Biochemical Pharmacology, Vol. 33, No. 14, pp. 2193–2197, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

OXYGEN-DEPENDENT CONJUGATION OF DOPA WITH CYSTEINE CATALYSED BY IRON–EDTA COMPLEX

SHOSUKE ITO* and KEISUKE FUJITA

Institute for Comprehensive Medical Science, School of Medicine, Fujita-Gakuen University, Toyoake, Aichi 470–11, Japan

(*Received* 17 *November* 1983; *accepted* 20 *January* 1984)

Abstract—Cytotoxicity of catechols has been ascribed to their binding with proteins through sulfhydryl groups. The possibility that iron–protein complexes catalyse this type of covalent binding was studied with a model system. Reaction of dopa and cysteine catalysed by iron–EDTA complexes at physiological pH resulted in the formation of not only cystine but also conjugation products, cysteinyldopas among which 5-S-cysteinyldopa was the major product. The reaction required iron ion, EDTA, and molecular oxygen. Fe^{3+} and Fe^{2+} were equally effective, while other transition metal ions examined had no effect on the formation of cysteinyldopas. Catalase, superoxide dismutase, and scavengers of hydroxyl radical inhibited to some extents the formation of 5-*S*-cysteinyldopa. Addition of both catalase and superoxide dismutase resulted in approximately 60% inhibition. These results indicated that the iron–EDTA-catalysed conjugation of dopa with cysteine was mainly mediated by hydroxyl radical.

Catechols are widely distributed in nature. Cytotoxicity of catechols has been a subject of extensive studies. The catecholic amino acid dopa is known to exert cytotoxicity to melanocytes [1, 2]. Catecholamines may also have cytotoxic effect to the cells where they are synthesized. Graham *et al.* [3] have postulated that Parkinson's disease may result from life-long exposure of dopaminergic neurons to cytotoxic dopamine.

Several catechols have been used as drugs. In recent years, dopa has been extensively used to treat Parkinson's disease. Another catecholic amino acid α-methyldopa is a widely used antihypertensive drug. Dopa and its analogs have been evaluated as antimelanoma agents [1, 4, 5]. However, cytotoxic, adverse effects of these catechols have also been reported. For example, α-methyldopa produces mild, clinically covert, hepatic injury in up to 36% of patients, and it initiates chronic active hepatitis in a smaller percentage of patients [6]. Catechols are very labile compounds, being readily oxidized to highly electrophilic *o*-quinones. Thus, the cytotoxicity of catechols has been ascribed to covalent binding of the *o*-quinones with proteins through nucleophilic sulfhydryl groups [5–9]. However, most of previous studies on the cytoxicity of catechols dealt with covalent binding of catechols with proteins. Thus, direct evidence for the nature of the covalent bonds has been limited.

The nucleophilic addition of cysteine to dopaquinone produces cysteinyldopas among which 5-*S*-cysteinyldopa is the major product [10]. This catecholic amino acid is excreted at a high level in the urine of melanoma patients and at a low level in normal subjects [11]. Previously, we have shown that the conjugation of dopa with cysteine to form cysteinyldopas is mediated by tyrosinase [10], peroxidase–H_2O_2 [12], superoxide radical formed by the reaction of hypoxanthine with xanthine oxidase [13], and hydroxyl radical formed by the reaction of H_2O_2 with iron–EDTA complex [14]. Inasmuch as iron–protein complexes are present in most biological systems, it is of interest to know whether iron–EDTA complexes can catalyse the conjugation of dopa with cysteine. We report here that the reaction can take place and that it requires molecular oxygen and appears to involve hydroxyl radical as an ultimate oxidizing species.

MATERIALS AND METHODS

Materials. Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this research to avoid contamination of metal ions. Superoxide dismutase, L-dopa, and L-cysteine were obtained from Sigma Chemical Co. (St. Louis, MO) and catalase was from Boehringer Mannheim GmbH (Mannheim, F.R.G.). 5-*S*-, 2-*S*-, and 6-*S*-cysteinyldopa and 2,5-*S*,*S*-dicysteinyldopa were prepared by us [15]. The other chemicals were of the highest purity commercially available.

Method. A typical reaction mixture contained in 4 ml of 0.05 M potassium phosphate buffer (pH 7.4): L-dopa (500 μM), L-cysteine (1000 μM), L-glutamic acid (100 μM; internal standard for amino acid analysis), EDTA·2Na (500 μM), and $FeCl_3$ or $FeSO_4$ (50 μM). The reaction was started by adding iron salt, continued for 1 hr at 30°, and stopped by adding 1 ml of 20% trichloroacetic acid containing 5 mM EDTA·2Na. The reaction mixture was assayed in duplicate with a JEOL JLC-6AH amino acid analyser

* Correspondence should be addressed to Dr. Shosuke Ito, Institute for Comprehensive Medical Science, School of Medicine, Fujita-Gakuen University, Toyoake, Aichi 470-11, Japan.

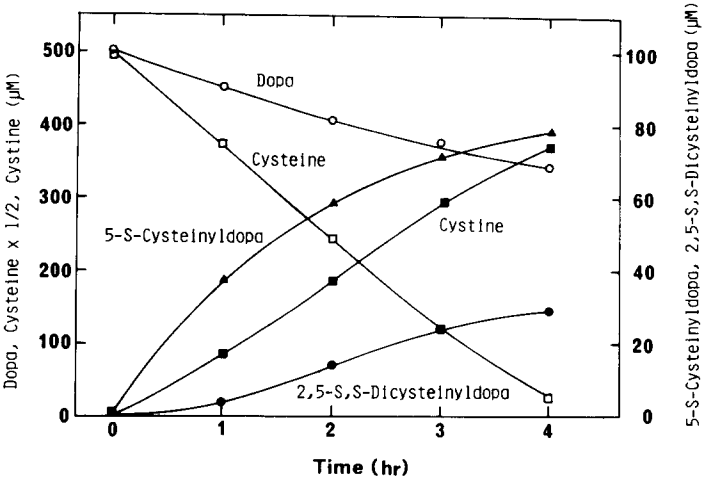

Fig. 1. Time course of the reactions between dopa and cysteine catalysed by Fe^{3+}–EDTA complex. The reactions were carried out as described in Materials and Methods for the periods of times indicated. Each point represents the mean for two separate experiments.

using 4 lithium citrate buffers as mobile phases. Dopa, 5-*S*-, 2-*S*-, and 6-*S*-cysteinyldopa, and 2,5-*S*,*S*-dicysteinyldopa appeared at 249, 296, 269, 266, and 286 min, respectively. Under the same conditions, methionine, leucine, tyrosine, and phenylalanine were eluted out at 247, 267, 276, and 299 min, respectively.

RESULTS

Time course of the reaction between dopa and cysteine catalysed by Fe^{3+}*–EDTA complex.* The reaction between dopa and cysteine catalysed by Fe^{3+}–EDTA complex was followed by amino acid analysis (Fig. 1). Dopa and cysteine decreased almost linearly with time, and most of cysteine disappeared at 4 hr. Major products were found to be 5-*S*-cysteinyldopa and cystine; production of pigments was not observed. 2-*S*-Cysteinyldopa and 2,5-*S*,*S*-dicysteinyldopa were also found in much lower concn; the yields of 5-*S*- and 2-*S*-cysteinyldopa and 2,5-*S*,*S*-dicysteinyldopa were 38, 6.7, and 3.6 μM in the first 1 hr, respectively. 6-*S*-Cysteinyldopa production was negligible. The ratio of cysteinyldopa isomers paralleled closely that with tyrosinase oxidation of dopa plus cysteine [10]. The total yield of cysteinyldopas was nearly equal to the decrease of dopa, when assayed at 1 hr. Furthermore, the rate of 5-*S*-cysteinyldopa formation decreased gradually in the course of the reaction; 38 μM in the first 1 hr to 7 μM in the last 1 hr (Fig. 1). From these results, 1 hr was chosen as the reaction time in the following studies.

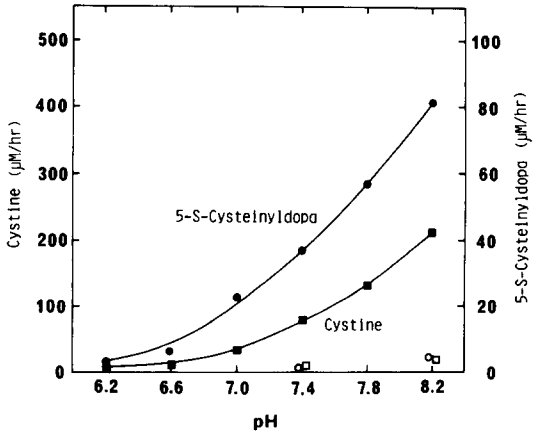

Fig. 2. Effect of pH on the formation of 5-*S*-cysteinyldopa and cystine. The reactions were carried out as described in Materials and Methods in the presence of Fe^{3+}–EDTA complex. Open circle (5-*S*-cysteinyldopa) and square (cystine) represent results obtained in the absence of Fe^{3+}. Each point represents the mean for two separate experiments.

Effects of pH, EDTA, and various metal ions on the formation of 5-S-*cysteinyldopa and cystine.* As indicated in Fig. 2, the rates of formation of both 5-*S*-cysteinyldopa and cystine increased with pH. This is consistent with the knowledge that autoxidation of catechols and sulfhydryl compounds proceeds faster at higher pH. A physiological pH of 7.4 was used in the following studies.

In the absence of EDTA, neither Fe^{3+} nor Fe^{2+}

Table 1. Effect of EDTA on the formation of 5-*S*-cysteinyldopa and cystine*

EDTA (μM)	Iron ion added	Products (μM/hr) 5-*S*-Cysteinyldopa	Cystine
0	Fe^{3+}	1.1 ± 0	6.8 ± 1.0
0	Fe^{2+}	2.1 ± 1.6	23.4 ± 10.8
100	Fe^{3+}	37.3 ± 1.1	99.9 ± 17.2
500	Fe^{3+}	37.6 ± 3.9	85.9 ± 18.5
1000	Fe^{3+}	33.3 ± 1.4	60.3 ± 11.6

* The reactions were carried out as described in Materials and Methods except that various concns of EDTA were added. The results represent mean ± S.D. for two separate experiments.

Table 2. Effect of various metal ions on the formation of 5-*S*-cysteinyldopa and cystine*

Metal ion added	Products (μM/hr) 5-*S*-Cysteinyldopa	Cystine
None	1.4 ± 0.6	11.2 ± 5.4
Fe^{3+} 50 μM	37.0 ± 2.0	79.4 ± 9.6 (n = 10)
Fe^{2+} 50μM	40.0 ± 1.3	109.5 ± 11.2 (n = 6)
Mn^{2+} 50 μM	1.2 ± 0.1	25.5 ± 9.9
Ni^{2+} 50 μM	1.3 ± 0.2	24.0 ± 7.8
Co^{2+} 50 μM	2.0 ± 0.5	30.3 ± 5.0
Cu^{2+} 5 μM	*ca.* 0.2	450 ± 4
0.5 μM	*ca.* 0.5	243 ± 18
Zn^{2+} 50 μM	1.6 ± 0.1	30.3 ± 0.6

* The reactions were carried out as described in Materials and Methods except that various metal ions were added. Except for Fe^{2+}, the results represent mean ± S.D. for two separate experiments.

promoted the formation of 5-*S*-cysteinyldopa (Table 1). Addition of EDTA caused a great increase in the formation of both 5-*S*-cysteinyldopa and cystine.

As seen in Table 2, no significant formation of 5-*S*-cysteinyldopa was detected in the absence of metal ion added. Fe^{3+} and Fe^{2+} had similar degrees of catalytic effect on the formation of cystine. Other transition metal ions, such as Mn^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+}, had little catalytic effect. Among them, Cu^{2+} showed a different behaviour with a rapid production of cystine. This is consistent with a recent finding that oxygen consumption by cysteine was strongly stimulated by Cu^{2+} [16].

Effects of O_2 exclusion and scavengers of active oxygens on the formation of 5-S-cysteinyldopa and cystine. The fact that Fe^{3+} and Fe^{2+} had similar and catalytic effects on the formation of 5-*S*-cysteinyldopa and cystine suggested that O_2 is required for the reaction. Therefore, the effects of O_2 exclusion and scavengers of active oxygens on the oxidation were examined. As seen in Table 3, the iron-catalysed oxidation of dopa to 5-*S*-cysteinyldopa was almost completely inhibited by omitting O_2, and the cystine formation was also greatly suppressed.

Catalase, a scavenger of H_2O_2, inhibited the formation of both 5-*S*-cysteinyldopa and cystine with a greater effect on the latter. Addition of a higher concn (100 μg/ml) of catalase lowered the inhibitory effect; this is possibly due to the peroxidase activity of catalase itself [17]. Superoxide dismutase, a scavenger of superoxide radical ($O_2^{\cdot-}$), weakly inhibited the formation of 5-*S*-cysteinyldopa, while it greatly accelerated the cystine formation. This augumentation was also observed with the enzyme in the absence of iron salts (data not shown). The same type of augumentation of disulfide formation by superoxide dismutase has been reported by Misra [18]. Addition of both catalase and superoxide dismutase had a synergistic effect on the inhibition of 5-*S*-cysteinyldopa formation. D-Mannitol and formate, scavengers of hydroxyl radical (·OH) [18], also decreased the oxidation rates to some extents.

Table 4. Formation of 2,5-*S*,*S*-dicysteinyldopa from 5-*S*-cysteinyldopa and cysteine*

Metal ion added	Products (μM/hr) 2,5-*S*,*S*-Dicysteinyldopa	Cystine
Fe^{3+} 50 μM	36.5 ± 0.4	57.7 ± 3.6
Fe^{2+} 50 μM	44.2 ± 3.9	72.6 ± 4.3

* The reactions were carried out as described in Materials and Methods except that 5-*S*-cysteinyldopa was used in place of dopa. The results represent mean ± S.D. for two separate experiments.

Formation of 2,5-S,S-dicysteinyldopa from 5-S-cysteinyldopa and cysteine. Oxidation of 5-*S*-cysteinyldopa in the presence of cysteine under similar conditions as for dopa gave 2,5-*S*,*S*-dicysteinyldopa in yields comparable to those of 5-*S*-cysteinyldopa from dopa. Considering that 2-*S*-cysteinyldopa and 2,5-*S*,*S*-dicysteinyldopa were also formed from dopa in addition to 5-*S*-cysteinyldopa, it appeared that the

Table 3. Effects of O_2 exclusion and scavengers of active oxygens on the formation of 5-*S*-cysteinyldopa and cystine*

Conditions	Percentage of control 5-S-Cysteinyldopa	Cystine
1. Fe^{3+} (50 μM)	100	100
− O_2 (N_2 bubbling)	5 ± 0	20 ± 1
+ Catalase (10 μg/ml)	73 ± 3	40 ± 5
+ Catalase (100 μg/ml)	76 ± 0	66 ± 4
+ Superoxide dismutase (10 μg/ml)	78 ± 1	265 ± 16
+ Superoxide dismutase (100 μg/ml)	73 ± 2	500 ± 59
+ Catalase (10 μg/ml) + superoxide dismutase (10 μg/ml)	44 ± 6	93 ± 9
+ D-Mannitol (10 mM)	86 ± 1	78 ± 8
+ Sodium formate (10 mM)	84 ± 1	75 ± 12
2. Fe^{2+} (50 μM)	100	100
− O_2 (N_2 bubbling)	2 ± 0	12 ± 3
+ Catalase (10 μg/ml)	65 ± 3	23 ± 0
+ Superoxide dismutase (10 μg/ml)	88 ± 4	201 ± 18
+ Catalase (10 μg/ml) + superoxide dimutase (10 μg/ml)	37 ± 7	91 ± 1
+ D-Mannitol (10 mM)	88 ± 3	82 ± 5
+ Sodium formate (10 mM)	82 ± 3	65 ± 4

* The reactions were carried out as described in Materials and Methods except that air was replaced by nitrogen bubbling or various scavengers were added. The results represent mean ± S.D. for two separate experiments.

reactivity of 5-*S*-cysteinyldopa is comparable to or slightly lower than that of dopa.

DISCUSSION

The present study shows that iron–EDTA complexes can catalyse at physiological pH the conjugation of dopa with cysteine to form cysteinyldopas among which 5-*S*-cysteinyldopa is the major isomer. Fe^{2+} and Fe^{3+} had similar degrees of catalytic effect. Cystine was also formed in yields two to three times those of 5-*S*-cysteinyldopa. The reaction requires iron ion, EDTA, and molecular oxygen, and was partially inhibited by catalase, superoxide dismutase, and scavengers of ·OH. These results indicate that iron–EDTA complexes mediate the oxidation of dopa to a reactive intermediate such as dopaquinone through the formation of active oxygens, H_2O_2, $O_2^{\cdot-}$, and ·OH. The following reactions (1)–(4) may be feasible between iron–EDTA complexes and O_2 or O_2-derived species [19–21].

$$Fe^{2+}\text{–EDTA} + O_2 \rightarrow Fe^{3+}\text{–EDTA} + O_2^{\cdot-} \quad (1)$$

$$Fe^{2+}\text{–EDTA} + O_2^{\cdot-} + 2H^+ \rightarrow Fe^{3+}\text{–EDTA} + H_2O_2 \quad (2)$$

$$Fe^{2+}\text{–EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{–EDTA} + \cdot OH + OH^- \quad (3)$$

$$Fe^{3+}\text{–EDTA} + O_2^{\cdot-} \rightarrow Fe^{2+}\text{–EDTA} + O_2 \quad (4)$$

When the reaction was initiated with Fe^{3+}, direct reduction of Fe^{3+} with dopa or cysteine can generate Fe^{2+}. After initial phase of the reaction, the same equilibrium of Fe^{2+} and Fe^{3+} states may be achieved regardless of the oxidation state of iron salt added. Combination of reactions (2) and (4) implies a superoxide dismutase activity of iron–EDTA complexes [19, 21]. Reaction (3) generates a powerful oxidising species, ·OH (Fenton reaction) [22]. It has recently reported that ·OH is also produced during cysteine autoxidation [16] and that sulfhydryl compounds promote ·OH production in an iron-H_2O_2 system [23].

The fact that superoxide dismutase had only a weak inhibitory effect may be ascribed to the superoxide dismutase activity of iron–EDTA complexes [19, 21]. Reaction (3) generates a powerful oxidizing reaction. Although the inhibitory effect of catalase alone on the formation of 5-*S*-cysteinyldopa was also weak, it acted synergistically with superoxide dismutase to prevent the reaction by approximately 60%. The synergistic effect of catalase and superoxide dismutase has been considered as evidence for the involvement of ·OH [24]. The fact that scavengers of ·OH did not strongly inhibit the 5-*S*-cysteinyldopa formation may be due to an extremely rapid reaction of dopa with ·OH. Although the rate constant for dopa has not been reported, that for dopamine has been analysed to be $5.9 \times 10^9\,M^{-1}s^{-1}$ at pH 4.7 and is likely to be similar [25]. The rate constant would be much higher at pH 7.4 where the present study was undertaken [25]. Those for mannitol and formate were reported to be 1.0×10^9 and $2.7 \times 10^9\,M^{-1}s^{-1}$, respectively [26].

As shown in our previous studies [13, 14], both $O_2^{\cdot-}$, and ·OH can mediate the conjugation of dopa with cysteine. The rate of oxidation of dopa by $O_2^{\cdot-}$ was shown to be comparable to that of reduction of nitro-blue tetrazolium ($k_1 = 6 \times 10^4\,M^{-1}s^{-1}$ at pH 9.8 in ref. 27) [13]. On the other hand, the rate constants for the reactions (2) and (4) were reported to be 6×10^5 and $2 \times 10^6\,M^{-1}s^{-1}$, respectively at pH 7.8 [21]. Thus, it is likely that most of $O_2^{\cdot-}$ formed is rapidly consumed by reactions (2) and (4). From these considerations, the ultimate oxidizing species in the conversion of dopa to a reactive intermediate appeares to be ·OH. The reaction of ·OH with dopa may produce initially a semiquinone radical [25]. It is, however, not known which of the semiquinone radical of dopa or the *o*-quinone of dopa (dopaquinone) is the ultimate oxidizing species producing cysteinyldopas.

The formation of 5-*S*-cysteinyldopa was less sensitive to the inhibition by catalase and by ·OH scavengers than that of cystine. Furthermore, Fe^{3+}–EDTA complex was less affected by O_2 exclusion than was Fe^{2+}–EDTA complex. These results suggest that direct oxidation of Fe^{3+}–EDTA complex may also contribute to the formation of 5-*S*-cysteinyldopa to a small extent (up to 30–40%).

Gillette *et al.* [28] have examined the effect of transition metal ions on the oxidation of catechols including dopa and found that Mn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} catalysed the oxidation, but Fe^{2+} had only a weak effect. The apparent discrepancy between our results and theirs may be attributed to the effect of EDTA; they stated that Fe^{3+} formed a stable complex with catechol (in the absence of EDTA), thus preventing further oxidation.

Iron–protein complexes are present in most biological systems. Examples include transferrin, ferritin, cytochromes, and hemoglobin. McCord and Day [29] have shown that iron-chelated transferrin is as effective as iron–EDTA complexes in catalysing the reactions (2)–(4) to form ·OH. Ambruso and Johnston [30] have reported that iron-saturated lactoferrin is extremely effective in enhancing ·OH generation by human neutrophiles. Methemoglobin is also shown to catalyse the formation of ·OH in the presence of ascorbic acid [31, 32]. ADP may also act as a physiological chelator of irons ions [33]. Thus, it appears likely that the reaction described in the present report is also relevant to biological systems.

Cytotoxicity of catechols has been ascribed to covalent binding of *o*-quinones with proteins through sulfhydryl groups [5–9]. However, most of previous studies examined the binding of radioactive catechols with proteins and thus, the nature of the covalent binding between catechols and proteins remained speculative. Our present study as well as the previous ones [10, 12–14] have identified cysteinyldopas as the products of the reactions between dopa and cysteine catalysed by tyrosinase, peroxidase ·H_2O_2, $O_2^{\cdot-}$, and ·OH. These results afford chemical evidence that the covalent binding of catechols with proteins results from the reaction of sulfhydryl groups of proteins with *o*-quinone or semiquinone radical form of catechols.

Finally, another problem which may be relevant to the present study is the genesis of cysteinyldopas

in mammals. It is generally accepted that urinary excretion of 5-*S*-cysteinyldopa reflects tyrosinase activity in melanocytes. However, Fehling *et al.* [34] have shown that after administration of dopa to albino rats, 5-*S*-glutathionedopa was formed in the spleen and they attributed the formation to non-specific oxidation of dopa. Furthermore, the high urinary excretion of 5-*S*-cysteinyldopa in the patients of Parkinson's disease undergoing dopa + carbidopa therapy [35] may also result from tyrosinase-independent oxidation such as one catalysed by iron–protein complexes.

REFERENCES

1. M. M. Wick, L. Byers and E. Frei III, *Science* **197**, 468 (1977).
2. J. M. Pawelek, *J. invest. Dermat.* **66**, 201 (1977).
3. D. G. Graham, S. M. Tiffany, W. R. Bell Jr. and W. F. Gutknecht, *Molec. Pharmac.* **14**, 644 (1978).
4. M. M. Wick, *J. natn. Cancer Inst.* **63**, 1465 (1979).
5. M. M. Wick, *Cancer Res.* **40**, 1414 (1980).
6. E. Dybing, S. D. Nelson, J. R. Michell, H. A. Sasame and J. R. Gillette, *Molec. Pharmac.* **12**, 911 (1976).
7. A. Rotman, J. W. Daly and C. R. Creveling, *Molec. Pharmac.* **12**, 887 (1976).
8. A. Rotman, J. W. Daly, C. R. Creveling and X. O. Breakefield, *Biochem. Pharmac.* **25**, 383 (1976).
9. D. G. Graham, S. M. Tiffany and F. S. Vogel, *J. invest. Derm.* **70**, 113, (1978).
10. S. Ito and G. Prota, *Experientia* **33**, 1118 (1977).
11. G. Agrup, P. Agrup, T. Andersson, L. Hafström, C. Hansson, S. Jacobsson, P.-E. Jönsson, H. Rorsman, A.-M. Rosengren and E. Rosengren, *Acta Dermatovener. (Stockholm)* **59**, 381 (1979).
12. S. Ito and K. Fujita, *Biochim. biophys. Acta* **672**, 151 (1981).
13. S. Ito and K. Fujita, *Biochem. Pharmac.* **31**, 2887 (1982).
14. S. Ito, *Bull. chem. Soc. Jpn.* **56**, 365 (1983).
15. S. Ito, S. Inoue, Y. Yamamoto and K. Fujita, *J. med. Chem.* **24**, 673 (1981).
16. G. Saez, P. J. Thornalley, H. A. O. Hill, R. Hems and J. V. Bannister, *Biochim. biophys. Acta* **719**, 24 (1982).
17. K. L. Kremer, *Biochim. biophys. Acta* **198**, 199 (1970).
18. H. P. Misra, *J. biol. Chem.* **249**, 2151 (1974).
19. B. Halliwell, *FEBS Lett.* **92**, 321 (1978).
20. A. M. Michelson, *Biochimie* **55**, 465 (1973).
21. C. Bull, J. A. Fee, P. O'Neill and E. M. Fielden, *Archs. Biochem. Biophys.* **215**, 551 (1982).
22. C. Walling, *Account chem. Res.* **8**, 125 (1975).
23. D. A. Rowley and B. Halliwell, *FEBS Lett.* **138**, 33 (1982).
24. E. W. Kellogg III and I. Fridovich, *J. biol. Chem.* **252**, 6721 (1977).
25. H. W. Richter and W. H. Waddell, *J. Am. chem. Soc.* **105**, 5434 (1983).
26. M. Anbar and P. Neta, *Int. J. appl. Radiat. Isot.* **18**, 495 (1967).
27. B. H. Bielski and H. W. Richter, *J. Am. chem. Soc.* **99**, 3019 (1977).
28. J. R. Gillette, D. Watland and G. Kalitsky, *Biochim. biophys. Acta* **15**, 526 (1954).
29. J. M. McCord and E. D. Day Jr., *FEBS Lett.* **86**, 138 (1978).
30. D. R. Ambruso and R. B. Johnston Jr., *J. clin. Invest.* **67**, 352 (1981).
31. U. Benatti, A. Morelli, L. Guida and A. De Flora, *Biochem. biophys. Res. Commun.* **111**, 980 (1983).
32. G. Agrup, C. Hansson, G. Rorsman, E. Rosengren and E. Tegner, *Acta Dermatovener. (Stockholm)* **63**, 152 (1983).
33. K.-L. Fong, P. B. McCay, J. L. Poyer, B. B. Keele and H. Misra, *J. biol. Chem.* **248**, 7792 (1973).
34. C. Fehling, C. Hansson, J. Poulsen, H. Rorsman and E. Rosengren, *Acta Dermatovener. (Stockholm)* **61**, 339 (1981).
35. R. M. Stewart, S. Miller and M. Gunder, *Acta Dermatovener. (Stockholm)* **63**, 97 (1983).