Weak Chemiluminescence at an Early Stage of the Maillard Reaction

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Because a fairly stable free radical was observed to form at an early stage of the Maillard reaction, the development of chemiluminescence (CL) in the Maillard reaction was examined by using a highly sensitive photon counting system. It was demonstrated that marked CL developed generally at an early stage in the Maillard reaction prior to browning. The CL intensity followed the order methylglyoxal ≫ glyoxal > xylose > glucose in the carbonyl compounds and alkylamine > lysine > β-alanine > α-alanine in the amino compound. CL was observed even at pH 6.0 and increased markedly with increase in pH. These features are almost the same as those observed in the free-radical formation and browning. CL was negligibly weak in Ar or N_2 and greatly increased in air; it was suppressed by addition of ascorbic acid and cysteine. The mechanism by which CL develops is not yet clear but is assumed to be attributable to the production of electronically excited species through the reaction of oxygen with the free-radical product(s) of low molecular weight dicarbonyl products or pyrazinium compounds and these may also directly contribute to browning.

INTRODUCTION

Attention has recently been focused on the Maillard reaction not only in studies on food quality changes but also in those on the mechanisms of the potential causes of aging (Namiki, 1988). Concerning the mechanism of the Maillard reaction, the scheme proposed by Hodge in 1953 (Hodge, 1953), involving Amadori rearrangement as a key step, has been accepted widely as being the most reasonable. However, development of novel free-radical products at an early stage of the reaction has been found (Namiki *et al.*, 1973; Namiki and Hayashi, 1975; Hayashi *et al.*, 1977), and on the basis of structural analysis a new mechanism has been demonstrated that involves fragmentation of the Schiff base product at the first stage of the Maillard reaction to give a very reactive low molecular weight enaminol product followed by the formation of a pyrazinium free-radical product as well as browning products (Namiki and Hayashi, 1983; Namiki, 1988).

On the other hand, Szent-Györgyi has studied the problem concerning the energy-transfer complex formed by the reaction of amino acid and protein with α-dicarbonyl compounds, particularly methylglyoxal, as related to the mechanism of cancerization of normal cells (Szent-Györgyi, 1976, 1980). He reported that reaction of methylglyoxal with methylamine, cysteine, and other compounds gives a charge-transfer complex as well as free-radical products (Gascoyne *et al.*, 1982).

Recently the development of a highly sensitive photon counting system has made possible the detection of very weak chemiluminescence (CL) (Inaba *et al.*, 1982). This is utilized as an ultramicro detector for electronically excited oxygen species such as breakdown products of lipid peroxide (Miyazawa *et al.*, 1981, 1988; Boveris *et al.*, 1981; Sato *et al.*, 1991). On the other hand, the generation of weak CL during the amino–carbonyl reaction has been reported (Børdalen, 1984), and that was also demonstrated as a generation mechanism of weak CL observed in the extracts of stored Chinese drugs such as *Glycyrrhizae radix* (Edo *et al.*, 1985). Moreover, the effects of amino and carbonyl compounds, pH, molar ratio, and oxygen on CL generation have been examined and a possible generation mechanism has been proposed (Kurosaki *et al.*, 1989, 1991a,b).

On the basis of our finding on the development of novel free-radical products at a very early stage of the Maillard reaction, our research group has also investigated the development of CL in the Maillard reaction. This paper deals with the development and some properties of weak CL at an early stage of various amino–carbonyl reaction systems in correlation with free-radical formation and browning.

EXPERIMENTAL PROCEDURES

Chemiluminescence Measurement. CL was measured mainly using the Tohoku Electronic Industry Co. (Sendai, Japan) CLD 100 chemiluminescence detector system (sensitivity, 10^{-14} W; wavelength region, 300–650 nm). In this case, a solution (usually 5.0 mL) in a sample cell (stainless steel, 5.0 cm i.d. open dish, except for the experiment under anaerobic conditions) was placed in a temperature-controlled sample room of a black box of the CLD 100. CL was recorded using a special data analyzer, CLC-10 (Tohoku Electronic Industry). The counts per second shown on the vertical axis in the figures is a CL intensity unit measured by the CLD 100 chemiluminescence detector. It is not a direct count of the number of photons but a unit by which one count corresponds to approximately 10 photons.

CL was also determined by a JASCO Co. (Tokyo, Japan) Model 825 chemiluminescence detector. In this case, a solution was circulated through the flow cell of the detector and CL was recorded as relative intensity.

Materials. All reagents used were of guaranteed grade.

Experimental Method. *Method A.* A solution of an amino compound in phosphate buffer or Merczen buffer and a solution of a carbonyl compound were mixed in a sample cell of the CLD 100 apparatus and heated to a given temperature in a sample room. Chemiluminescence was recorded as a function of time.

Method B. A mixture of amino and carbonyl compounds was heated in a flask, and aliquots taken at time intervals during heating were used for the determination of browning with

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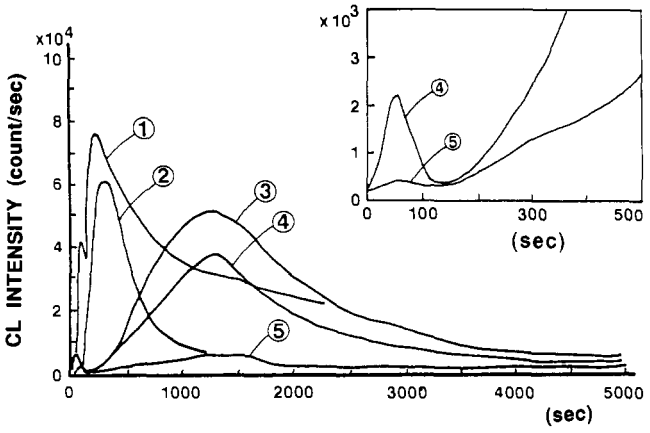

Figure 1. Chemiluminescence in various Maillard reaction systems (pH 8.0, 90 °C, in air): (1) glyoxal–methylamine (0.025 M each); (2) xylose–β-alanine (1.0 M each); (3) glucose–lysine (1.0 M each); (4) glucose–β-alanine (1.0 M each); (5) glucose–α-alanine (1.0 M each).

absorption at 420 nm (LKB Ultraspec 4050); CL was determined using the CLD 100 system.

Method C. A mixture of an amino compound and a carbonyl compound was heated in a flask and circulated in a Model 825 CL detector by HPLC pump, and development of CL recorded.

Method D. CL Measurement of the Reaction under Oxygen-Free Condition. Each solution of amino and carbonyl compound previously deaerated was exchanged with pure argon gas by repeated degasifications *in vacuo* of the frozen sample solution followed by saturation with pure argon gas after defreezing. These reactant solutions were mixed in a glass cuvette under argon gas atmosphere, and CL was measured instantaneously with heating using the CLD 100 apparatus.

RESULTS AND DISCUSSION

Chemiluminescence of Various Maillard Reaction Systems (Method A). Figure 1 shows the results obtained from the various Maillard reaction mixtures at pH 8.0 and 90 °C. It was demonstrated that CL clearly developed in every amino–carbonyl reaction system very early in the reaction and increased with heating time. No CL was detected in the solution of an amino or a carbonyl compound alone at any pH value. The development of CL was especially prominent in the glyoxal–methylamine system: CL was observed as soon as the reaction was started and increased very rapidly with heating time, giving a first small peak within 100–150 s and then increasing again until a maximum was reached at about 300 s. This two-step-type increase in the development of CL was also observed in other cases including the glucose–β-alanine system, whose initial process is shown in the inset of Figure 1. After a main maximum peak at around 1200 s, CL decreased with further heating. A comparison of the reaction time and the CL intensity at each maximum point indicated that the development followed the order Lys > β-Ala ≫ α-Ala in amino acid and D-xylose > D-glucose in sugar. The order for both was almost the same in browning and in free-radical formation (Namiki and Hayashi, 1975).

Chemiluminescence and Browning in Maillard Reaction (Method B). The relationship between CL and browning was examined with the glucose–β-alanine system at different pH values by method B. As shown in Figure 2, development of CL was observed at an early stage of the reaction prior to the browning reaction, and the intensity increased rapidly with heating time, especially in the alkaline pH mixtures, and showed a maximum peak at about 30 min, along with a simultaneous increase of browning. Integrated CL values calculated from the CL curves are shown by broken lines in Figure 2. Although the increase in the integrated CL seems to precede slightly

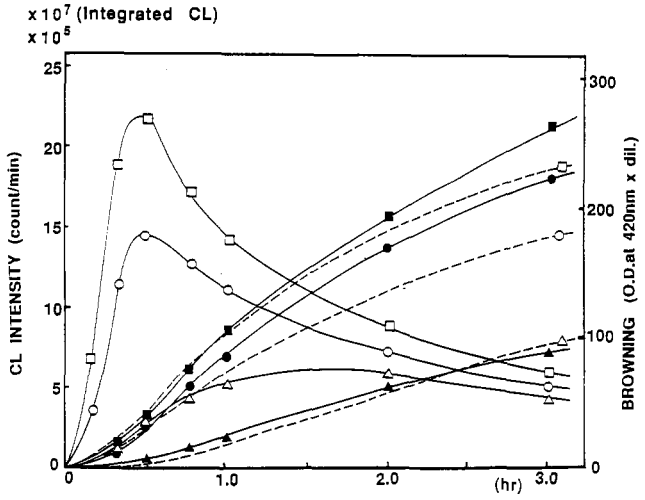

Figure 2. Chemiluminescence and browning in Maillard reaction. Glucose–β-alanine (1.0 M each, in air) was heated in a boiling water bath. Chemiluminescence was measured at 30 °C, and browning was determined at 420 nm. CL: pH 6 (–△–); pH 8 (–○–); pH 11 (–□–). Integrated CL: pH 6 (- -△- -); pH 8 (- -○- -); pH 11 (- -□- -). Browning: pH 6 (–▲–); pH 8 (–●–); pH 11 (–■–).

that of browning, every increasing curve of the integrated CL corresponds approximately to each curve of the browning at the same pH, indicating that the CL-forming reaction product(s) may directly participate in further reaction, giving rise to melanoidin. The tendency in the integrated CL curves to saturate at a later stage may be partly due to absorption of CL light emitted by the browning product.

The results shown here and in Figure 1 clearly indicate that the CL in the Maillard reaction occurs prior to the browning reaction. This is the same result observed in earlier studies on free-radical development in the Maillard reaction (Namiki and Hayashi, 1975) but differs from the results reported by Kurosaki *et al.* (1989).

Effect of pH on Development of CL (Method A). As shown in Figure 2, the development of CL was prominent in alkaline solution, so the effect of pH during reaction on the development of CL was examined in the reaction systems of xylose–β-alanine (0.1 M each) and glucose–β-alanine (1.0 M each) of 90 °C. The results shown in Figure 3 indicate that CL could be detected even at pH 6.0 and increased nearly logarithmically with increasing pH values until near pH 8.0, indicating that CL develops especially strongly in alkaline pH reactions.

Relationship between CL and Reactant Concentration. The development of CL in the reaction of the glyoxal–methylamine system at pH 8.0 and 90 °C in air was measured at different molar concentrations. As shown in Figure 4, a linear relationship in log–log scale was observed between the intensity of CL and the molar concentration of the reactants, and it was shown that the CL in this system is detectable even at a concentration on the order of 1 mM of reactant.

Effect of Oxygen on the Development of Chemiluminescence. It is commonly recognized that CL is generated from excited molecules such as singlet oxygen and excited carbonyl compounds and that oxygen plays an important role in the formation of such excited molecules, usually through the formation of peroxide product by the reaction of a free radical and triplet oxygen (Campell, 1988). In fact, Kurosaki *et al.* (1989) reported that almost all of the CL of the Maillard reaction disappeared under nitrogen gas bubbling. However, the ESR signal of the Maillard reaction can be detected even in an open test tube and disappears rapidly by bubbling

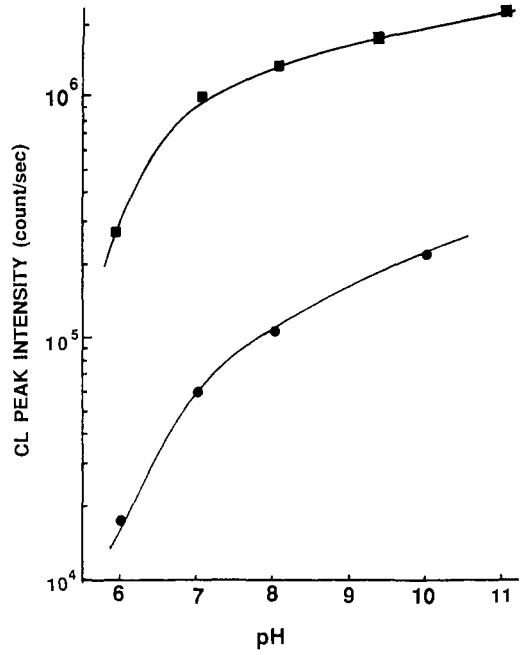

Figure 3. Effect of pH on development of chemiluminescence in Maillard reaction. Xylose–β-alanine (0.1 M each) (●) and glucose–β-alanine (1.0 M each) were heated at 90 °C in air. Each spot indicates the maximum peak value of chemiluminescence in each reaction system.

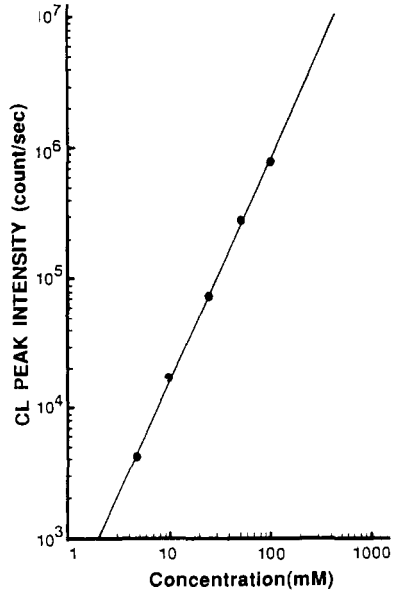

Figure 4. Relationship between chemiluminescence and reactant concentration in Maillard reaction. Glyoxal–methylamine, pH 8.0, was heated at 90 °C in air.

of air, indicating that the free-radical product is fairly stable in the reaction mixture (Namiki and Hayashi, 1975). On the other hand, browning in the Maillard reaction is known to proceed even in the absence of oxygen, although the effect of oxygen on the reaction remains to be elucidated (Namiki, 1988). Moreover, the possibility of the development of CL in the Maillard reaction independent of oxygen, e.g., from an excited carbonyl or Schiff group, is indisputable. Thus, we examined the effect of oxygen on the CL of the Maillard reaction by preparing a strictly anaerobic reaction system using method D. As shown in Figure 5, the reaction of xylose–β-alanine in this system showed negligibly weak CL at an early stage, about 200 counts/s compared to the background (about 100 counts/s with β-alanine solution), and it increased dramatically with the introduction of air. The result indicates that almost all of the CL observed in the Maillard reaction

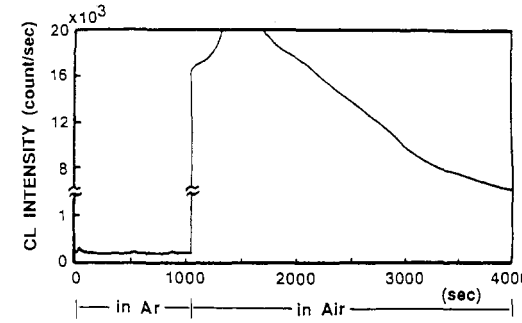

Figure 5. Effect of oxygen on chemiluminescence in Maillard reaction. Xylose–β-alanine (1.0 M each, pH 8.0, in argon) was heated at 90 °C.

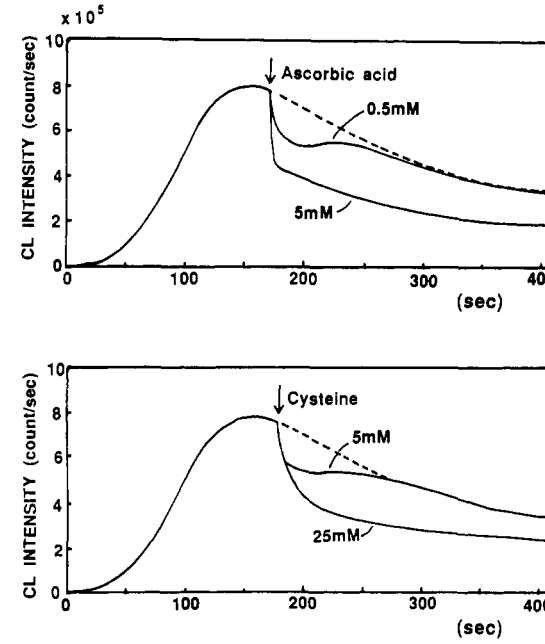

Figure 6. Effect of ascorbic acid and cysteine on development of chemiluminescence in Maillard reaction. Glyoxal–methylamine (0.1 M each, pH 8.0, 5.0 mL) was heated at 90 °C in air, and 1 mL of ascorbic acid or cysteine in distilled water was added.

is caused by the reaction of oxygen with some reactive reaction product(s) such as a free-radical compound or a highly oxidizable compound.

Effect of Ascorbic Acid and Cysteine on the Development of CL. To observe the effect of a reducing or oxygen radical scavenging agent on the development of CL in the Maillard reaction, a solution of ascorbic acid or cysteine was added to the reaction mixture of glyoxal–methylamine proceeding at 90 °C by method A at around the maximum point of the development of CL. As shown in Figure 6, the CL instantaneously and markedly decreased by the addition of ascorbic acid as well as cysteine and then gradually decreased with further heating. The suppressive effect seemed somewhat stronger with ascorbic acid than with cysteine. The mechanism is not yet clear for either case, but it is assumed to be due to their reducing activity on the oxygen-related excited molecule and/or to the scavenging effect on the free-radical product. It should be noted that ascorbic acid is a representative enediol reductone compound and the addition of ascorbic acid caused the marked decrease in the CL. Ascorbic acid has been known to produce some active oxygen species such as superoxide, H_2O_2, and OH radical by the reduction of the oxygen molecule, especially in the presence of Fe or Cu ion (Fridovich, 1979; Seib, 1982). Thus, oxidative damage to some protein and enzymes caused by the ascorbic acid–metal ion system (Shinar *et al.*, 1983) as well as the Amadori compound–metal ion system (Kawak-

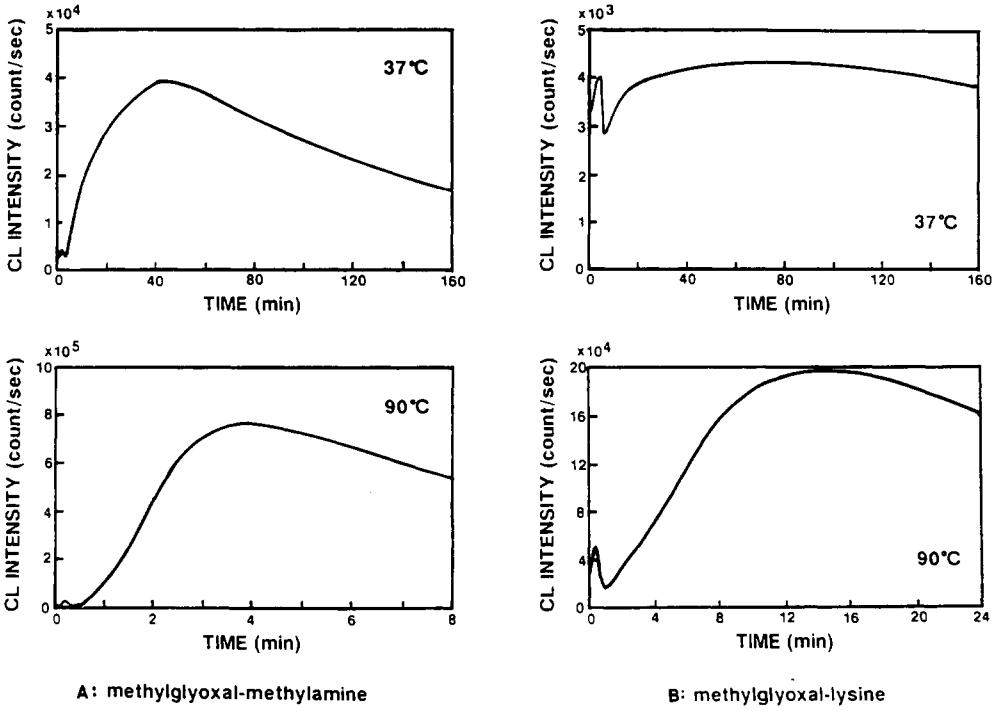

Figure 7. Chemiluminescence of methylglyoxal–amino compound systems (0.1 M each, at pH 8.0 in air): (A) methylglyoxal–methylamine; (B) methylglyoxal–lysine.

ishi *et al.*, 1990) was assumed to be caused by the reaction of such an active oxygen species. However, no clear evidence of formation of singlet oxygen, the most probable origin of CL, in such reaction systems has yet been demonstrated.

The fact that the addition of ascorbic acid caused a decrease in CL suggests no generation of singlet oxygen contributing to CL from ascorbic acid. It was shown in our experiment that when CL was measured on a solution of ascorbic acid (50 mM in phosphate buffer, pH 8.0, heated in air) with or without the presence of Fe and Cu ion (50 μm), no detectable CL was observed. These facts suggest that no appreciable amount of singlet oxygen giving clear CL is formed by the reaction of a reductone compound with oxygen even in the presence of a metal ion. We also conducted a preliminary experiment to measure CL development during heating of the solutions of Amadori product alone and with amino acid using *N*-(1-deoxy-D-fructosyl)-L-leucine [prepared by the method of Hodge and Fisher (1963)] and 1-deoxy-1-β-alanino-D-fructose [prepared by the method of Anet (1957)]. In this experiment, the CL was weak with the Amadori compound alone as compared with the reaction with amino acid. Thus, we considered that there is no notable contribution of Amadori product to the CL in the Maillard reaction to support the mechanism proposed by Kurosawa *et al.* (1989).

Effect of Temperature on the Development of CL. It was generally observed that the development of CL was prominent at higher temperatures, and in most cases the CL was measured under heating at above 60 °C and undetectable in the reaction at room temperature or at 37 °C (Kurosaki *et al.*, 1989). However, in the case of methylglyoxal–amino compound systems such as methylamine and lysine, the reaction proceeded violently even at room temperature, and CL was detectable immediately after the reaction was started and clearly increased with marked coloration of yellow to red and deep blood red, as shown in Figure 7. In this system, the development of CL was also observed for proteins such as serum albumin at 37 and 90 °C, as shown in Figure 8.

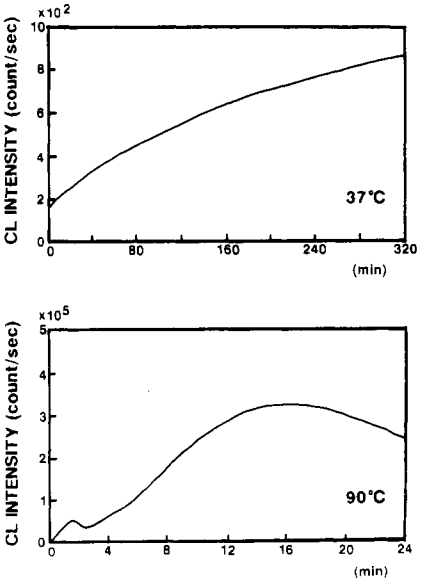

Figure 8. Chemiluminescence of methylglyoxal–serum albumin system (methylglyoxal, 0.1 M; serum albumin, 2.5 %; at pH 8.0, in air).

Chemiluminescence, Coloration, and ESR Spectra of Various Amino–Carbonyl Reaction Systems. To examine the effect of the chemical structure of the reactant on the development of CL, the coloration (mostly browning) and free-radical formation (ESR signal) of various amino–carbonyl reaction systems conducted mainly in alkaline solution are summarized in Table I.

Concerning amino compounds, development of CL was observed with primary amino compounds but was undetectable with secondary and tertiary amino compounds; this effect is the same as observed for the formation of free radicals (Hayashi *et al.*, 1977). Among the alkylamines, CL intensity was normal > secondary > tertiary derivative, as shown in Figure 9.

The development of CL was observed in most amino acids by the reaction with methylglyoxal at room temperature. Among the amino acids, arginine showed a somewhat strong CL, but it was undetectable in cysteine,

Table I. Chemiluminescence (CL), Coloration, and Free-Radical Formation (ESR) of Various Maillard Reaction Systems

carbonyl compd	amino compd	pH	temp, °C	CL	color	ESR	ref
			Monocarbonyl Compounds				
glycoaldehyde	methylamine	8.0	RT	+	−		a
	methylamine	9.7	50	+	+		b
	methylamine	8.0	90	+++	+++		a
	α-Ala	8.0	80		++	++	c
	β-Ala	9.0	60	++	++		a
	β-Ala	8.0	80		+++	+++	c
glyceraldehyde	methylamine	9.4	RT	+	−		b
	methylamine	8.0	90	++	++		a
	β-Ala	8.0	60		++	++	c
	Lys	9.0	80	+++	+++		b
xylose	methylamine	8.0	80	++	++		b
	diethylamine	11.0	60	−	+		b
	triethylamine	11.0	60	−	+		b
	propylamine	11.0	60	+++	+++		b
	β-Ala	8.0	80	++	++		c
	β-Ala	8.0	90	+++	+++		a
glucose	methylamine	11.0	60	−	−		b
	α-Ala	8.0	90	+	+		a
	β-Ala	8.0	90	++	++	+	a, c
fructose	methylamine	11.0	60	+	+		b
	β-Ala	8.0	80		++	+	c
			α,β-Dicarbonyl Compounds				
glyoxal	methylamine	8.0	RT	+	−		a
	methylamine	8.0	90	+++	++		a
	β-Ala	8.0	80		++	+	c
	Cys	8.0	RT	+	−		a
methylglyoxal	methylamine	8.0	RT	+++	++		a
	methylamine	8.0	RT		+++		a
	methylamine	8.0	90	++++	+++		a
	Gly	9.0	RT	+++	+++		b
	α-Ala	10.0	RT	+	++		b
	β-Ala	8.0	80		++	−	c
	β-Ala	9.0	RT	++	+++		b
	Cys	11.0	RT	−	−		b
	Cys	8.0	90	−	−		a
	Lys	9.0	RT	+++	+++		b
	Arg	9.8	RT	++	+		b
	His	11.0	RT	+	++		b
	Tyr	10.0	RT	+	+		b
	Trp	10.0	RT	+	+		b

[a] Tohoku Electronic Industry Model CLD-100. [b] JASCO Model 825 CL detector. [c] Namiki and Hayashi (1975). [d] M. Namiki, unpublished data (1992).

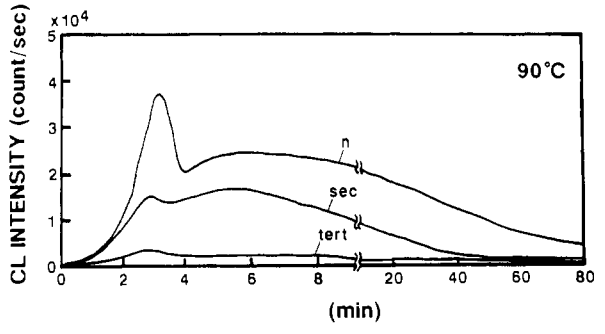

Figure 9. Chemiluminescence of glyoxal–*n*-, *sec*-, *tert*-butylamine systems (0.1 M each, at pH 8.0, at 90 °C).

probably due to the inhibitory effect of the SH group on the development of CL. Among the carbonyl compounds examined, methylglyoxal was especially reactive in the development of CL, but it has been classified as a compound inactive in giving a free-radical product at an early stage of the Maillard reaction (Hayashi *et al.*, 1977). This time we were able to detect a weak ESR signal only very early in the reaction of the methylglyoxal–methylamine system (unpublished data). This short-lived free-radical formation seems to be correlated with high activity in the development of CL. Other carbonyl compounds were effective in the formation of CL at heating temperature, and the activity followed the order glycolaldehyde = glyoxal > glyceraldehyde > xylose > fructose = glucose, which is almost the same as that in the case of free-radical formation (Namiki and Hayashi, 1983).

In summary, it was demonstrated that the development of CL was observed from an early stage of the Maillard reaction and the effects of chemical properties of the reactants, pH, and temperature on the development of CL resembled well those observed in the formation of free radicals as well as in browning reactions. This and the notable fact that the increasing curve of the integrated CL closely resembled that of browning in the sugar–amino acid reaction suggest that CL development is an important phenomenon directly related not only to the free-radical formation but also to the formation of browning products. An important feature of CL development is the significant role of oxygen, suggesting a possible mechanism for the development of CL whereby the reaction of oxygen with the free-radical product formed at an early stage of the Maillard reaction produces some excited-state molecule(s) such as singlet oxygen and/or a peroxy compound which causes the CL. Development of CL from singlet oxygen has been recognized in the direct processes in itself or in the indirect processes via the formation of dioxetane derivative or hydroperoxide derivative by the reaction with some double-bond compound. The formation of singlet oxygen during the Maillard reaction as a source of CL is considered to be the most probable source of the CL, and Kurosaki *et al.* (1989) proposed its formation on the basis of the visible spectral data of CL of Maillard reaction reported in a review note (Mizugaki and Sato, 1989, no experimental details). We also measured similar visible spectra with various Maillard reaction mixtures, but it should be noted that the assumption that the visible spectrum is due to a singlet oxygen (Khan and Kasha, 1970) was recently considered to be inadequate as evidence for the presence of singlet oxygen in the reaction (Kanofsky, 1989); further investigation to elucidate whether a singlet oxygen is produced or not is under way. While we recognized the presence of a singlet oxygen in the Maillard reaction, what kinds of products in the Maillard reaction act as reactant to produce the singlet oxygen remains to be elucidated. Kurosaki *et al.* (1989) proposed the formation of an active carbon radical to produce the singlet oxygen in the Amadori product or melanoidin. However, as mentioned above, CL was undetectable with the heating of the solutions of ascorbic acid, a representative enediol, as well as the Amadori product. Moreover, in our previous studies on the formation of a free radical at an early stage of the Maillard reaction (Hayashi and Namiki, 1981), no significant ESR signal was detected during heating of the solution of Amadori compound (1-β-alanino-1-deoxyfructose) alone or with amino acid (β-alanine), while marked development of ESR signal as well as browning was noted with the solution of glucosyl-β-alanine or glucose plus β-alanine. Usually the Amadori compound is considered to be present in an aqueous solution as a pyranose ring structure and shown to be much slower in browning than small molecular weight dicarbonyl compounds such as glycoaldehyde, glyoxal, and methylglyoxal, fragmental products of glucosylamino compounds (Hayashi and Namiki, 1985). This tendency in reactivity is the same in CL development as shown above. Thus, the possibility of Amadori product or melanoidin as a reactant to generate the CL via production of singlet oxygen is very unlikely, and the CL in the Maillard reaction is considered to be due mainly to the reaction of oxygen with free-radical product(s) of low molecular weight dicarbonyl products of sugar fragmentation or pyrazinium compound; these may also directly contribute to the browning. The importance of their role is substantiated by the fact that

methylglyoxal and glyoxal were very effective in CL development (Figure 7) as well as browning.

The fact that a weak but clear small peak of CL was observed as soon as the reaction was started in the cases of the methylglyoxal–lysine (Figure 7) and glyoxal–methylamine systems (Figure 1) suggests the existence of another mechanism for the development of CL at an initial stage of the reaction, e.g., formation of some excited-state molecule related to the Schiff base product initially formed by amino–carbonyl reaction, though such mechanisms are speculative at present.

Moreover, the fact that the reaction of methylglyoxal with amino acid as lysine and protein as serum albumin produced CL even at 37 °C is very interesting in light of the potential effect of methylglyoxal *in vivo* correlated to carcinogensis that Szent-György *et al.* have especially emphasized, because this may provide evidence that methylglyoxal produces some highly reactive molecule(s) such as active oxygen by the mechanism of the development of CL. Coloration in the reaction of methylglyoxal with protein and studies on the chemical identification of the products in the reaction with amino acid are under way.

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