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Evidence for the possible involvement of the Superoxide radicals in the photodegradation of bilirubin

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Abstract. The photodecomposition of bilirubin follows first order kinetics with a k_B value of $12{\cdot}5 \times 10^{-3}$ min^{-1}. In the presence of a model system generating superoxide anions, such as xanthine-xanthine oxidase, the k_B value was 103×10^{-3} min^{-1} This ten-fold enhancement of k_B value by xanthine-xanthine oxidase was abolished when the reaction mixture was supplemented with a superoxide ion scavenger—superoxide dismustase. Further, known singlet oxygen quenchers like *ß*–carotene and bistidine did not prevent the enhancement of bilirubin oxidation by xanthine-xanthine oxidase, thereby ruling out the obligatory conversion of superoxide anion to singlet oxygen. It is concluded that radical oxygen mediated bilirubin degradation might be a natural catabolic route for the bile pigment degradation during oxygen stress.

Keywords. Bilirubin catabolism; photodegradation; superoxide anion; singlet oxygen.

Introduction

Photo-oxidation of bilirubin involves photo-oxygenation in which bilirubin acts as a photosensitiser of its own decomposition (McDonagh, 1971; Bonnett and Stewart, 1972). It was suggested that singlet oxygen, a high energy form of molecular oxygen, may be mediating the photo-oxygenation of bilirubin by the following evidences: (i) stimulation of photodecomposition by singlet oxygen sensitisers like dyes, (ii) suppression of photodecomposition of *ß*-carotene, a well-known singlet oxygen quencher, (iii) suppression by 2 : 5 dimethyl furan which traps singlet oxygen.

Based on the above studies with model systems, it is currently believed that the biotransformation of bilirubin in the skin of children suffering from jaundice undergoing phototherapy is mediated by singlet oxygen. Evidence is presented in this paper suggesting the possible role of superoxide anion in the biotransformation of bilirubin.

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Materials and methods

Commercially available bilirubin with an ε value of 5·9-6·1 × 10^4 $litre^{-1}$ mol^{-1} cm^{-1} at 450 nm in chloroform was dissolved in a few drops of 0·1 N NaOH and the pH of the solution adjusted to 8·3 with 0·1 M sodium pyrophosphate buffer. Xanthine oxidase (E.C. 1.2.3.2) was prepared from milk cream according to Waud *et al.* (1975) and activity assayed according to Massey *et al.* (1969).

Superoxide anion dismutase (E.C. 1.15.1.1) of bovine red blood cell origin with an activity of 2,900 units/mg protein marketed by Sigma Chemical Company, St. Louis, Missouri, USA, was a generous gift of Dr. M. K. Sahib, Division of Biochemistry, Central Drug Research Institute, Lucknow. Phenazine methosulphate was procured from Sigma Chemical Company and nitroblue tetrazolium and reduced nicotinamide adenine dinucleotide were purchased from Centron Laboratories, Bombay. *ß*-Carotene was purchased from E. Merck, Dermstadt, Germany. It was dissolved in a few drops of methanol followed by addition of sodium pyrophosphate buffer before use. Histidine was procured from British Drug House, Poole, England.

Bilirubin was assayed by the procedure of Malloy and Evelyn (1937) or by directly measuring the absorbance at 450 nm. Protein was estimated by the colorimetric method of Lowry *et al.* (1951) using crystalline serum albumin as the standard.

Irradiation of bilirubin

Solutions were kept in open petri dishes and exposed to an 80 W shaded mercury lamp from a distance of 30 cm. The dishes were kept in crushed ice during irradiation.

The reduction of tetrazolium blue by reduced nicotinamide adenine dinucleotide was quantitated by measuring the decrease in absorbance of 550 nm.

Results

(i) *Role of Superoxide radical in bilirubin oxidation*

Evidence for a role of Superoxide anion in decomposition of bilirubin was sought by exploring the effect of a Superoxide generating system as well as Superoxide anion dismutase on the rate of oxidation. The results are summarised in table 1. Xanthine-xanthine oxidase was used to generate Superoxide anion (Fridovich, 1975). The first order rate constant, k_B for bilirubin oxidation in the presence of xanthine or xanthine oxidase when added separately was unaltered as compared to controls but the combination of the two led to a ten-fold stimulation of k_B.

The addition of Superoxide anion dismutase to the above system restored the k_B values to control levels. However, *ß*-carotene and histidine failed to inhibit the ten-fold stimulation of the rate of the reaction by xanthine-xanthine oxidase system.

The linear correlation between the amount of Superoxide anion generated by the xanthine-xanthine oxidase system and stimulation of photocatabolism. of bilirubin is shown in table 2.

Table 1. Effect of xanthine-xanthine oxidase (superoxide anion generating system) on bilirubin degradation.

Additions	k_B* (min^{-1}) × 10^{-3}
Xanthine[a]	12·5
Xanthine oxidase[b]	12·3
Xanthine + xanthine oxidase	103·1
Xanthine + xanthine oxidase + superoxide dismutase[c]	12·8
Xanthine + xanthine oxidase + histidine[d]	102·8
Xanthine + xanthine oxidase + β-carotene[e]	103·2

* k_B is first order rate constant for bilirubin degradation.
The reaction mixture in final volume of 3 ml consisted of 21·3 mols of bilirubin in 0·1 M pyrophosphate buffer, pH 8·3.
[a] 300 nmol xanthine.
[b] 15·6 milliunits xanthine oxidase.
[a] 15 μg superoxide dismutase.
[d] 0·5 mM histidine.
[e] 30 μM β-carotene.

Table 2. Effect of increasing concentration of xanthine oxidase in xanthinexanthine oxidase system on the rate of bilirubin degradation.

Xanthine oxidase added (milliunits)	k_B (min^{-1})* × 10^{-3}
3·9	36·1
7·8	53·1
11·7	78·0
15·6	103·1

The reaction system comprised of 21·3 nmol of bilirubin in 0·1 M sodium pyrophosphate buffer pH 8·3 and 300 nmol of xanthine in a total volume of 3 ml.

* A linear correlation between xanthine oxidase (rate limiting component of xanthine oxidase catalysed-superoxide anion generating system) and rate of bilirubin degradation with a correlation coefficient of 1·77 was obtained.

(ii) *Effect of bilirubin on nitroblue tetrazolium reduction initiated by Superoxide anion*

The reduction of nitroblue tetrazolium by reduced nicotinamide adenine dinucleotide is known to be mediated by Superoxide radical generated *in situ* (Nishikimi *et al.,* 1972). The effect of increasing concentration of bilirubin was, therefore, studied on this reaction and as evident from results presented in figure 1, bilirubin appears to compete with nitroblue tetrazolium for the superoxide radical.

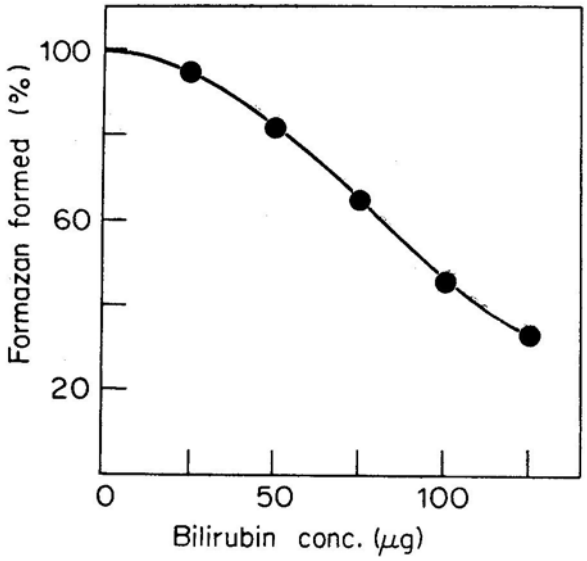

Figure 1. Effect of bilirubin on reduction of nitrobluetetrazolium.
The reaction in a final volume of 3 ml consisted of 78 μM NADH, 50 μM. Nitrobluetetrazolium and 28 μM phenazine metaosulphate in sodium pyrophosphate buffer 0·017 M, pH 8·3 containing different concentration of bilirubin.

(iii) *Effect of Superoxide anion dismutase on photocatabolism of bilirubin*

The effect of Superoxide dismutase was studied on photodecomposition of bilirubin. Results presented in figure 2 would reveal that when the reaction mixture exposed to light was supplemented with Superoxide dismutase, there was inhibition of photooxidation of the bile pigment by nearly 50%.

Discussion

Bilirubin catabolisim has hitherto been found to follow two pathways: one involveing conjugation (Axelrod *et al.,* 1957) and the other singlet oxygen mediated *in vitro* photooxidation (McDonagh 1971; Bonnett and Stewart, 1972). We have accumulated sufficient evidence to invoke superoxide anion-mediated oxidation of bilirubin as an important pathway for its degradation. This is borne out by stimulation of k_B of bilirubin from 12·5 × 10^{-3} min^{-1} to 103 × 10^{-3} min^{-1} by inclusion of xanthine-

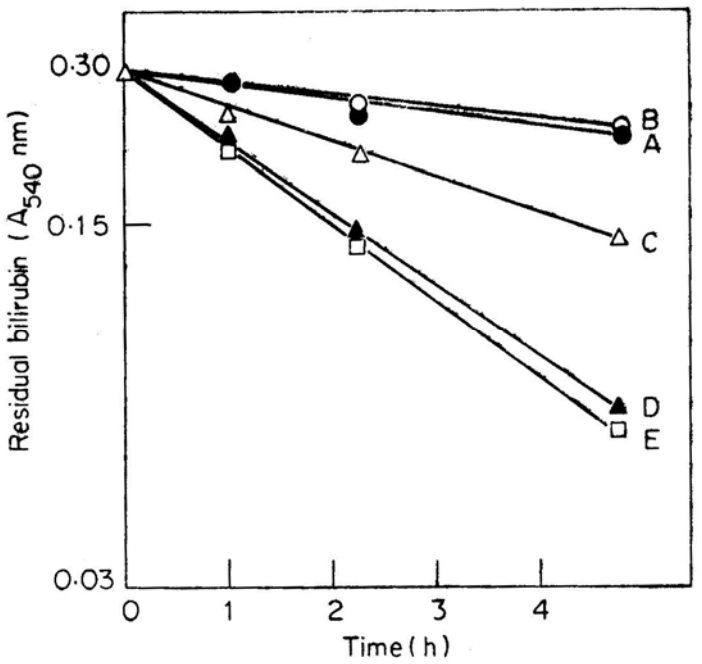

Figure 2. Effect of superoxide anion dismutase on bilirubin photodegradation. The reaction was carried out in a final volume of 2 ml consisting of: (A) 850 nmol of bilirubin in 0·017 M sodium phosphate buffer pH 8·3 incubated in the dark. (B) 50 μg (144 units) superoxide anion dismutase added to reaction mixture A and kept in the dark. (C) Same as in B, but exposed to light. (D) Same as in A, but exposed to light. (E) 50 μg (144 units) boiled enzyme added to reaction mixture A and exposed to light. Aliquots (0·1 ml) were withdrawn at various times and analysed for bilirubin. Values of residual bilirubin at different intervals of time expressed as absorbance at 540 nm were plotted against time on a semilog plot.

xanthine oxidase in the reaction mixture. Stimulation of bilirubin decomposition by superoxide anion generating system and complete inhibition of the accelerated rate by superoxide anion dismutase further supports the hypothesis.

Superoxide anions could be converted into singlet oxygen (Kellogg and Fridovich, 1977) and the latter then could initiate a chain reaction culminating in bilirubin degradation. However this possibility was ruled out by the failure of β-carotene and histidine to inhibit the enhancement in the bilirubin decay caused by superoxide radical. Bilirubin induced concentration-dependent inhibition of formazon formation in a coupled system would further support the role of superoxide radical in bilirubin decay.

Different systems may contribute *in vivo* to generation of radical species of oxygen. Recent observations of Kapitulnik and Ostrow (1977) that cytochrome P-448 mediates bilirubin oxidation *in vivo* and stimulation of the rate of bilirubin degradation by xanthine-xanthine oxidase would support the involvement of $O_2^{\cdot-}$ It would appear thus that radical oxygen-mediated bilirubin decay may be a normal route of bilirubin catabolism.

Our observations support the concept that bilirubin is degraded by radical species of oxygen (superoxide anion) besides the well-documented singlet oxygen-mediated pathway. Only 50% inhibition of bilirubin degradation, achieved by superoxide anion dismutase, would suggest that at least 50% of photooxidation reaction is contributed by superoxide anion. Foote and Ching (1975) while studying the kinetics of bilirubin degradation obtained 83% of singlet oxygen quenching by bilirubin. However, Matheson *et al.* (1974 a, b) failed to observe any inhibition of bilirubin degradation by addition of 1:3 diphenylisobenzfuran–a known singlet oxygen quencher. They proposed that this compound reacts essentially without any physical quenching. However, Lightner and Cu (1977) gave some plausible evidence for free radical reactions in the photodecompsition of bilirubin based on the observation that bilirubin half-life increased when oxygen was associated with chloroform because oxygen acted as a free radical inhibitor.

It remains to be clarified whether light induced photooxidation of bilirubin follows Haber-Weiss reaction. In contrast, xanthine-xanthine oxidase generated superoxide anion does not follow the above reaction to achieve bilirubin degradation. Evidence at hand would support that radical oxygen is an active species involved in bilirubin catabolism. In fact its role may be of relevance to the normal route of breakdown of bilirubin to polar metabolites in contrast to the conjugated metabolites produced by hepatic bilirubin diglucuronide conjugase. Water soluble metabolites of bilirubin are known to be excreted through the kidneys by jaundiced neonates subjected to phototherapy (Bajpai *et al.,* 1976). Some products present in *in vitro* photooxidation reaction of bilirubin resemble urinary metabolites of control jaundiced neonates as well as jaundiced neonates subjected to phototherapy. Similarly, Gunn rats known to suffer from the genetic deficiency of hepatic bilirubin conjugase excrete into bile and urine, a few metabolites with close similarity to the *in vitro* photodecomposition products of bilirubin (Berry *et al.,* 1972).

Apparently when there is a deficiency of UDP-glucuronyl transferase, the mammals excrete polar metabolites of bilirubin which resemble the degradation products obtained by *in vitro* photodecomposition. It is suggested that superoxide anion may be the mediator of such reactions and superoxide anion can originate *in vivo* from xanthine-xanthine oxidase and similar enzymatic mechanisms or from oxygen-haemoglobin exchange reaction.

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