medium containing $15 \mu\text{M}$ -riboflavin. In addition, as the dismutase produces ground-state (eqn. 2) rather than singlet-state (1O_2) oxygen (Goda *et al.*, 1974), which is produced via the non-enzymic dismutation (eqn. 1), it seems that the production of singlet oxygen by the non-enzymic dismutation of the superoxide ion is also not responsible for stimulating the mono-oxygenase.

Certain reactions of systems that are known to generate O_2^- can be ascribed to hydroxyl radicals which are produced by the interaction of O_2^- with H_2O_2 , the product of O_2^- dismutation as shown in eqn. (3) (Beauchamp & Fridovich, 1970).

However, superoxide dismutase usually inhibits these reactions (Beauchamp & Fridovich, 1970). The lack of effect of superoxide dismutase on the mono-oxygenase induction suggests that hydroxyl radicals are not responsible for initiating the induction of benzo[a]pyrene mono-oxygenase. This conclusion is confirmed by the lack of effect of hydroxyl-radical scavengers, such as ethanol, mannitol and sodium benzoate, which when incorporated into the culture medium at concentrations found to be effective in other systems (Fong *et al.*, 1973) had no effect on the stimulation of the mono-oxygenase by the riboflavin-and-light system (Table 3).

Effect of singlet oxygen quenchers on mono-oxygenase induction

Triethylenediamine (1,4-diazabicyclo[2,2,2]octane) and β -carotene have been shown specifically to quench singlet oxygen (Ouannès & Wilson, 1968; Foote *et al.*, 1970). Culture medium containing $1 \mu\text{M}$ - β -carotene or 5 mM -triethylenediamine had no effect on the rate of Nitro Blue Tetrazolium reduction during a 1h illumination of culture medium containing $15 \mu\text{M}$ -riboflavin, confirming that these quenchers do not scavenge the superoxide ion.

However, when rat liver cells were illuminated for 1h in a medium containing $15 \mu\text{M}$ -riboflavin and 1 mM -triethylenediamine, the stimulation of benzo[a]-pyrene mono-oxygenase was inhibited by 40%. Increasing the triethylenediamine concentration to

Table 3. *Lack of effect of superoxide dismutase and hydroxyl-radical scavengers on the stimulation of benzo[a]pyrene mono-oxygenase*

Superoxide dismutase, mannitol, ethanol or sodium benzoate were added to rat liver epithelial cells cultured in Dulbecco's modification of Eagle's medium containing 5% calf serum at the same time as riboflavin or benz[a]anthracene. Then duplicate flasks of cells were illuminated as described in Table 1 for 1 h, followed by 23 h in the dark before assay of the mono-oxygenase activity (expressed as pmol of 3-hydroxybenzopyrene formed/30min per mg of protein). Cells cultured with 17.5 μ M-benz[a]anthracene were maintained in the dark throughout the experiment. Variation in mono-oxygenase activity between duplicate flasks of cells from the same subculture rarely exceeded 10%.

	Addition to medium	...	Benzo[a]pyrene mono-oxygenase activity		
			None	15 μ M-riboflavin+light	17.5 μ M-benz[a]anthracene—light
Expt. 1	None		22	100	84
	Superoxide dismutase (0.3 μ M)		20	99	86
	Superoxide dismutase (0.6 μ M)		20	98	78
	Superoxide dismutase (1.2 μ M)		27	95	77
Expt. 2	None		24	96	115
	3mM-Mannitol		23	96	112
	8mM-Ethanol		24	91	107
	3mM-Sodium benzoate		23	100	121

Table 4. *Effect of the singlet-oxygen quencher triethylenediamine on the stimulation of the mono-oxygenase*

Duplicate flasks of rat liver epithelial cells cultured in Dulbecco's modification of Eagle's medium containing the respective concentration of triethylenediamine were illuminated with and without riboflavin for 1 h as described in Table 1, and then placed in a dark incubator for 23 h before harvesting the cells and assaying the mono-oxygenase activity (expressed as pmol of 3-hydroxybenzopyrene formed/30min per mg of cell protein). Cells cultured with 17.5 μ M-benz[a]anthracene were kept in the dark throughout the experiment. Variation in mono-oxygenase activity between duplicate flasks of cells from the same subculture rarely exceeded 10%. Repetition of this experiment on two other separate occasions produced similar results.

Treatment	Concn. of triethylenediamine in medium	...	Benzo[a]pyrene mono-oxygenase activity		
			0	1 mM	5mM
None			22	24	25
15 μ M-Riboflavin and light			192	114	84
17.5 μ M-Benz[a]anthracene without light			166	164	182

5mM produced 56% inhibition of the mono-oxygenase activity enhanced by the riboflavin-and-light system (Table 4). In three experiments carried out on separate occasions, the presence of 1mM-triethylenediamine in a culture medium containing 15 μ M-riboflavin consistently inhibited the stimulation of the mono-oxygenase by 40%. Although culture medium containing 10mM-triethylenediamine produced cytotoxic effects, the finding that culture medium containing 1 mM- or 5 mM-triethylenediamine

is without effect on the mono-oxygenase activity induced by the classical inducer benzanthracene (Table 4) suggests that triethylenediamine does not inhibit the stimulation of the mono-oxygenase by the illumination of a culture medium containing 15 μ M-riboflavin by its cytotoxic effects.

Table 5 shows that the other singlet oxygen quencher, β -carotene, is even more effective in preventing the enhancement of the mono-oxygenase by the riboflavin-and-light system. Culture medium containing 15 μ M-riboflavin and the highest practical concentration of β -carotene (1 μ M) inhibited the stimulation of the mono-oxygenase by 48%. This 1000-fold difference in the concentration of β -carotene and triethylenediamine required to produce the same degree of inhibition in the stimulation of the mono-oxygenase is in accord with their respective rate constants for singlet oxygen quenching (Foote *et al.*, 1970).

Although the β -carotene concentration of the culture medium is dependent on the concentration of Triton X-100 used as a surfactant, higher concentrations of β -carotene could not be obtained, as medium containing more than 20 μ g of Triton X-100/ml was cytotoxic. However, Table 5 shows that lower concentrations of Triton are without effect on the stimulation of the mono-oxygenase by the riboflavin-and-light system or the classical inducer benz[a]anthracene.

Homogenates from cells cultured in media containing β -carotene were yellow, owing to adhering β -carotene. Although many compounds structurally similar to vitamin A have been shown to inhibit benzo[a]pyrene mono-oxygenase activity *in vitro* (Hill & Shih, 1974), β -carotene was not found to inhibit the mono-oxygenase activity. Indeed if

Table 5. *Effect of culture medium containing β -carotene and Triton X-100 as a surfactant on the stimulation of the mono-oxygenase in cultured liver cells*

Duplicate flasks of rat liver epithelial cells cultured in Dulbecco's modification of Eagle's medium containing 5% calf serum, with or without Triton X-100 (17 μ g/ml) and β -carotene (1 μ M), were illuminated (as described in Table 1) with and without riboflavin for 1 h followed by 23 h incubation in the dark before harvest and assay of the mono-oxygenase activity (expressed as pmol of 3-hydroxybenzopyrene formed/30 min per mg of protein). Cells cultured in medium containing 17.5 μ M-benz[a]anthracene with or without Triton X-100 and β -carotene were incubated in the dark for 24 h. Before illumination the β -carotene concentration of the culture medium was determined as described in the Materials and Methods section. Variation in mono-oxygenase activity between duplicate flasks of cells from the same subculture rarely exceeded 10%. Repetition of this experiment on two other separate occasions produced similar results.

Treatment	Triton X-100	β -Carotene	Mono-oxygenase activity (pmol/30 min per mg of protein)
None	—	—	22
None	+	—	25
None	+	+	20
15 μ M-Riboflavin+light	—	—	116
15 μ M-Riboflavin+light	+	—	106
15 μ M-Riboflavin+light	+	+	60
17.5 μ M-Benzanthracene—light	—	—	98
17.5 μ M-Benzanthracene—light	+	—	88
17.5 μ M-Benzanthracene—light	+	+	89

Table 6. *Effect of Tiron on the stimulation of mono-oxygenase activity by the riboflavin-light system and by the classical inducer benz[a]anthracene*

Tiron (disodium 1,2-dihydroxybenzene-3,5-disulphonate) previously neutralized with 1M-NaOH was added to rat liver epithelial cells cultured in a special Dulbecco's modification of Eagle's medium without Fe^{3+} ions plus 5% (v/v) calf serum at the same time as the riboflavin. Then duplicate flasks of cells were illuminated, as described in Table 1, for 1 h, followed by 23 h in the dark, before assay of the mono-oxygenase activity (expressed as pmol of 3-hydroxybenzo[a]pyrene formed/30 min per mg of protein). Cells cultured with 17.5 μ M-benz[a]anthracene were maintained in the dark throughout the experiment. Variation in mono-oxygenase activity between duplicate flasks of cells from the same subculture rarely exceeded 10%.

Treatment	0.1 mM-Tiron	Mono-oxygenase activity (pmol/30 min per mg of protein)	
		Expt. 1	Expt. 2
None	—	23	24
None	+	31	Not determined
15 μ M-Riboflavin+light	—	99	96
15 μ M-Riboflavin+light	+	47	27
17.5 μ M-Benzanthracene	—	114	Not determined
17.5 μ M-Benzanthracene	+	120	Not determined

β -carotene, and for that matter triethylenediamine, carried across to the assay were responsible for inhibiting the mono-oxygenase activity induced by

the riboflavin-and-light system, they should also decrease the induction by benzanthracene, an effect which was not observed (Tables 4 and 5).

Effect of Tiron on the stimulation of the mono-oxygenase

Tiron (1,2-dihydroxybenzene-3,5-disulphonic acid disodium salt), like superoxide dismutase, has been shown to inhibit several of the actions of xanthine oxidase (EC 1.2.3.2), all of which are secondary consequences of superoxide generation by this enzyme (Fridovich & Handler, 1962; Greenlee *et al.*, 1962). Tiron, like superoxide dismutase, is also an effective inhibitor of Nitro Blue Tetrazolium reduction by photo-reduced tetra-acetyl-riboflavin, a known source of the superoxide ion (Miller, 1970). These studies suggest that Tiron directly scavenges the superoxide ion. However, in contrast with the experiments with superoxide dismutase, Table 6 shows that when rat liver cells were illuminated with 15 μ M-riboflavin in a special Dulbecco's modification of Eagle's medium, which was without Fe^{3+} ions, as Tiron is also an iron chelator (Fridovich & Handler, 1962), the presence of 0.1 mM-Tiron completely prevented the stimulation of the mono-oxygenase in one experiment and inhibited the stimulation by more than 80% in another, although it was without effect on the induction of the mono-oxygenase benz[a]-anthracene.

Further, 0.1 mM-Tiron inhibits Nitro Blue Tetrazolium reduction by the riboflavin-and-light system by 80%. As Tiron and superoxide dismutase are both effective inhibitors of Nitro Blue Tetrazolium reduction, and hence superoxide production by the

Scheme 1. Some of the possible excited states of oxygen generated by the riboflavin-and-light system

riboflavin-and-light system, whereas only Tiron inhibits the increase in mono-oxygenase activity it seems that Tiron and the dismutase must prevent superoxide generation by different mechanisms. If, as suggested by the results above, singlet oxygen is responsible for initiating the stimulation of the mono-oxygenase activity, then Tiron must also prevent the production of this species. Thus it seems probable that Tiron quenches the energy of the riboflavin molecule sensitized by visible-light absorption and hence prevents the production of both singlet oxygen and superoxide ions rather than quenching free superoxide ions in solution, as is the case for the dismutase. Although it is possible to determine singlet-oxygen production by measuring the disappearance of molecules which react with singlet oxygen, it has not been possible to make use of these 'trapping' molecules in the present work, as they are either not oxidized at an appreciable rate by the riboflavin-and-light system (i.e. tetracycline, described by Finazzi-Agrò *et al.*, 1973) or are light sensitive (i.e. 1,3-diphenylisobenzofuran, described by Ouannès & Wilson, 1968).

The way in which the compounds used in the present work are thought to interact with excited states of oxygen produced by the riboflavin-and-light system is summarized in Scheme 1.

Discussion

The induction of the hepatic microsomal cytochrome *P*-450 enzyme system, which includes benzo[*a*]pyrene mono-oxygenase by many pharmacological and structurally diverse compounds in the whole animal (Conney, 1967) and in liver cell culture

(Nebert & Gelboin, 1968; Gielen & Nebert, 1971, 1972; Owens & Nebert, 1975) involves DNA-dependent RNA synthesis and requires protein synthesis. However, the intracellular site of action, as well as the properties that allow many diverse compounds to induce the enzyme system, are unknown (Conney, 1967; Kuntzman, 1969). 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.* (1971). It seems more likely that some common denominator exists between the many inducers. The finding that the increase in mono-oxygenase activity by singlet oxygen studied in the present work appears to be similar to that produced by the classical inducer benz[*a*]anthracene (Paine & McLean, 1974b) suggests that singlet oxygen may be the common factor in induction by many diverse compounds. This proposal may be of special significance, as it seems likely that singlet oxygen can be produced inside cells during electron flow in the endoplasmic reticulum, as microsomal suspensions generate singlet oxygen during NADPH oxidation (King *et al.*, 1975). A possible source of singlet oxygen may be the non-enzymic dismutation of the superoxide intermediate (Khan, 1970) proposed in the mechanism of oxygen activation by cytochrome *P*-450 (Coon *et al.*, 1973; Estabrook *et al.*, 1973). It is therefore conceivable that inducers generate singlet oxygen inside cells by binding to cytochrome *P*-450 and stimulating electron flow from NADPH. Such a working hypothesis, which may account for the great diversity of inducers by utilizing the well-established versatility of cytochrome *P*-450, could provide a common

mechanism for all induction systems. Thus the classical inducers could generate singlet oxygen inside the cell during oxidation, whereas the non-substrate riboflavin plus light, in the model system *in vitro*, generates singlet oxygen outside the cell in this way; the same basic sequence of events can be initiated inside the cell by either means.

If inducers do produce singlet oxygen inside cells, then the singlet oxygen quenchers used in this work could be expected to inhibit the mono-oxygenase induction by the classical inducer. However, because an exposure to benz[a]anthracene as short as 1 min will induce the mono-oxygenase in cultured cells (Gelboin *et al.*, 1972) it seems probable that the simultaneous addition of inducer and singlet-oxygen quencher in the present work did not allow sufficient time for the quencher to reach the concentration inside cells that may inhibit induction by benz[a]-anthracene. When cells were cultured in the presence of the singlet oxygen quenchers for 24h before and during contact with benz[a]anthracene, the induction of the mono-oxygenase was indeed inhibited. However, after 48h contact with either β -carotene and Triton X-100 or triethylenediamine the cells were extremely vacuolated, making it difficult to interpret whether the inhibition of the mono-oxygenase induction by benz[a]anthracene was directly due to the singlet-oxygen quenching properties of these molecules.

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References

- Aust, S. D., Roerig, D. L. & Pederson, T. C. (1972) *Biochem. Biophys. Res. Commun.* **47**, 1133-1137
- Ballou, D., Palmer, G. & Massey, V. (1969) *Biochem. Biophys. Res. Commun.* **36**, 898-904
- Beauchamp, C. & Fridovich, I. (1970) *J. Biol. Chem.* **245**, 4641-4646
- Conney, A. H. (1967) *Pharmacol. Rev.* **19**, 317-366
- Coon, M. J., Strobel, H. W. & Boyer, R. F. (1973) *Drug Metab. Dispos.* **1**, 92-97
- Debry, P. & Balny, C. (1973) *Biochimie* **54**, 329-332
- Dulbecco, R. & Freeman, G. (1959) *Virology* **8**, 396-397
- Estabrook, R. W., Matsubara, T., Mason, J. I., Werrigloer, J. & Baron, J. (1973) *Drug Metab. Dispos.* **1**, 98-110
- Egerton, A. C., Everett, A. J., Minhoff, G. J., Rudrakanchana, S. & Salooja, K. C. (1954) *Anal. Chim. Acta* **10**, 422-428
- Finazzi-Agrò, A., De Sole, P., Rotilio, G. & Mondovi, B. (1973) *Ital. J. Biochem.* **22**, 217-231
- Fong, K. L., McCay, P. B., Poyer, J. L., Keele, B. B. & Misra, H. (1973) *J. Biol. Chem.* **248**, 7792-7797
- Foot, C. S., Denny, R. W., Weaver, L., Chang, Y. & Peters, J. (1970) *Ann. N.Y. Acad. Sci.* **171**, 139-148
- Fridovich, I. & Handler, P. (1962) *J. Biol. Chem.* **237**, 916-921
- Gelboin, H. V., Weibel, F. J. & Kinoshita, N. (1972) *Biochem. Soc. Symp.* **34**, 103-135
- Gielen, J. E. & Nebert, D. W. (1971) *J. Biol. Chem.* **246**, 5189-5198
- Gielen, J. E. & Nebert, D. W. (1972) *J. Biol. Chem.* **247**, 7591-7602
- Goda, K., Kimura, T., Thayer, A. L., Kees, K. & Scharp, A. P. (1974) *Biochem. Biophys. Res. Commun.* **58**, 660-666
- Greenlee, L., Fridovich, I. & Handler, P. (1962) *Biochemistry* **1**, 779-783
- Hartz, J. W. & Deutsch, H. F. (1969) *J. Biol. Chem.* **244**, 4565-4572
- Hill, D. L. & Shih, T. W. (1974) *Cancer Res.* **34**, 564-570
- Kasha, M. & Khan, A. U. (1970) *Ann. N.Y. Acad. Sci.* **171**, 5-23
- Khan, A. U. (1970) *Science* **168**, 476-477
- Khan, A. U. & Kasha, M. (1970) *Ann. N.Y. Acad. Sci.* **171**, 24-33
- King, M. M., Lai, E. K. & McCay, P. B. (1975) *J. Biol. Chem.* **250**, 6496-6502
- Knobloch, E. (1971) *Methods Enzymol.* **18**, 305-368
- Kuntzman, R. (1969) *Annu. Rev. Pharmacol.* **9**, 21-36
- Lippitt, B. & Fridovich, I. (1973) *Arch. Biochem. Biophys.* **159**, 738-741
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Luck, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 885-888, Verlag Chemie/Academic Press, London and New York
- Marshall, W. J. & McLean, A. E. M. (1971) *Biochem. J.* **122**, 569-573
- McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049-6055
- Miller, R. W. (1970) *Can. J. Biochem.* **48**, 935-939
- Montesano, R., Saint-Vincent, L. & Tomatis, L. (1973) *Br. J. Cancer* **28**, 215-220
- Nebert, D. & Gelboin, H. V. (1968) *J. Biol. Chem.* **243**, 6250-6261
- Ouannès, C. & Wilson, T. (1968) *J. Am. Chem. Soc.* **90**, 6527-6528
- Owens, I. S. & Nebert, D. W. (1975) *Mol. Pharmacol.* **11**, 94-104
- Paine, A. J. & McLean, A. E. M. (1974a) *Biochem. Soc. Trans.* **2**, 605-606
- Paine, A. J. & McLean, A. E. M. (1974b) *Biochem. Biophys. Res. Commun.* **58**, 482-486
- Paine, A. J. & McLean, A. E. M. (1974c) *Biochem. Pharmacol.* **23**, 1910-1913
- Paine, A. J. (1976) *Chem.-Biol. Interact.* **13**, 307-315
- Spikes, J. D. & MacKnight, M. L. (1970) *Ann. N.Y. Acad. Sci.* **171**, 149-162
- Venkatesan, N., Arcos, J. C. & Argus, M. F. (1971) *J. Theor. Biol.* **33**, 517-537