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Porphyrin-induced photooxidation of conjugated bilirubin

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Abstract

Visible light irradiation of 18 uM bilirubin ditaurate (BR-DT) at pH 7.0 for 30 min showed a 10% decrease in absorbance at 445 nm. When the reaction was carried out in the presence of a trace amount of uroporphyrin (UP), the spectrum of BR-DT disappeared without a concomitant formation of biliverdin. Photooxidation products were confirmed to be dipyrrole-containing compounds. Photobleaching of BR-DT was accelerated by the increasing concentration of UP and was inhibited, when UP was replaced by Cu^{2+}UP. Formation of 2,2,6,6-tetramethylpiperidine N-oxyl through the irradiation of UP was diminished by sodium azide, a potent scavenger of singlet oxygen. The efficiency of singlet oxygen formation through visible light irradiation was in the order UP, coproporphyrin > Cu^{2+}UP. Both bilirubin and BR-DT bound to human serum albumin were photooxidized effectively in the presence of UP. The results indicate that irradiation of UP produces singlet oxygen with high efficiency which then rapidly oxidizes free and conjugated bilirubin.

Keywords

uroporphyrin; bilirubin; conjugated bilirubin; singlet oxygen; phototherapy; hyperbilirubinaemia.

Introduction

In neonatal hyperbilirubinaemia, phototherapy for the patients is a simple and safe method for lowering elevated levels of free bilirubin [1, 2]. Almost all bilirubin is present in serum as conjugated form or bound to albumin. When the concentration of free bilirubin is increased, the risk of neurological dysfunction develops due to deposition of bilirubin in brain [2]. Since the free bilirubin molecule forms internal hydrogen bond, free bilirubin is insoluble in water [3]. In rare instances, patients treated with phototherapy develop a discoloration of their skin described as Bronze Baby Syndrome [1]. The discoloration of skin is mainly explained by the accumulation of photoisomers and pigments derived from bilirubin which are not excreted in bile and urine [1, 2]. It has been confirmed that phototherapy causes the phototransition of free bilirubin to more polar photoisomers such as bilirubin IXαE [4-8].

Recent studies have shown that both free and conjugated bilirubin effectively scavenge oxygen radicals [9-17] so that bilirubins are classified as antioxidants [13, 15-17]. Large amounts of porphyrins and abnormal concentrations of copper are found in neonatal hyperbilirubinaemia patient serum [18, 19]. Irradiation of bilirubin *in vitro* elicits the oxidation of bilirubin [9] and the photooxidation of bilirubin is markedly increased in the presence of photosensitizers [12, 14, 20]. There is evidence that these oxidations involve singlet oxygen generated through the energy transfer from excited sensitizers to oxygen [10-12]. Oxidative stress to animals, which causes the formation of reactive oxygen species *in vivo*, is known to result in the fragmentation of bilirubins [21, 22].

In this study we have observed the porphyrin-induced photooxidation of free and conjugated bilirubin. Effects of copper and human serum albumin upon the porphyrin-induced photooxidation of free and conjugated bilirubin have been also investigated. Our results support the possibility that the fragmentation of bilirubin would occur during the phototherapy of neonatal hyperbilirubinaemia patients.

Materials and Methods

Bilirubin IX was purchased from Nacalai Tesque,Kyoto,Japan. Biliverdin, bilirubin ditaurate (BR-DT), uroporphyrin and coproporphyrin were purchased from Frontier Scientific, Inc., Tokyo, Japan. The molecular extinction coefficients used for the estimation of concentrations of bilirubin and BR-DT are $45x10^3$ [21] and $49.5x10^3$ $M^{-1}cm^{-1}$ [14], respectively. The concentrations of uroporphyrin and coproporphyrin were determined from published molecular extinction coefficients [25,26]. Diethylenetriaminepenta-acetic acid and human serum albumin were obtained from Sigma, St.Louis, MO, USA. 2,2,6,6-tetramethylpiperidine and 2,2,6,6-tetramethylpiperidine N-oxyl were obtained from Tokyokasei, Tokyo, Japan and Aldrich, St.Louis, MO, USA, respectively. Bilirubin oxidation products assay kit was purchased from Dojindo Laboratories, Kumamoto, Japan. Other reagents used were of analytical grade.

Copper-uroporphyrin (Cu-UP) was prepared as follows. Reactions were carried out in 10 mM phosphate (pH 7.0) containing 1.1 uM UP and varied concentrations of $CuCl_2$. The reaction mixtures were incubated for 10 min and diethylenetriaminepenta-acetic acid solution was added last to give a final concentration of 50 uM for removing free copper. Binding of copper to UP was confirmed by quenching of the fluorescence of UP.

For photooxidation experiments, 1.0 ml solutions containing bilirubin or BR-DT were exposed to a fluorescent lamp (intensity of incident light, 12000 lux). After various time intervals the reaction mixtures were withdrawn for spectral and ESR measurements.

Porphyrin-induced photooxidation products of bilirubin and BR-DT were analyzed by enzyme-linked immunosorbent assay (ELISA) [23]. In the assay, the anti-bilirubin monoclonal antibody (24G7) has an epitope in the dipyrrole moiety of bilirubin [24], which enables to recognize both non-bilirubin compounds and bilirubin.

Singlet oxygen was observed by a detection of the 2,2,6,6-tetramethylpiperidine N-oxyl ESR signal produced in the reaction of 2,2,6,6-tetramethylpiperidine with singlet oxygen [25].

ESR measurements were performed with a JEOL JES-TE300 ESR spectrometer (Tokyo, Japan) with 100 KHz modulation. Spectral changes were followed with a Shimadzu MPS 2000 spectrometer.

All reactions were carried out at 25℃ in 10 mM phosphate buffer, pH7.0 unless otherwise noted.

Results

The photooxidation of BR-DT was carried out in the absence (Fig. 2A) or presence (Fig. 2B) of UP. When the reaction mixture containing 18 uM BR-DT was exposed to near UV light for 30 min, a 10 % decrease in absorbance at 450 nm was observed (Fig. 2A). Figure 2Ba shows the spectrum of 18 uM BR-DT and 1.1 uM UP. The spectrum consists of a sum of the spectrum of BR-DT (Fig. 1a) and that of UP (Fig. 1c). The rate of initial decrease in absorbance at 450 nm was markedly accelerated by the addition of 1.1 uM UP. In this case the spectrum of BR-DT was abolished in 15 min and the spectrum of UP remained (Fig. 2Bc). The velocity of initial decrease in absorbance increased with increasing concentration of UP (data not shown). Neither increase in absorbance at 650 nm due to biliverdin nor decrease in absorbance at 398 nm due to UP was found during the period of illumination. Because the absorbance at 450 nm is ascribed to BR-DT, UP remained unchanged until BR-DT was exhausted. Biliverdin was also photooxidized in the presence of UP (Fig. 3), whereas no significant spectral change was observed in the absence of UP during 30 min illumination (data not shown). In the presence of 2.2 uM UP, the absorbance at 650 nm was decreased by 60 % within 30 min (Fig. 3b). These results indicate that BR-DT reacts more rapidly with singlet oxygen than biliverdin.

Neonatal serum contains a high concentration of bilirubin, abnormal concentration of copper and porphyrins (UP and coproporphyrin) [18, 19]. Therefore, we have carried out photooxidation of BR-DT in the presence of UP and copper. The inhibitory effect of copper was concentration dependent. The addition of one equivalent of copper to UP gave photooxidation in which the BR-DT concentration was reduced to 40 % (Fig. 4d). Separate experiments demonstrated one-to-one Cu binding to UP (data not shown). The results indicated that one mole of copper is tightly ligated to UP (Cu-UP), which ineffectively photooxidizes BR-DT.

Figure 5 shows the time courses for photo-oxidation of BR-DT in the absence (Fig. 5a) or presence of UP. BR-DT disappeared within 15 min in the presence of 1.1 uM UP (Fig. 5b). The UP-induced photooxidation of BR-DT is inhibited by the addition of NaN_3, a quencher of singlet oxygen. On the addition of 10 mM NaN_3, the concentration of BR-DT was reduced to 40 % of its initial value (Fig. 5c) and the time course was similar to that in the

presence of Cu-UP: (ratio of copper to UP =1.0) (Fig. 5d).

Formation of singlet oxygen was confirmed by observation of the ESR signal of 2,2,6,6-tetramethylpiperidine N-oxyl formed through the oxidation of 2,2,6,6-tetramethylpiperidine in the presence of UP (Fig. 6b). The ESR signal intensity was low in the absence of UP (Fig. 6a) and the intensity of the triplet signal due to nitroxide was markedly increased by the addition of UP (Fig. 6b). UP-induced formation of ESR signal was decreased by the addition of 10 mM NaN_3 (Fig. 6c). Formation of the ESR signal due to 2,2,6,6-tetramethylpiperidine N-oxyl was also observed in the presence of Cu-UP (Fig. 6d, e). Yield of nitroxide formation decreased with increasing concentrations of copper. The intensity was estimated to be reduced to about 40 % as the molar ratio of copper to UP was increased to 1.0.

Bilirubin is tightly bound to human serum albumin (HSA) and transported within the blood circulation [28]. Therefore, we examined the bilirubin-HSA complex for photooxidation similar to the experiments above. Bilirubin bound to HSA was photooxidized in the presence or absence of UP (Table 1A). About 70% of HSA-bound BR-DT was photooxidized after 30 min irradiation in the presence of 0.45 uM UP, whereas nearly all BR-DT disappeared in the absence of HSA. About 40 % of protection was also observed with HSA-bound biliverdin. These results, however, imply that singlet oxygen formed through UP activation oxidizes BR-DT effectively whether it is free or bound to HSA. Time courses of spectral change for free and bound BR-DT clarified that UP-induced photooxidations of HSA-bound bilirubin and BR-DT proceeded in a similar manner to those of free bilirubin and BR-DT.

Photooxidation products of bilirubin and BR-DT in the presence of UP were examined by ELISA [23,24]. Absorbance at 450 nm assigned to bilirubin and BR-DT disappeared in the presence of UP (Table IA and B). Anti-bilirubin monoclonal antibody (24G7) assay showed that bilirubin epitope with 24G7 still remained in the reaction mixtures after 30 min irradiation (data not shown). The results indicate that UP-induced photooxidation of bilirubin and BR-DT results in the formation of dipyrrole-containing compounds.

Discussion

The results clearly show that photooxidation of conjugated bilirubin (BR-DT) is accelerated in the presence of a small amount of UP or coproporphyrin. In this oxidation, biliverdin was not concomitantly formed. Biliverdin was also photooxidized in the presence of UP. The rate constant for the reaction of biliverdin with singlet oxygen is slower than that for BR-DT [12, 29]. The kinetic results are consistent with the previous reports that biliverdin is not an intermediate in the formation of final photooxidation products of bilirubin [10, 11].

Porphyrins and bilirubin are photosensitizers that produce singlet oxygen through irradiation with visible or near UV light at wavelengths absorbed by porphyrins and bilirubin. ESR experiments indicate the formation of singlet oxygen during exposure of UP to near UV light, whereas no formation ESR signal was observed during exposure of bilirubin and BR-DT (data not shown). The rate constants for the reactions of tetramethylpiperidine with superoxide and hydroxyl radical are 73 and $1.5 \times 10^9\ M^{-1}s^{-1}$, respectively [30]. However, the rate constant for the reaction of singlet oxygen with tetramethylpiperidine is so slow [30] that the method is not so sensitive for detecting singlet oxygen. The present ESR results are in good agreement with the reports that quantum yield for the singlet oxygen formation of UP is relatively higher than that of bilirubin and BR-DT [26]. Bilirubin and BR-DT are not good photosensitizers compared to UP and coproporphyrin, but are good singlet oxygen quenchers and reactants than UP and coproporphyrin. The present results and kinetic results have ruled out the possibility that bilirubin-induced photooxidation of porphyrins should occur in Bronze Baby Syndrome sera [18, 19].

Neonatal jaundice serum contains porphyrins and copper porphyrins [18]. UP-induced photooxidation of BR-DT was inhibited with increasing concentration of copper. Separate experiments show that a fluorescence of UP is completely quenched by the addition of a stoichiometric amount of copper and indicate one mole of copper is tightly bound to UP (data not shown). Photooxidation of UP itself was followed by a spectral change of Soret peak. Irradiation of UP shows that spectrum of UP was diminished with time at a half life of 2 hours under the same experimental conditions. The decrease in absorbance at 398 nm which indicates the degradation of porphyrin ring was inhibited in the presence of equimolar copper to UP (data not shown). Figure 6 shows that the yield of singlet oxygen formation by

UP was higher than that from Cu-UP. The results show that UP was degraded through selfquenching of singlet oxygen.

Bilirubin serves as a substrate for peroxidase, resulting in the formation of biliverdin [31]. A substantial amount of biliverdin is formed through reactions of bilirubin with superoxides and peroxyradicals [16, 17]. Biliverdin is also formed through a reaction of BR-DT with HOCl [13]. The abstraction of a C10 hydrogen atom of bilirubin would occur in these reactions. Myeloperoxidase present in polymorphonuclear leukocytes catalyzes the formation of hypohalous acids at the expense of H_2O_2. Superoxides are generated by stimulated polymorphonuclear leukocytes and undergo dismutation reaction into H_2O_2. Reaction of biliverdin with HOCl results in the decrease of absorbance at 365 and 665 nm which indicates the fragmentation of biliverdin (data not shown).

Specific reaction of singlet oxygen with the double bond of bilirubin, which leads to the fragmentation of bilirubin, has been pointed out [12]. The rate constant for the reaction of singlet oxygen with BR in methanol is 1.5×10^9 $M^{-1}s^{-1}$ [12]. Singlet oxygen, formed by bilirubin or BR-DT as sensitizer, has been shown to react rapidly with bilirubin or BR-DT. Bilirubin and BR-DT were decomposed in the presence of good sensitizer [Fig. 2B and Table 1].

UP-induced photooxidation of bilirubin bound to HSA was observed (Table 1). Several lines of evidence have pointed out that unconjugated bilirubin not associated with HSA readily diffuses in the brain and causes kernicterus. From fluorometric titrations it is concluded that HSA has two binding sites for bilirubin [28]. The affinity constant of fluorescent binding site for bilirubin is 7×10^6 M^{-1} and one hundred times stronger than the other non-fluorescent binding site. HSA is photooxidized in the presence of the sensitizers, rose bengal or methylene blue, whereas no photooxidation is observed in the presence of bilirubin [20]. In this report bilirubin bound to HSA undergoes phototransition from Z form to E form of bilirubin IXα, which is unable to form internal hydrogen bond and is water-soluble. The resulting E form is easily excreted in urine or bile. This phototransition is followed by a slow oxidation of the pigments including oxygen uptake. The present results suggest that UP is accessible to bilirubin bound to HSA and photooxidizes it.

Recent reports suggested that reactions of bilirubin with reactive oxygen species *in vivo* result in the fragmentation of bilirubin, generating biopyrrins [21, 22]. BR-DT and bilirubin are, therefore, characterized as antioxidants.

Biopyrrins are found to increase in urine after reperfusion injury of rat liver [21]. In neonatal hyperbilirubinaemia, phototherapy is used for lowering elevated levels of unconjugated bilirubin. Phototherapy causes the formation of bilirubin photoisomers. Insufficient excretion of bilirubin isomers by the liver in the Bronze Baby Syndrome, the photoisomers accumulate in patient serum. In periods of low concentration of bilirubin isomers in the urine, fluorescent porphyrins are often detected [32]. These results suggest that porphyrin-induced singlet oxygen formation and superoxide formed through redox cycling of porphyrin [33] contribute to the fragmentation of bilirubin whether free or conjugated.

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References

1. Kopelman, A. E., Brown, R. S., and Odel, G. B. (1972). The bronz baby syndrome: a complication of phototherapy., *J. Pediatr.* **81**, 466-472.
2. Lucey, J., Ferreiro, M., and Hewitt, J. (1968). Prevention of hyperbilirubinemia of prematurity by phototherapy., *Pediatrics* **41**, 1047-1054.
3. Bonnett, R., Davies, J. E., and Hursthouse, M.B. (1976). Structure of bilirubin., *Nature* **262**, 326-328.
4. McDonagh, A. F., and Ramonas, L. M. (1978) Jaundice phototherapy: micro flow-cell photometry reveals rapid biliary response of Gunn rats to light., *Science* **201**, 829-831.
5. McDonagh, A. F., Palma, L. A., Trull, F. R., and Lightner, D. A. (1982). Phototherapy for neonatal jaundice. Configurational isomers of bilirubin., *J. Am. Chem. Soc.* **104**, 6865-6867.
6. McDonagh, A. F., Palma, L. A., and Lightner, D. A. (1982). Phototherapy for neonatal jaundice. Stereospecific and regioselective photoisomerization of bilirubin bound to human serum albumin and NMR characterization of intramolecularly cyclized photoproducts., *J. Am. Chem. Soc.* **104**, 6867-6869.
7. Onishi, S., Itoh, S., Isobe, K.,Togari, H., Kitoh, H.,and Nishimura, Y. (1982). Mechanism of development of bronze baby syndrome in neonates treated with phototherapy., *Pediatrics* **69**, 273-276.
8. Onishi, S., Miura, I., Isobe, K., Itoh, S., Ogino, T., Yokoyama, T., and Yamakawa, T. (1984). Structure and thermal interconversion of cyclobilirubin IXα., *Biochem. J.* **218**, 667-676.
9. Ostrow, J. D., and Branham, R. V. (1970). Photodecomposition of bilirubin and biliverdin *in vitro*., *Gastroenterology* **58**, 15-25.
10. McDonagh, A. F. (1971). The role of singlet oxygen in bilirubin photo-oxidation., *Biochem. Biophys. Res. Commun.* **44**, 1306-1311.
11. McDonagh, A. F. (1972). Evidence for singlet oxygen quenching by biliverdin IXα dimethyl ester and its relevance to bilirubin photo-oxidation., *Biochem. Biophys. Res. Commun.* **48**, 408-415.
12. Foote, C. S., and Ching, T-Y. (1975). Chemistry of singlet oxygen. XXI. Kinetics of bilirubin photooxygenation., *J. Am. Chem. Soc.* **97**, 6209-6214.
13. Stocker, R., and Peterhans, E. (1989). Antioxidant properties of conjugated bilirubin and biliverdin: biologically relevant scavenging of

hypochlorous acid., *Free Radic. Res. Commun.* **6**, 57-66.

14. Jirsa, M. Jr., Sip, M.,and Jirsa, M. (1990). Influence of hyperbaric oxygenation on bilirubin and ditaurobilirubin auto-oxidation and porphyrin-sensitized photo-oxidation., *J. Photochem. Photobiol. B: Biology* **5**, 295-302.
15. Stocker, R., Yamamoto, Y., McDonagh, A. F., Glazer, A. N., and Ames, B. N. (1987). Bilirubin is an antioxidant of possible physiological importance., *Science* **235**, 1043-1046.
16. Stocker, R., Glazer, A. N., and Ames, B. N. (1987). Antioxidant activity of albumin-bound bilirubin., *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5918-5922.
17. Stocker, R., and Ames, B. N (1987). Potential role of conjugated bilirubin and copper in the metabolism of lipid peroxides in bile., *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8130-8134.
18. Rubaltelli, F. F., Jori, G., and Reddi, E. (1983). Bronze baby syndrome: a new porphyrin-related disorder., *Pediatr. Res.* **17**, 327-330.
19. Jori, G., Reddi, E., and Rubaltelli, F. F. (1990). Bronze baby syndrome: an animal model., *Pediatr. Res.* **27**, 22-25.
20. Pedersen, A. O., Schonheyder, F., and Brodersen, R. (1977). Photooxidation of human serum albumin and its complex with bilirubin., *Eur. J. Biochem.* **72**, 213-221.
21. Yamaguchi, T., Horio, F., Hashizume, T., Tanaka, M., Ikeda, S., Kakinuma, A., and Nakajima, H. (1995). Bilirubin is oxidized in rats treated with endotoxin and acts as physiological antioxidant synergistically with ascorbic acid *in vivo*., *Biochem. Biophys. Res. Commun.* **214**, 11-19.
22. Yamaguchi, T., Terakado, M., Horio, F., Aoki, K., Tanaka, M., and Nakajima, H. (1996). Role of bilirubin as an antioxidant in an ischemia-reperfusion of rat liver and induction of heme oxygenase., *Biochem. Biophys. Res. Commun.* **223**, 129-135.
23. Izumi,Y.,Yamazaki,M.,Shimizu,S., Shimizu,K.,Yamaguchi,T., and Nakajima,H.(1988). Anti-bilirubin monoclonal antibody II. Enzyme-linked immunosorbent assay for bilirubin fractions by combination of two monoclonal antibodies., *Biochim.Biophys.Acta.* **967**,261-266.
24. Yamaguchi,T., Shioji,I., Sugimoto,A., Komoda,Y., and Nakajima,H. (1996). Epitope of 24G7 anti-bilirubin monoclonal antibody., *Biochim.Biophys.Acta.* **1289**,110-114.

25. Rimington, C. (1960). Spectral-absorption coefficients of some porphyrins in the Soret-band region., *Biochem. J.* **75**, 620-623.
26. Blum, A., and Grossweiner, L. I. (1985). Singlet oxygen generation by hematoporphyrin IX, uroporphyrin I and hematoporphyrin derivative at 546 nm in phosphate buffer and in the presence of egg phosphatidylcholine liposomes., *Photochem. Photobiol.* **41**, 27-32.
27. Lion, Y., Delmelle, M., and van de Vorst, A. (1976). New method of detecting singlet oxygen production., *Nature* **263**, 442-443.
28. Lamola, A. A., Eisinger, J., Blumberg, W. E., Patel, S. C., and Flores, J. (1979). Fluorometric study of the partition of bilirubin among blood components: basis for rapid microassays of bilirubin and bilirubin binding capacity in whole blood., *Anal. Biochem.* **100**, 25-42.
29. Stevens, B., and Small, R. D. Jr. (1976). The photoperoxidation of unsaturated organic molecules-XV. O2 quenching by bilirubin and biliverdin., *Photochem. Photobiol.* **23**, 33-36.
30. Ando, T., Yoshikawa, T., Tanigawa, T., Kohno, M., Yoshida, N., and Kondo, M. (1997). Quantification of singlet oxygen from hematoporphyrin derivative by electron spin resonance., *Life Sci.* **61**, 1953-1959.
31. Brodersen, R., and Bartels, P. (1969). Enzymatic oxidation of bilirubin., *Eur. J. Biochem.* **10**, 468-473.
32. Meisel, P., Jahrig, D., and Meisel, M. (1987). Detection of photobilirubin in urine of jaundiced infants supporting the diagonosis of 'bronze baby syndrome'., *Clin. Chim. Acta* **166**, 61-65.
33. Morehouse, K. M., Moreno, S. N., and Mason, R. P. (1987). The one-electron reduction of uroporphyrin I by rat hepatic microsomes., *Arch. Biochem. Biophys.* **257**, 276-284.

Figure legends

Figure 1.
Spectra of BR-DT, biliverdin and uroporphyrin (UP).
Solutions contained 18 uM BR-DT(a), 22 uM biliverdin (b) or 2.4 uM UP (c) in 10 mM phosphate buffer, pH 7.0.

Figure 2.
Photooxidation of BR-DT in the absence (A) or presence (B) of UP.
A, spectra were obtained at 0 (a) and 30 (b) min after irradiation had been started. Reaction mixture contained 18 uM BR-DT. B, Spectra were obtained at 0 (a), 12(b) and 15 (c) min after irradiation had been started. Reaction mixture contained 18 uM BR-DT and 1.1 uM UP.

Figure 3.
Photooxidation of biliverdin in the presence of UP.
Spectra were obtained at 0 (a) and 30 (b) min after irradiation had been started. Reaction mixture contained 20 uM biliverdin and 2.2 uM UP.

Figure 4.
Effects of copper concentrations upon the UP-induced photooxidation of BR-DT.
Spectra were obtained at 30 min after irradiation had been started. Reaction mixtures contained 18 uM BR-DT, 1.1 uM UP and 0 (b), 0.60 (c), 1.2 (d) or 2.5 (e) uM $CuCl_2$. Irradiation was started by the addition of BR-DT solution to reaction mixtures containing UP and copper (Materials and Methods).

Figure 5.
Effects of azide and copper upon the UP-induced photooxidation of BR-DT.
Reaction mixtures contained 18 uM BR-DT and 1.1 uM UP in the absence (b) or presence of 10 mM NaN_3 (c) or 1.2 uM $CuCl_2$ (d). Trace a shows the photooxidation of BR-DT alone.

Figure 6.
ESR spectra generated during UP-induced photooxidation of 2,2,6,6-tetramethylepiperidine.
Reaction mixtures contained 100 mM 2,2,6,6-tetramethylepiperidine.

Reactions were carried out in the absence (a) or presence of 1.1 uM UP (b-e). Reaction mixtures were irradiated for 30 min. The effects of 10 mM NaN_3 (c), 0.61 uM $CuCl_2$ (d) or 2.53 uM $CuCl_2$ (e) were tested. Instrumental conditions were gain, 500; power, 5.0 mW; modulation amplitude, 0.1 mT; time constant, 0.3 sec; scan rate, 2.5 mT min^{-1}.

Table I

Effect of human serum albumin (HSA) upon the photooxidation of bilirubin (A), BR-DT (B) or biliverdin (C) in the absence or presence of UP.
Reactions were carried out in 10 mM phosphate buffer, pH 7.0.
UP was added last to reaction mixtures containing bilirubin and HSA.

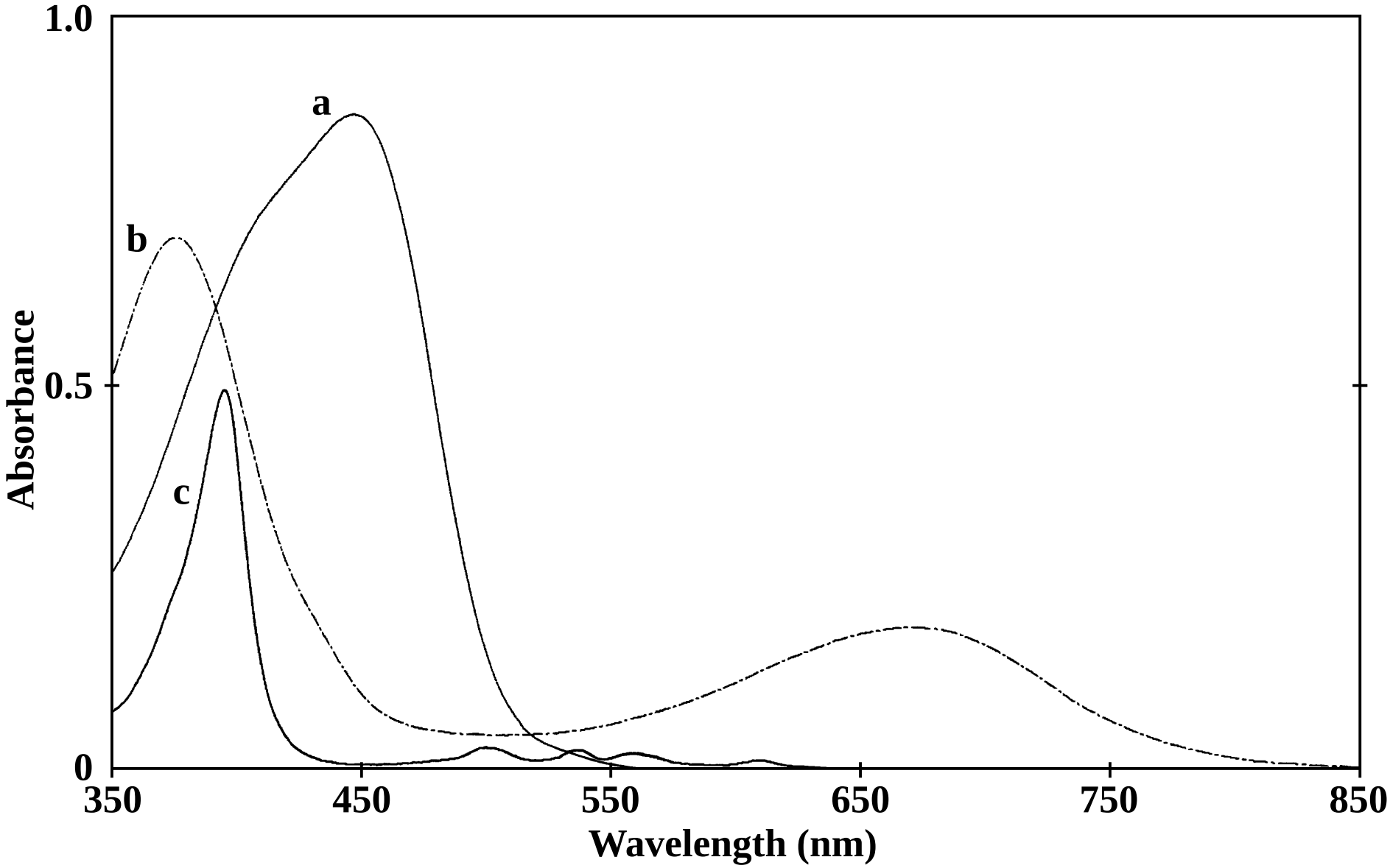
Reaction mixture	Oxidation (%) [d]
A, bilirubin [a]	
+ none	62
+ 20 μM HSA	18
+ 0.45 μM UP	91
+ 20 μM HSA and 0.45 μM UP	58
B, BR-DT [b]	
+ none	11
+ 20 μM HSA	9
+ 0.45 μM UP	98
+ 20 μM HSA and 0.45 μM UP	70
C, biliverdin [c]	
+ none	5
+ 20 μM HSA	5
+ 0.45 μM UP	58
+ 20 μM HSA and 0.45 μM UP	14

a,b,c

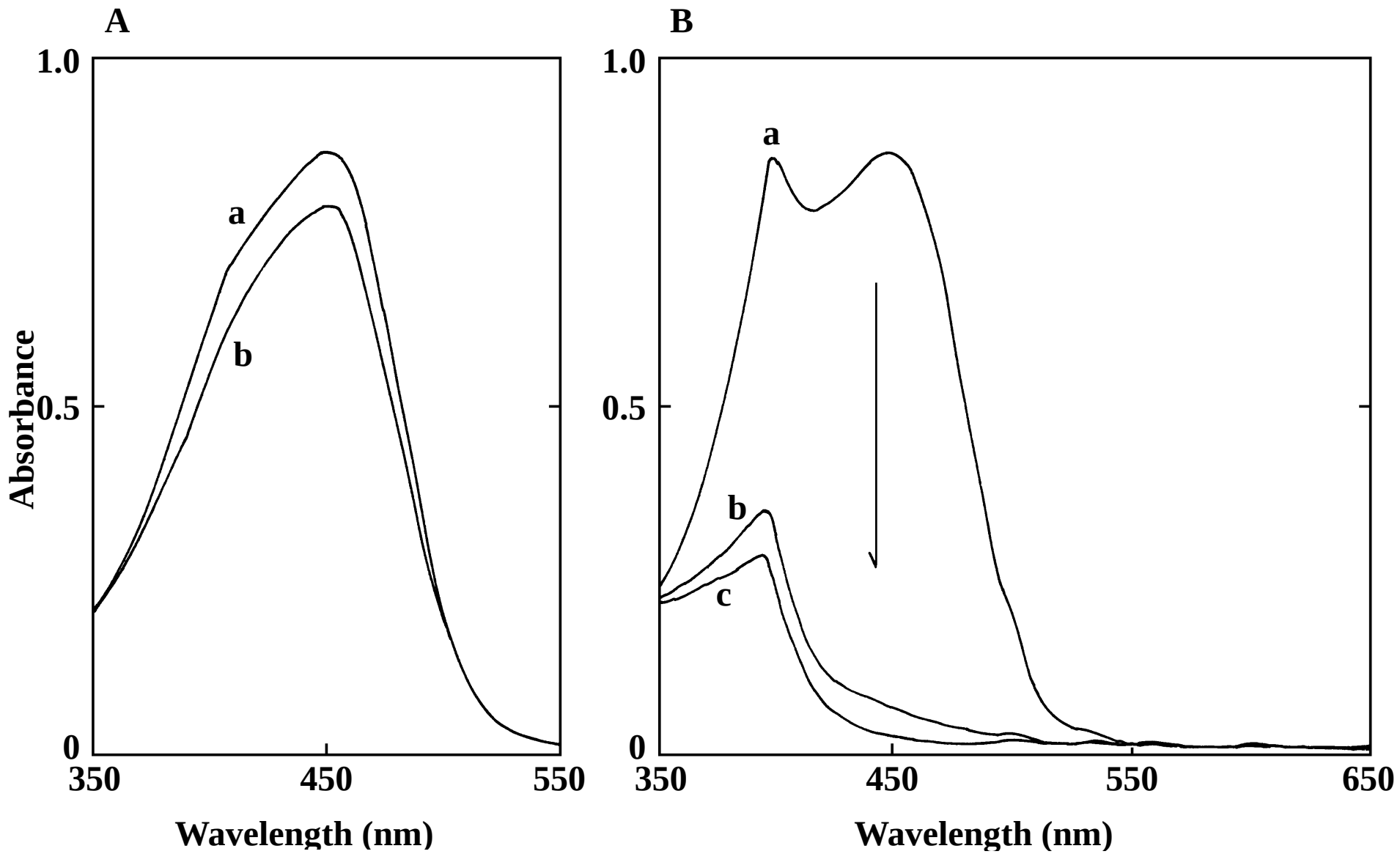
The reaction mixtures contained 16 uM bilirubin (A), 14 uM BR-DT (B) or 16 uM biliverdin (C).

d,

The reaction mixtures were irradiated for 30 min. Each value represents the mean of three experiments.



ShishidoFig.1



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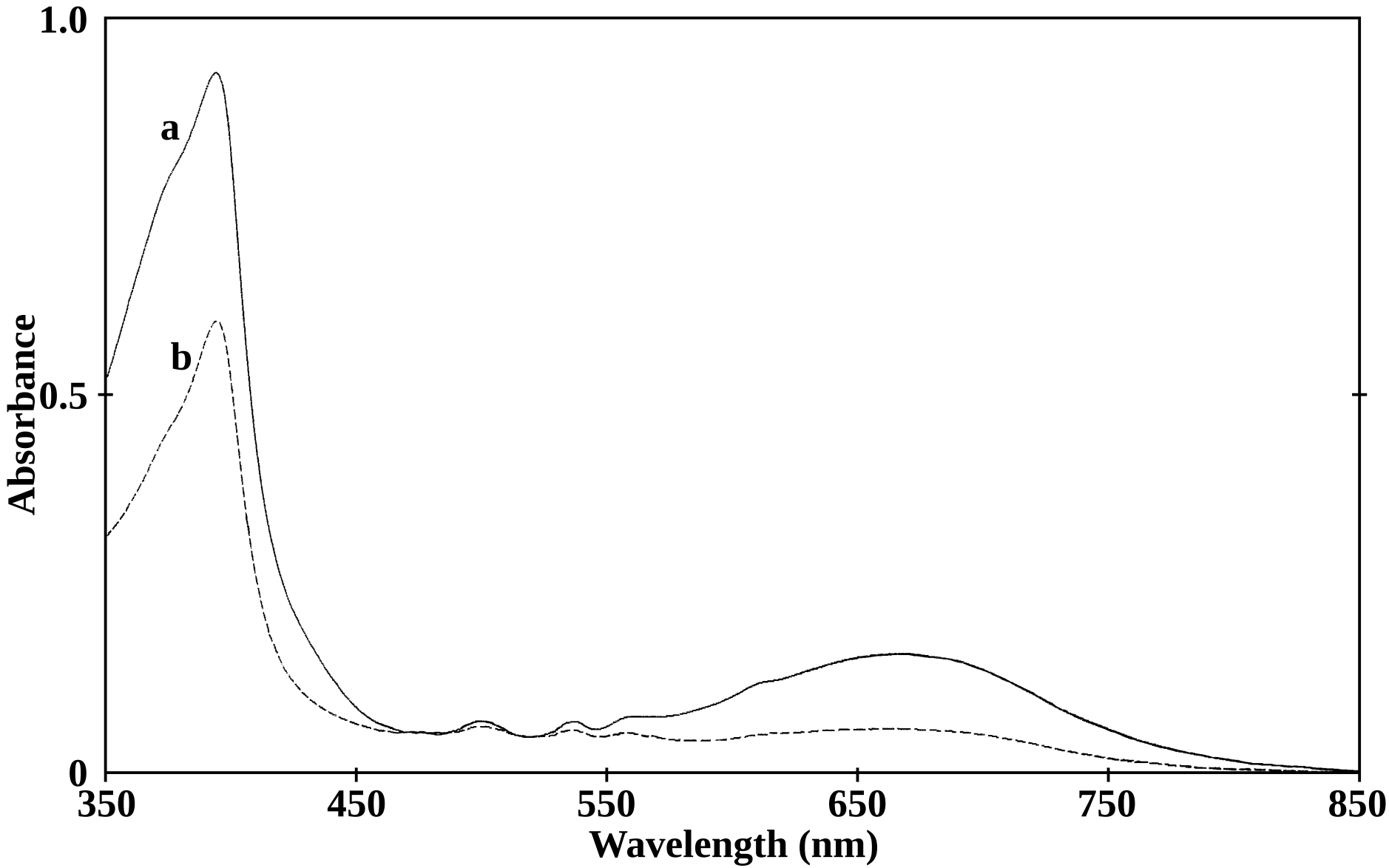

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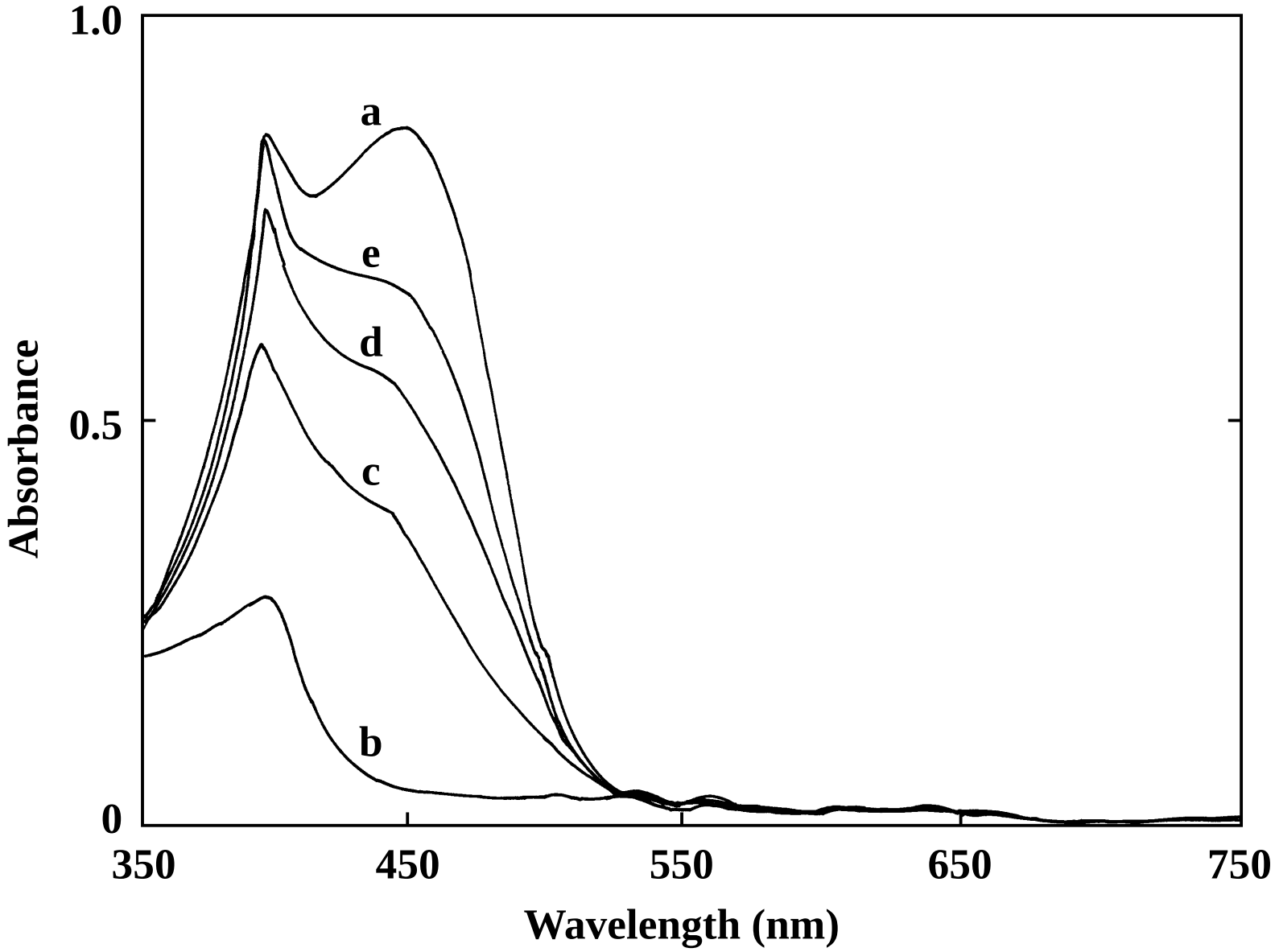

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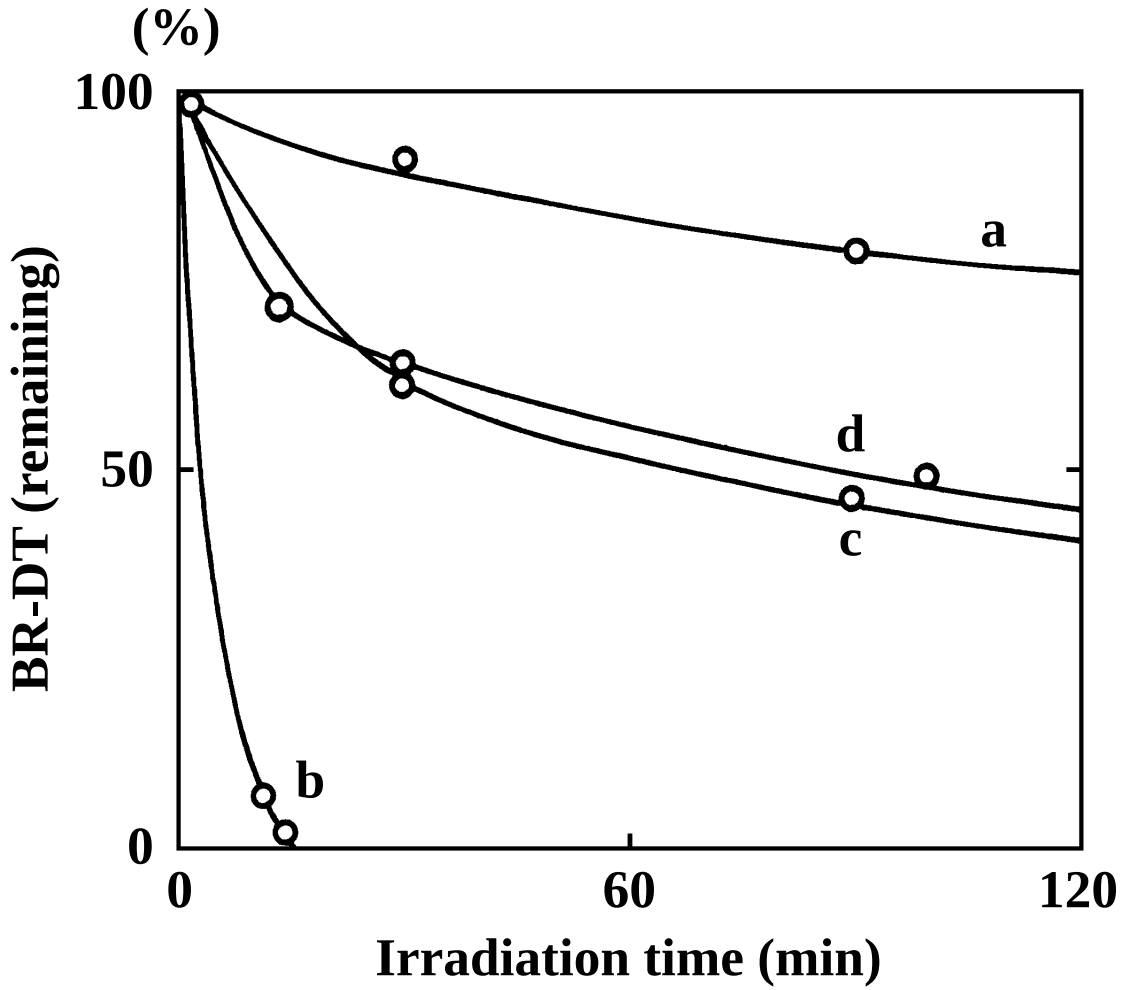
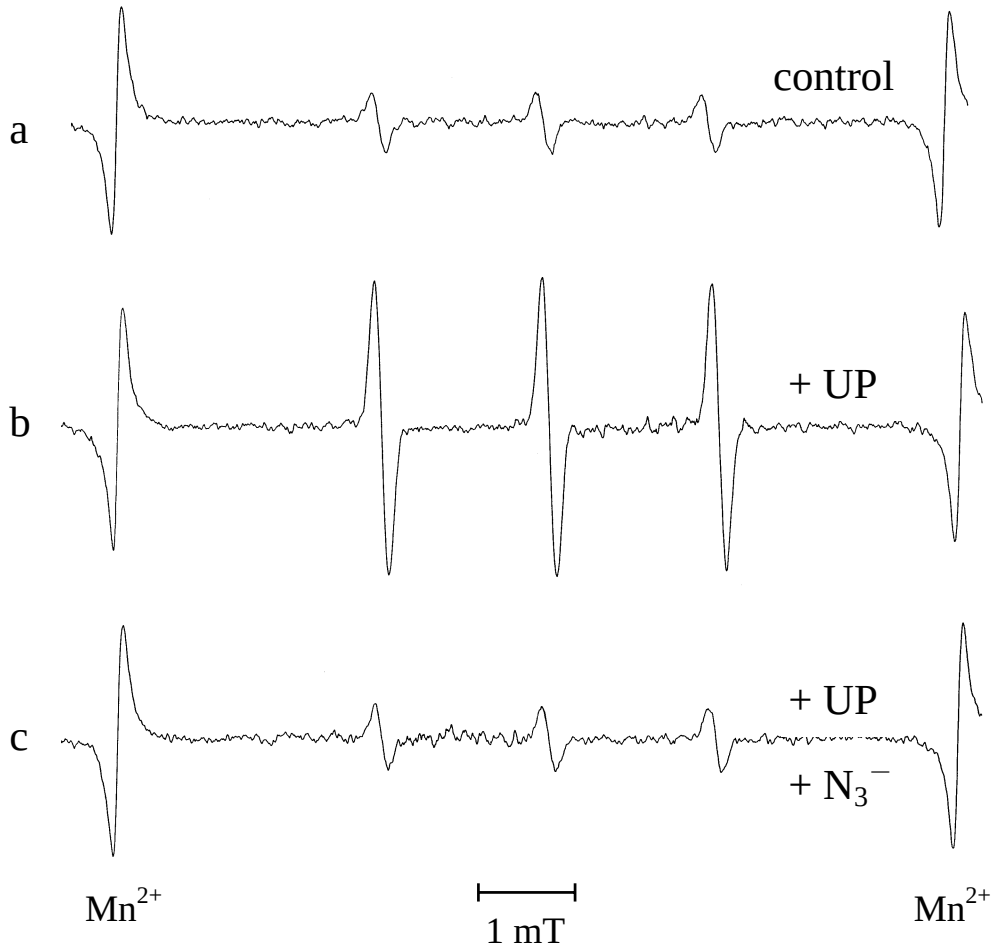
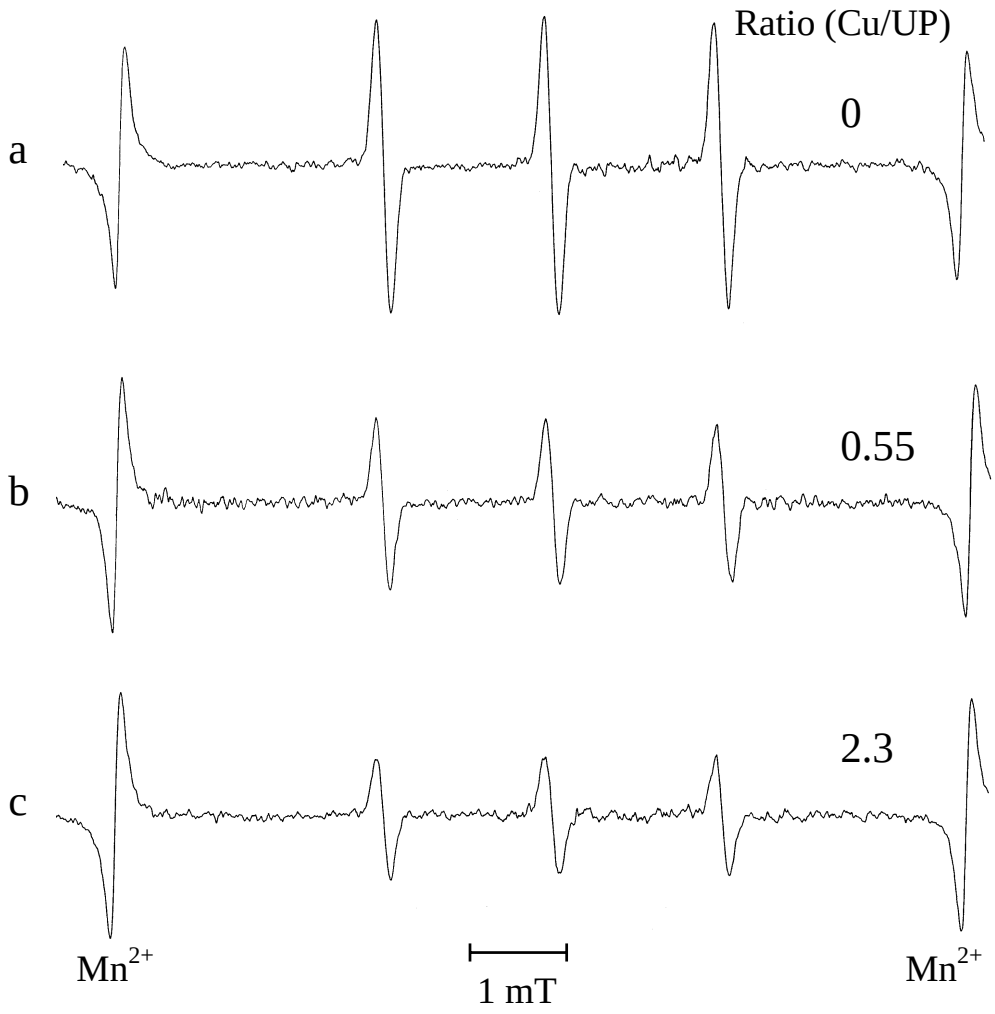


ShishidoFig.5



ShishidoFig.6



ShishidoFig.7