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Aldehyde-enhanced photon emission from crude extracts of soybean seedlings

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The photon emission from a soybean seedling was remarkably enhanced with the addition of acetaldehyde. The emission spectrum in a seedling had peaks at around 670 and 610–615 nm, with a shoulder at 530–540 nm. In the crude extracts of seedlings, enhancement of photon emission depended on the aldehyde chain length; acetaldehyde gave a maximal photon emission intensity. The photon emission intensity in crude extracts reduced by hydrosulfite showed an initial rapid increase followed by a quick decay in the first phase and a slow decay in the second phase, in the presence of oxygen and aldehyde. The emission spectrum in a whole soybean seedling was observed similarly in crude extracts, with the addition of acetaldehyde. Since a similar photon emission pattern appears in the supernatants of autoclaved extracts, concomitant with the same emission spectra, it is concluded that this photon emission occurs nonenzymatically. Furthermore, when the sample has decayed once photon emission is reduced, the photon emission appears again, upon mixing the sample with oxygen and aldehyde. This result suggests that a hydroperoxide intermediate is an emitter reacting with aldehyde. An energy transfer from triplet carbonyl may not be involved in this reaction.

Introduction

It is known that photon emission from intact soybean seedlings is relatively higher than those from other living organisms. Especially, wounded seeds or seedlings [1–4] show an enhanced photon emission with the addition of indole acetic acid [3] or hydrogen peroxide [2]. Salin and Bridges [3] have suggested that peroxidase is involved in such an enhanced chemiluminescence in injured soybean. Cilento [5] has proposed that peroxidase or α-oxidase generates triplet carbonyl via oxidation of aldehyde or carboxylic acid [6] through dioxetane formation by activated oxygen. Triplet carbonyl transfers the excited energy to a fluorescent compound, such as chlorophyll [7–9]. Activated oxygen may be produced in vivo by peroxidase (oxidase) in wounds. Boveris et al. [10] observed photon emission from a mass of soybean seeds upon imbibition. Approx. 50–70% of the photon emission was contributed by emissions in wavelengths greater than 600 nm.

We found that acetaldehyde remarkably enhanced photon emission from intact soybean seedlings, and that it also enhanced chemiluminescence even in supernatants of autoclaved crude extracts of seedlings. Boh et al. [11] and Steel et al. [12] have also found that acetaldehyde stimulates the photon emission from mitochondria. They have proposed either that a reaction of cyanohydrin with acetaldehyde induces chemiluminescence, or that the emitter has been produced from reactions of superoxide or hydrogen peroxide. In plants, however, there has so far been no report about aldehyde-enhanced photon emissions.

In this report, the reaction mechanism of aldehyde-enhanced photon emission from crude extracts of soybean seedlings was examined with respect to the reactivity of the emitter molecule with oxygen and with respect to the photon emission spectra. It seems likely that triplet carbonyl may not contribute to this reaction. According to the change in spectral profile observed during measurement, it is suggested that at least two emitters are involved.

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Materials and Methods

Sample

Soybean (*Glycine max* var. Tanrei) seeds were imbibed in tapwater for 2 h prior to cultivation on quartz sand immersed with water. One of the soybean seedlings, grown in complete darkness for 4 days at 25 °C, was used for the photon emission imaging in the presence or absence of acetaldehyde. Emission spectra were also measured from a whole soybean seedling in the presence of aldehyde.

For the preparation of crude extracts of the seedlings, parts of the cotyledon and parts of the root and hypocotyl (further referred to as root) were segmented. Cotyledons and roots were homogenized separately with a blender at room temperature in 50 mM potassium phosphate buffer (pH 7.0). A ratio of 2.5 ml of buffer to 1 g wet weight was used for roots, whereas 10 ml of buffer to 6 g wet weight was used for cotyledons.

Crude extracts used in the measurements of photon emission were obtained after centrifugation at 12000 × *g* for 30 min. Supernatants obtained from cotyledons and roots were further autoclaved for 5 min at 1.2 atm and 120 °C. Following this, heat-denatured materials in crude extracts were removed by centrifugation at 12000 × *g* for 30 min. Supernatants from autoclaved crude extracts were also used in the photon emission measurements. All autoclaved supernatants and crude extracts were kept on ice before measurements.

Photon counting measurements

For measurements of photon emission imaging, an image intensifier-videocamera system (Argus 100/VIM; Hamamatsu Photonics) [14] was used. Photon emission images were obtained for a 20 min exposure and then photographed. For spectral analyses of weak photon emission, a filter-equipped photon-counting type spectrometer was developed in our laboratory [13]. The rotatory filter-disc is faced on photomultiplier (R 1333 type; Hamamatsu Photonics). In these measurements, the gate time was 1 s for each filter, and a filter disc was circulated for 1 min. Total photon counts per second (without filter) in aerobic and anaerobic conditions were also recorded during each rotation of the disc. Aerobic and anaerobic conditions were produced by introducing oxygen and argon gases to samples in a light-tight chamber, respectively. Using a microcomputer, the spectral distribution of the emission was calculated and corrected to intensity changes. The measurements using a filter-equipped spectrometer were carried out at 37 °C. For analyses of reaction mechanisms, the time-course of photon emission was measured by a Lumiphotometer TD 4000 (Labo Science) [15] at 25 °C.

Reagents

Acetaldehyde was purchased from Merck, NJ, U.S.A. Propanal, butanal, and pentanal were obtained from Tokyo Kasei Kogyo, Tokyo, Japan. The other aldehydes were obtained from Nacalai Tesque, Kyoto, Japan. Sodium azide, mannitol, and xanthine were from Wako Pure Chemicals, Osaka, Japan. Catalase, superoxide dismutase and β-carotene were obtained from Sigma, MO, U.S.A. β-Carotene was dissolved in benzene and acetone, and then diluted by 50 mM phosphate buffer (pH 7.0). Reagents were dissolved in the same buffer. Aldehydes, ethanol and acetic acid were added into the reaction mixture without dilution. In some experiments, however, an aldehyde solution diluted 10-times in the 50 mM phosphate buffer was used. For reduction of the

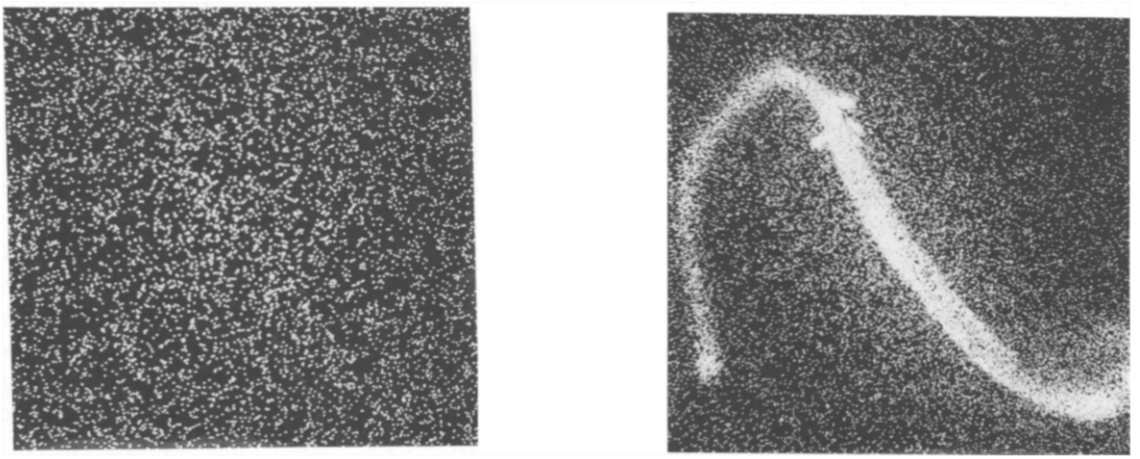

Fig. 1. Photon emission profile of a whole soybean seedling. (a) One intact seedling was laid on a black plate and the photon emission from the whole seedling was accumulated for 20 min, using an image intensifier-videocamera system. (b) To the same seedling, a small amount of acetaldehyde solution was added and photon emission from a whole seedling with acetaldehyde was also taken for 20 min.

sample, a small amount of hydrosulfite powder was added. All reagents used were of analytical grade.

Results

Photon emission from a etiolated soybean seedling grown in the dark for 4 days was observed by using an image-intensifier and a videocamera system [14]. Fig. 1a shows the image of photon emission from an intact whole seedling accumulated for 20 min. Obscure photon emission from hypocotyl was observed. When the seedling was immersed in acetaldehyde solution, an enhanced photon emission from a whole seedling occurred within the same acquisition time as shown in Fig. 1b. This photon emission was intense, so that a clear image of the whole seedling was formed by its own photon emission.

The emission spectrum and its intensity depending on the time from a whole seedling with its root immersed in 6.8 M (30 %) acetaldehyde in 50 mM phosphate buffer are presented in Fig. 2. This spectrum indicates peaks at around 670 nm and 610–615 nm with a shoulder at 530–540 nm. Intensities of emission spectra also increased depending on the measurement time. In the absence of acetaldehyde, photon emission was too weak to obtain the spectrum. The spectrum obtained with the addition of acetaldehyde showed the peaks at longer wavelengths, similar to that obtained in wounded seeds [10].

In order to investigate the emitter(s) from seedlings which evolved photons in the presence of acetaldehyde, the enhancement effect of aldehydes has been studied with crude extracts of soybean cotyledon and root. The stimulation of photon emission by acetaldehyde was compared to the other chemicals. As shown in Table I, acetaldehyde and formaldehyde specifically stimulated photon emission, with respect to peak intensities and total photon emissions in 1 min. However, a long-chain aliphatic aldehyde, decanal, showed no effect. Endogenous xanthine oxidase in crude extracts apparently did not work on acetaldehyde to produce superoxide, or triplet carbonyl, because the addition of xanthine did not enhance photon emission. The stimulation by aldehydes in crude extracts of cotyledons, was also apparent in roots (data not shown). Ethyl alcohol enhanced photon emission in an intact seedling, but there was no effect in crude extracts. The effect of the aldehyde chain length on the enhancement of photon emissions was examined in crude extracts of the cotyledon and the root, respectively. In roots, acetaldehyde showed the most effective stimulation on the peak intensity and total photon emission in 1 min (Fig. 3). For comparison, benzaldehyde was tested; the stimulative effect was also observed (Fig. 3). In crude extracts of cotyledons, a similar response to the aldehyde chain length appeared. It was of interest that the supernatants from autoclaved crude extracts of roots or cotyledons showed almost the same stimulation effect along with the aldehyde chain length upon photon emission (data not shown).

TABLE I

Enhancement effects of some chemicals on the photon emission from crude extracts of soybean seedlings

1 ml of crude extracts of cotyledons was added into 200 μl of chemicals. The same volume of phosphate buffer was added instead of chemicals as control (None). In a case of decanal, 50 μl was added. Measurements were carried out for 1 min; peak intensity and total photon emission were recorded and expressed as the ratio to the control.

	Peak intensity	Total photon
None	1.0	1.0
Acetaldehyde	37.8	355.7
Ethyl alcohol	1.3	5.5
Acetic acid	0.8	0.6
Formaldehyde	7.2	70.7
Decanal	1.2	0.6
Xanthine (10 mM)	0.8	0.6

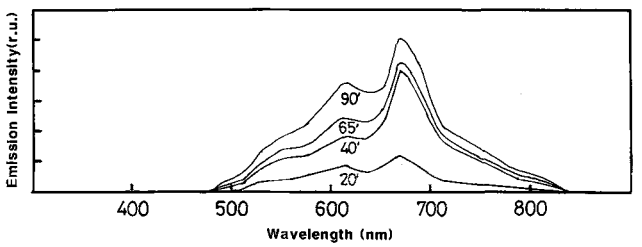

Fig. 2. Spectra of photon emission from a whole soybean seedling in the presence of acetaldehyde. The sample was set in a light tight chamber of a filter-equipped spectrophotometer [13]. The time dependency of spectral intensity change was measured. Each spectrum is presented as the summation of five scans. The elapsed time (20 to 90 min) from the start of measurements is indicated. The ordinate shows the relative emission intensity of the summation of the five scans for the spectrum. The abscissa shows the wavelength (nm).

The emission spectra from crude extracts of roots and supernatants of autoclaved cotyledons were examined, in the presence of 225 mM acetaldehyde (data not shown). Peaks in both spectra appeared at around 670 nm and 610–615 nm, with a shoulder at 530–540 nm. It highly resembles the spectrum of a whole seedling immersed in 6.8 M acetaldehyde (Fig. 2). In crude extracts of roots, the highest emission spectrum was attained at 9 min, while in supernatants of autoclaved cotyledons, the intensity of emission spectrum was gradually increased. A disproportional increase in both peak intensities between 610–615 nm and 670 nm suggests that, in part, the emitter at 670 nm is formed slowly in the presence of acetaldehyde, comparing it at 610–615 nm.

In order to examine the role of active oxygens on the reaction mechanisms of photon emissions, scavengers

were tested with oxidized (non-reduced) supernatants from autoclaved roots and cotyledons, in the presence of acetaldehyde (Table II). Obvious inhibition for photon emission from supernatants was observed with the addition of 50 mM sodium azide to acetaldehyde solution before injection of supernatants ('azide before' in Table II). However, in the case which the supernatants were mixed first with acetaldehyde, then azide immediately added ('azide after' in Table II), the inhibition of azide disappeared in both supernatants of autoclaved roots and cotyledons. This suggests that azide and acetaldehyde are competitive for binding to the emitter in supernatants. Therefore, singlet oxygen may not be involved directly in the photon emissive reactions.

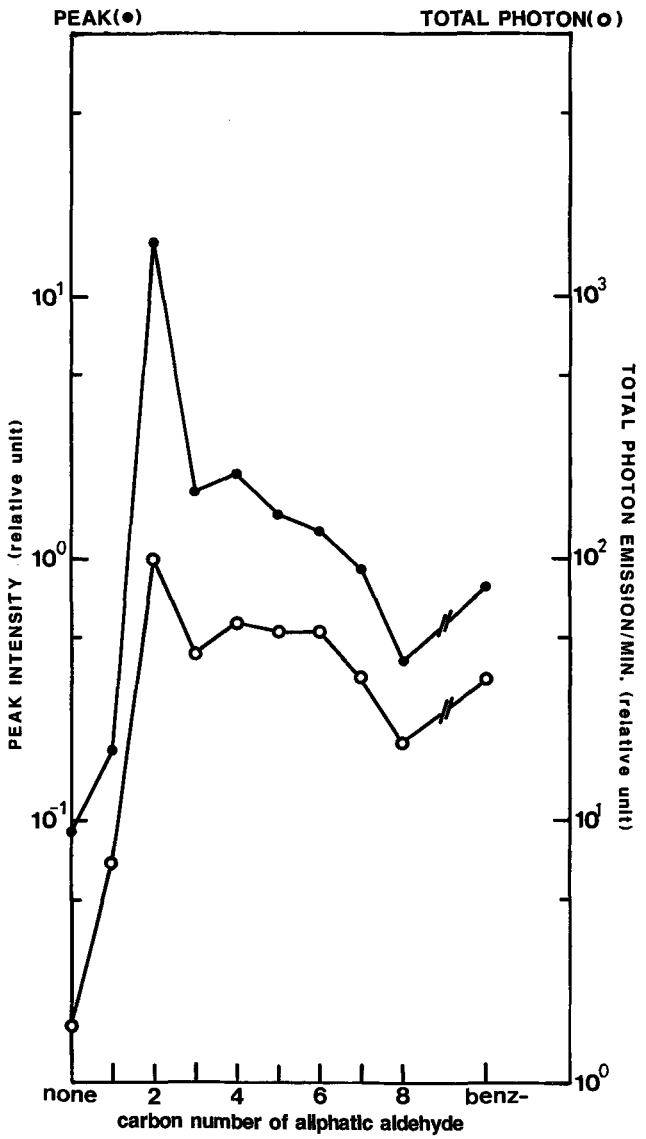

Fig. 3. Dependence of photon emission efficiency of crude extract on the chain length of aliphatic aldehydes. Measurements were started by injection of 1 ml of supernatants of autoclaved roots into the vial containing 100 μl of aldehyde. Peak intensity (peak; •) and total photon emission (total photon;o) in 1 min were indicated in relative units, at left ordinate and right, respectively. Abscissa shows carbon numbers of aldehydes. For control, 100 μl of 50 mM phosphate buffer (pH 7.0) (none) were added instead of aldehyde. Benzaldehyde (benz-) was tested for comparison.

TABLE II

The effect of scavengers for active oxygen species and radicals on supernatants of autoclaved crude extracts of seedlings

1 ml of supernatants was added into the mixtures of 100 μl of 22.7 M acetaldehyde and 100 μl of scavengers. The concentration of scavengers is a final concentration in reaction mixtures. Azide was mixed with acetaldehyde before addition of the sample (before), or it was added later after mixing the sample with acetaldehyde (after). The peak intensity of photon emission in 1 min was expressed as percentage (%).

	Cotyledon	Root
Control	100	100
Azide (50 mM) (before)	59.3	66.0
Azide (50 mM) (after)	117.5	90.5
Mannitol (0.1 M)	173.1	95.7
Catalase (1250 U/ml)	106.8	87.9
SOD (500 U/ml)	85.6	88.1
β-Carotene (2 μg/ml)	97.7	108.7

Another scavenger, superoxide dismutase, showed a small inhibitory effect on both autoclaved samples (Table II), but such inhibiton was less obvious. Mannitol, a hydroxyl radical scavenger, showed some stimulative effect on photon emission from supernatants of autoclaved cotyledon, but not in those of roots. In crude extracts of roots and cotyledons, a clear inhibition for photon emission was also observed with azide added to aldehyde solution before the injection of the sample (data not shown). In the oxidized samples, therefore, active oxygen species and any radical may not be involved in the formation of emitter molecules with aldehyde.

The oxygen dependency of the emission was further confirmed with supernatants of autoclaved roots. As shown in Fig. 4a, at the introduction of oxygen gas (No. 2 in Fig. 4a) photon emission intensity increased in the absence of aldehyde, then continued to decrease gradually despite continuous oxygen bubbling. When argon gas was introduced into the same sample during the decayed photon emission (No. 3 in Fig. 4a), the decay rate was scarcely influenced.

When acetaldehyde was mixed with supernatants prior to measurements of photon emissions, oxygen gas bubbling (No. 2 in Fig. 4b) again increased photon emission, and decayed during the bubbling of oxygen (Fig. 4b). At the introduction of argon gas (No. 3 in Fig. 4b), the decay rate slightly increased once as shown in Fig. 4b. Although an initial increase in photon emission with the addition of acetaldehyde was not recorded in Fig. 4b, these results showed that the decay rate was not changed significantly, even with the introduction of argon gas. This is probably due to a stable oxygenated

emitter(s). When a sample in the experiment of Fig. 4b was reduced by hydrosulfite after decayed photon emission (No. 4 in Fig. 4c), there was an increase in photon emission again, following a decay during oxygen bubbling (Fig. 4c), in which the replacement of oxygen to argon gas did not influence the decay rate.

In the presence of hydrosulfite in excess in the sample, photon emission was quenched promptly (data not shown). This suggests that the reduced intermediate of the emitter could bind to oxygen immediately, and thus the oxygenated intermediate could be decomposed by an excess reductant.

The changes of emission profile corresponding to the time-course of photon emissions indicated in Fig. 4a and b, were represented in Fig. 4d and e. The clear peak at 610–615 nm appeared concomitant with an increase in temperature to 37 °C (Fig. 4d at 9 min). During an introduction of oxygen gas, such an emission profile was not altered (Fig. 4d at 18 min). In the presence of aldehyde as shown in Fig. 4e, however, the spectral profile did not change throughout the time-course of photon emissions presented in Fig. 4b. Fig. 4e shows a reduction of emission spectra in intensity at 57 min, based on the decay of photon emission as shown in Fig. 4b. The introduction of pure oxygen gas showed the same emission spectra as those in the presence of acetaldehyde, but the intensity of photon emission in the absence of aldehyde was smaller than in the presence of aldehyde.

The reactivities of a reduced intermediate in photon emissions were also examined with supernatants of autoclaved roots reduced by hydrosulfite. As shown in Table III, oxidized (non-reduced) supernatants showed a broad emission during measurement (1 min) with a peak intensity at 45 s (line 1); the sample reduced by hydrosulfite showed a higher peak intensity after 4 s, and the total photon emission in 1 min was slightly increased (line 2,3). This rapid reactivity with acetaldehyde may reflect the prompt reaction of the reduced intermediate of emitter molecules with oxygen in the presence of aldehyde. On the other hand, when the oxidized sample was mixed with reduced aldehyde solution, the intensities of photon emission in peak and total was higher than the control (increased to 2.6 times) (line 5). This may be due to the continuous reduction of the intermediate and the supplement of

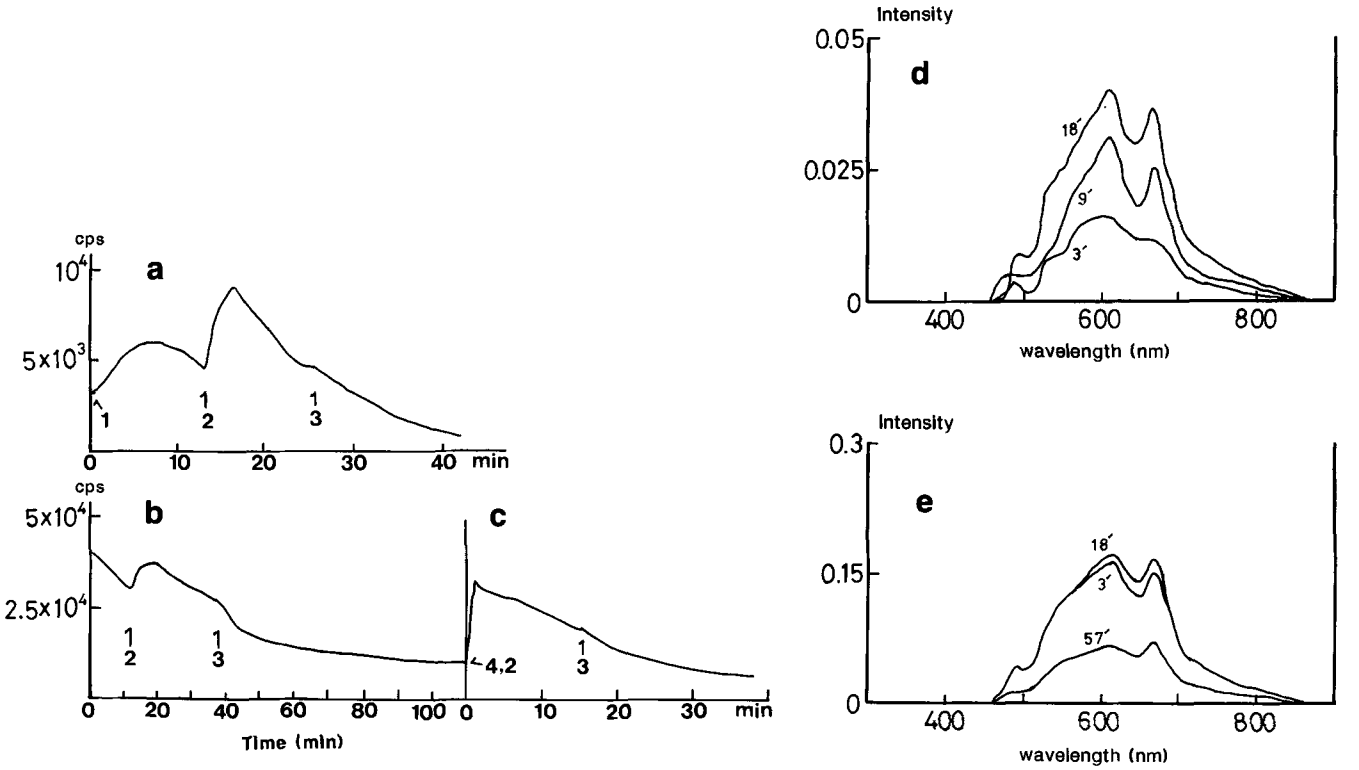

Fig. 4. Time course of photon emission and spectral change from the supernatants of autoclaved roots. (a), (b) and (d) show the time course of photon emission with the introduction of oxygen or argon gas, measured by a filter-equipped spectrometer. Photon emissions (cps) from 10 ml of supernatants were measured in 1 s without a filter at 1 min intervals. Numbers in (a)–(c) indicate the treatment to samples: (1) warming of the sample from room temperature to 37 °C controlled in a spectrophotometer; (2) the introduction of oxygen gas; (3) the introduction of argon gas, to replace oxygen; (4) reduction of the sample with hydrosulfite. The ordinates in (a), (b) and (c) show photon emission intensity. Abscissa in (a), (b) and (c) shows the time (min). In (b) and (c), 225 mM acetaldehyde was present in the samples. Figures (d) and (e) show the spectral changes in the same samples in (a) and (b), concomitant with the introduction of gases. (d) shows the change of spectra treated in (a); (e) shows the change of spectra treated in (b). The ordinates in (d) and (e) show the relative intensity of spectra; the abscissa in (d) and (e) show the wavelength (nm). Each spectrum is the summation of three scans and the respective time at the measurement of the spectrum is indicated in (d) and (e).

oxygen from the surface of the solution; a turnover of such reactions may occur. When hydrosulfite was added immediately after mixing the sample with aldehyde, photon emission was almost the same as in oxidized samples (line 4); this was due to the decomposition of the oxygenated intermediate and incomplete depletion of oxygen. When the sample was reduced with dithiothreitol (DTT), the emission intensity was similar to the oxidized sample (line 6). This suggested that the supernatants could be reduced only by a strong reductant, and that the reduced form of the intermediate rapidly reacted with oxygen, similar to reduced flavin mononucleotide ($FMNH_2$) in the presence of aldehyde [16]. Since this reactivity with oxygen in reduced form occurs even in the autoclaved supernatant (Fig. 4c), the formation of emitter from reduced intermediate and oxygen may not involve an oxidase catalyzed reaction.

The time-course of photon emission from reduced samples is represented in Fig. 5. Reduced samples from supernatants of autoclaved root showed a rapid increase and a quick decay of photon emission, followed by a slow decay in a second phase, while oxidized (non-reduced) samples showed a slow increase followed by a similar slow decay to the reduced samples. Azide inhibited photon emission in oxidized samples (as shown previously in Table II). In reduced samples, however, azide showed no inhibition – the same as mannitol – as indicated in Fig. 5. Since azide may compete with aldehyde for binding to the emitter, it exhibits the presence of two intermediates of emitters; one is a dihydro intermediate which reacts rapidly with oxygen in the presence of aldehyde and decays, and the other is a hydroperoxy intermediate which reacts with aldehyde

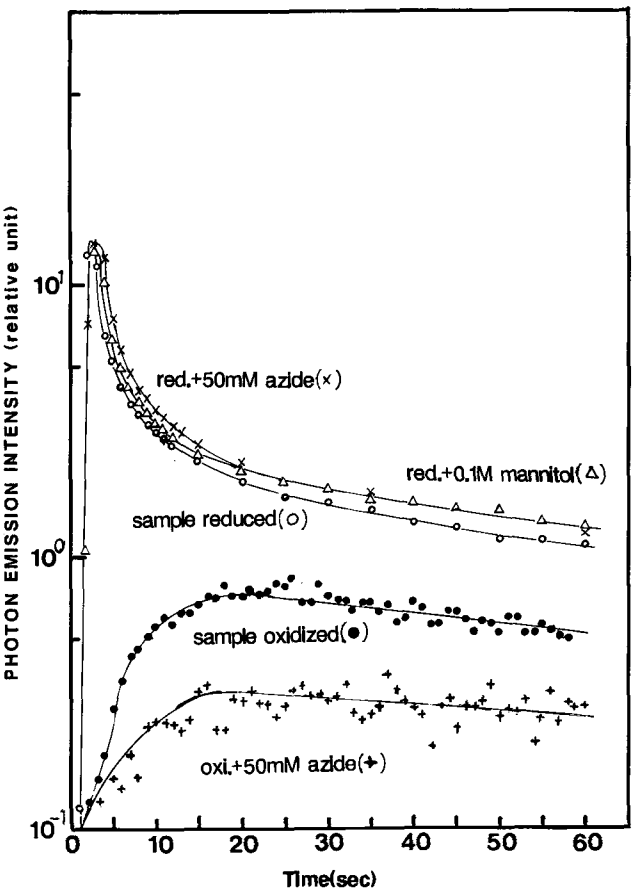

Fig. 5. The time course of photon emission from supernatants of autoclaved roots in the presence of acetaldehyde. The reaction was initiated by the injection of 1 ml of sample, into a vial containing one ml of 2.27 M acetaldehyde and 100 μl of scavengers. In reduced (○) and oxidized (non-reduced) (●) sample, 100 μl of phosphate buffer was added, instead of scavenger. Scavengers, 50 mM azide and 0.1 M mannitol were the final concentration in the reaction mixture. The reactions were as follows: reduced sample (○), reduced sample with 50 mM azide (x), reduced sample with 0.1 M mannitol (△), oxidized (non-reduced) sample (●) and oxidized sample with 50 mM azide (+). The ordinate shows relative intensity of photon emission. The abscissa shows the time (s).

TABLE III

Photon emission of reduced supernatants from autoclaved crude extracts of roots

1 ml of supernatants was injected with a syringe into a vial containing 1 ml of 2.27 M acetaldehyde. Reduction of supernatants or aldehyde solutions was done by the addition of a small amount of hydrosulfite. In line 6, dithiothreitol (DTT) was added into the sample instead of hydrosulfite. Measurements were carried out for 1 min. Peak intensity of photon emission and total photon intensities emitted in 1 min were recorded and expressed as the ratio to control (line 1); time attained to peak intensity was indicated in the table as peak time.

	Peak intensity	Peak time	Total photon
1 Sample oxidized	1.00	45	1.00
2 Sample reduced	6.57	4	1.27
3 Sample reduced + aldehyde red.	8.00	4	1.43
4 Sample + aldehyde reduced	0.90	40	1.04
5 Sample oxidized + aldehyde red.	2.66	53	2.64
6 Sample with DTT	0.91	54	0.70

slowly and also decays slowly, as the same as shown in oxidized form. An analogous situation was reported on the basis of light emission from the flavin molecule [16]. Only to this hydroperoxy intermediate may azide inhibit aldehyde binding.

Because the initial increase was so rapid in photon emissions, we could not observe the spectrum using a filter-equipped spectrometer. The biphasic decay of photon emission in the reduced samples, as indicated in Fig. 5, suggests the presence of two reduced intermediates, since intense reduction by hydrosulfite may decompose the hydroperoxy intermediate completely.

The decay rate of the emitter in reduced samples was affected by the aldehyde chain length as shown in Fig. 6. The decay rate at the initial phase was the largest with acetaldehyde among the aldehydes tested, concom-

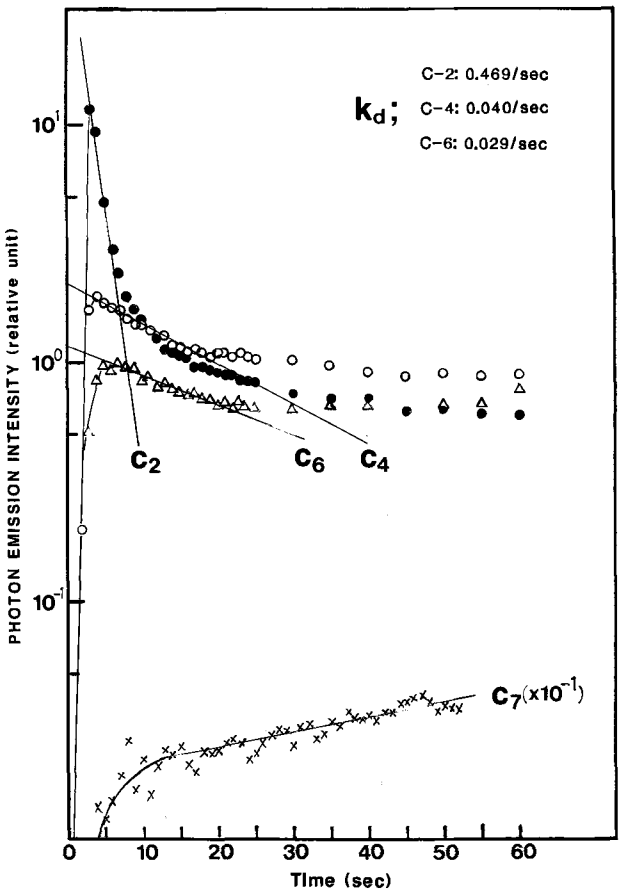

Fig. 6. Decay rate of photon emission from supernatants of autoclaved roots. The reaction was initiated by the injection of 1 ml of sample with a syringe into a vial containing 1 ml of 2.27 M aldehyde. Aldehydes added are: acetaldehyde (C_2), butanal (C_4), hexanal (C_6) and heptanal (C_7). Photon emission intensity with heptanal was expressed as one tenth of the observed. The ordinate shows the relative intensity of photon emission. The abscissa shows the time (s).

itant with its peak intensity of photon emissions. With heptanal, the initial decay was not observed but only a slow increase of photon emissions. Apparently heptanal may not react rapidly with the reduced sample in the presence of oxygen. As shown also in Fig. 3, the difference in reactivity as a function of the aldehyde chain length supports the assumption of the presence of a hydroperoxide intermediate which reacts with aldehyde through hemiacetal binding [17]. The intermediate (hydroperoxide) in crude extract might be relatively stable at room temperature, differing from flavin hydroperoxide [17].

Discussion

The emission spectra from soybean seedlings showed the peak at longer wavelengths, 670 and 610–615 nm, and produced a high emission intensity with aldehyde. The emission from imbibed soybean seeds at longer wavelengths greater than 600 nm has been reported previously by Boveris et al. [10]. They suggested that singlet oxygen is one of the emitters, but such emission had complexity. In this report, such a red emission from the soybean seedling was analyzed in the presence of aldehyde.

Acetaldehyde specifically enhanced the chemiluminescence of seedlings by interacting with the emitter. Based on a change of the spectra during measurements of the photon emission in crude extracts of seedlings, it was suggested that at least two emitters were present in a fraction of the slow decay phase shown in Fig. 6. For example, the peak at 670 nm was increased more than the peak at 610–615 nm in the spectra of crude extracts of roots with aldehyde. Conversely, in supernatants of autoclaved roots, emission at 610–615 nm was increased greater than it was at 670 nm by oxygen gas bubbling. Emission spectra from both the supernatants of autoclaved cotyledons and the crude extracts of roots in the presence of aldehyde were the same as that of a whole seedling with aldehyde. Therefore, a spectral profile in a seedling with aldehyde was indeed reproduced in both crude extracts.

Nassi and Cilento [8] reported that triplet carbonyl produced from isobutylaldehyde by a peroxidase reaction could excite chloroplasts to emit red light; however, fluorescence from a chloroplast had peaks at 685–695 nm and 730–735 nm [18,19] in their report. This differs from the spectral profile obtained with crude extracts of seedlings with aldehyde.

Aldehydes specifically enhanced the photon emission in crude extracts, differing from that of a whole seedling in which ethyl alcohol also enhanced photon emission. Aldehyde chain length affected the intensity (see Fig. 3) and the decay rate (see Fig. 6) of photon emissions in crude extracts; benzaldehyde also showed a stimulative effect. These results suggest that the aldehyde molecule can react directly with the emitter, without the formation of an excited carbonyl. A similar observation was reported with the emission from hydroperoxy flavin mononucleotide [16,20].

Reduced crude extracts showed a rapid increase in the photon emission intensity followed by a fast decay upon mixing with aldehyde and oxygen. This suggested that triplet oxygen (ground state) could react with a reduced intermediate of the emitter in the presence of aldehyde. This was supported further by the fact that azide and mannitol had no inhibition on the reactivity of a reduced intermediate with oxygen (Fig. 5). The inhibition of azide on oxidized (non-reduced) samples disappeared upon prior mixing of the sample with aldehyde as shown in Table II. Such a difference in the inhibition by azide between oxidized and reduced samples was supposed to be a conformational change between two intermediates, namely dihydro and hydroperoxy intermediates.

Alternatively, in the oxidized sample the intensity of

photon emission increased and decayed slowly, like the second phase of decay in the reduced samples with acetaldehyde (Fig. 5). Kemal and Bruice reported that 4a-hydroperoxy FMN showed a slow increase and a slow decay of light emission with formaldehyde in acetate buffer (pH 4.5), while 4a,5-dihydro FMN in the presence of formaldehyde and oxygen showed a faster reactivity and a faster decay of light emission [16]. These reactivities were similar to the photon emission in crude extracts of seedlings, although two phases of decay in photon emission were observed in reduced samples. Aldehyde may react with hydroperoxide intermediates of the emitter in a mode similar to the bacterial luciferase reaction [17].

Reaction pathways in reduced and oxidized samples are postulated as follows.

$$FH_2 + O_2 \rightarrow FHOOH + RCHO \rightarrow {}^{+}FHOH^{-} + RCOOH \rightarrow F^{*} + H_2O \rightarrow F + h\nu \quad (1)$$

$$F'H\cdot + \cdot O_2^{-} + H^{+} \rightarrow {}^{+}F'HOOH^{-} \rightarrow F'^{*} + H_2O_2 \rightarrow F' + h\nu \quad (2)$$

In the oxidized sample, the reaction may be initiated at binding aldehyde (RCHO) to hydroperoxide (FHOOH), as shown in formula 1, whereas in the reduced sample, the reaction is started with the formation of hydroperoxide from a dihydro-intermediate and oxygen. In the absence of aldehyde, the postulated hydroperoxide intermediate might be decomposed directly to hydrogen peroxide and excited emitter (F*) as presented in formula 2, or through a hydroxide intermediate by the abstraction of one atom of oxygen to an unknown acceptor in a sample such as in formula 1. However, it should be considered that the sample reduced by hydrosulfite might produce superoxide upon mixing with oxygen. In a case of the presence of superoxide in the reaction mixture, a formation of hydroperoxide (F'HOOH) from superoxide is also considered as shown in formula 2. Whether this type of hydroperoxide can excite chromophore (F') at the liberation of hydrogen peroxide in the absence of aldehyde, is unknown.

The emitter was postulated as hydroperoxide intermediate having reacted with triplet oxygen or superoxide. Although the reproduction of emitter required a strong reduction by hydrosulfite, the regenerated reactivity of the reduced sample with oxygen suggests that a dioxetane formation in emitter is not involved, since emission from the dioxetane intermediate is accompanied by its cleavage to two keto compounds [22]. It is also unlikely that triplet carbonyl formation in which aldehyde is oxygenated by peroxidase to dioxetane transfers energy to fluorescence compounds as chlorophyll [7]. Since supernatants of autoclaved seedlings also showed the same reaction pattern, a contribution of enzymatic oxidation to photon emissions can be excluded. An exhaustive reduction of the sample should decompose the lipid peroxide formed by lipoxygenase [24] to produce singlet oxygen through a Russell mechanism [23].

Woodstock and Taylorson reported [25] that acetaldehyde and ethanol accumulated in imbibing embryonic axes and seeds of soybeans. This fact suggests that the acetaldehyde produced in a seedling may react with emitter(s) and enhance photon emission in vivo.

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