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Computerized Analyzing System for Chemiluminescence

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We made a pneumatically-driven injection device and a computer analyzing system for chemiluminescence studies. This total instrument provided superior reproducibilities; *e.g.* within-run variabilities ($n=4$ to 6) of entire light emission were about 1 to 4%. The entire emission is calculated from both an integration of the signal and an approximation to a first-order reaction. It observed that rapid, intense light ($t_{1/2}=0.35$) was produced by luminol reaction catalyzed by concentrated hemin (10^{-5} M). Applications of the equipment, the analyzing method and hemin as a catalyst are discussed to analytical and clinical chemistry.

Keywords——luminescence; chemiluminescence; automated injection; computer analysis; personal computer; luminol; hemin

Recently, many luminometers have appeared on markets[1] and various devices have been made in laboratories.[2] In the region of analytical and clinical chemistry, they supply a relation between the amounts of analytes and quantities of light emitted from luminescent materials. Chemiluminescence (CL) of luminol and bioluminescence of luciferase are well-known. Luminol and its derivatives come to be often used as labeling materials in immunoassay in place of radioactive atoms. Chemiluminescent reaction of luminol is written:[3]

$$\text{luminol} + H_2O_2 \longrightarrow \text{aminophthalate} + N_2 + H_2O + h\nu$$

This reaction proceeds in the presence of catalysts such as hemin and hemoproteins in aqueous alkaline solutions.

For the purpose of analysis, the reaction is usually initiated by injection of hydrogen peroxide solution into a reaction vessel containing luminol and a catalyst and then the light emission is detected by a photomultiplier, amplified and recorded. The observed total quantity or the peak intensity connects directly with the amount of the luminescent material or the catalyst used as a label.

Commercial and home-built equipments have various characteristics and availabilities in clinical laboratories, *e.g.* easy manipulation, good sensitivity and long-term stability. However, they lack versatilities and flexibilities, when applied to analytical and physical chemistry:

(A) Few instruments equip with automated injection systems, although their precision will greatly exceed that of manual operations.

(B) Mixing style made up of shapes of cuvettes, reagent injection rates and volumes, *etc.* that is suitable for an assay system is not necessarily suitable for any other one; poor mixing can often give variable and spurious results. Thus adequate setting of the mixing mode is a prerequisite. However, most of the units use only reaction vessels of limited sizes.

(C) Observation time is important in laboratories dealing with many samples, and rapid assays need fast light production by a luminescent material and a recording unit with sufficient time-response. Commercial units, however, have poor response.

(D) Recorded data can not be calculated or analyzed easily by methods that one desires.

Intense emission is of great advantage to cheap, facile measurement, since even insensitive detectors can trace it. Many investigators have reported intensities of luminol CL using several catalysts, *e.g.* ferricyanide,[2f,3] hemin derivatives, [2l,4] hemoglobin,[5] peroxidase[2a] and microperoxidase.[2k,4a,6] The experiments showed that CL intensities increased to some extent as the concentrations of the catalysts increased. The rate of emission, however, can be expected to become so high that a usually available analog recorder with poor time-response cannot trace the luminescence phenomenon precisely. Furthermore the mixing mode will affect the apparent kinetics of the CL reaction more strongly. Hence the measurement of prompt light production requires a quick response recorder and a rapid mixing apparatus with high reproducibilities.

To improve these faults, we made a versatile detection system possessed of a pneumatically-driven injection device, utilized a transient memory to obtain accurate data for fast reaction and fitted it with a personal computer to analyze CL phenomena flexibly.

Experimental

Materials and Methods——Luminol and hemin were purchased from Tokyo Kasei Co., Ltd. (Tokyo) and used without further purification. All other reagents were of analytical grade and not further purified. Luminol stock solution was prepared in a glass vial at *ca.* 1 mM in 0.1 M Na_2CO_3, pH 10.5, and kept at 4 °C; hemin stock solution was made at *ca.* 0.3 mM in 0.1 M NaOH.[6,7] Stock solutions of luminol and hemin were diluted to desired concentrations with 50 mM NaOH. Commercially available 30% H_2O_2 was diluted with degassed double-distilled water and injection volume of H_2O_2 was 0.1 or 0.06 ml. Reaction mixtures (100 μl) were assembled: hemin solutions of varied concentrations were added to 2 volume of luminol solutions. Luminol solutions were 6.7×10^{-8} M in the abscence of description. Temperature was maintained at 23 to 25 °C.

Stopped-Flow Apparatus——Kinetics of luminol CL was traced by a stopped-flow spectrophotometer, Union Giken RA-401, without a light source. This instrument had a rapid mixing device of dead time 8 ms and a cell of optical path length 10 mm and was interfaced with SORD microcomputer M200 mark II.

Instrumentation——Figure 1 shows the block diagram of our total luminescence research instrument. An automated injection device rapidly added reagent solution to a reaction vessel containing a luminescent material and a catalyst to mix thoroughly and initiate light production. The light emitted from the vessel was converted into current by a photomultiplier (931B, Hamamatsu) that had cathode sensitivity of 71 μA/1 m (Sk, 100 V), anode sensitivity of 600 μA/μl m (Sp, 1000 V) and dark current of 0.092 nA (Idb, 750 V); a high-voltage power supply was a Hamamatsu C665. The output current was changed into voltage by an amplifier (C1556-01, Hamamatsu) and recorded by a transient memory (TMR-120, Kawasaki Electronica) with a memory of 4096 words, 12 bit word length and the shortest sampling interval of 2 μs. The automated equipment generated a trigger pulse simultaneously with the injection. Then the transient memory started recording the output signal according to a selected delay time; it provided 13 settings of delay times between 0 and $\pm$100% of an observation period. The digitally recorded reaction curve was transferred into a personal computer (MZ 2000, Sharp) through a GP-IB interface (MZ-8BI04, Sharp) and

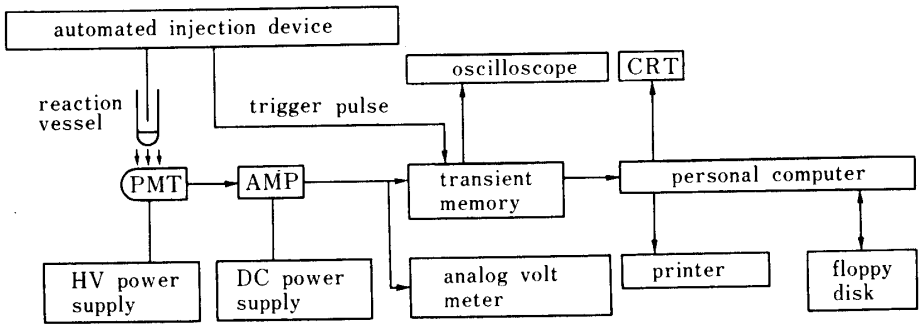

Fig. 1. Block Diagram of Total Luminescence Analyzing System

PMT represents photomultiplier; AMP, an amplifier; HV, high voltage; DC, direct current.

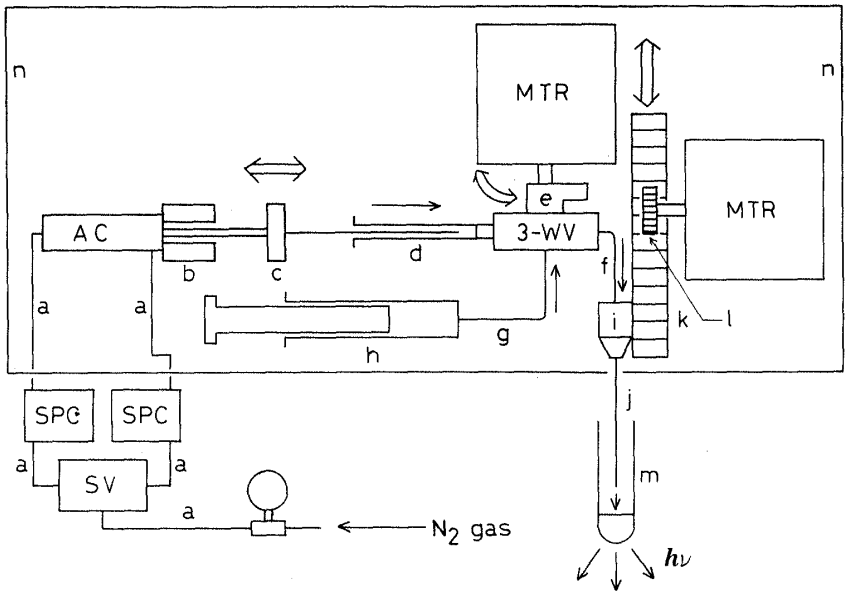

Fig. 2. Schematic Diagram of Automated Injection Device

SV represents as solenoid air valve (050-4E1, Koganei Ltd.); SPC, a speed controller (SCO-F, Koganei Ltd.); AC, an air cylinder (KAOD6×45, Koganei Ltd.); 3-WV, a three-way valve (disposable L type, Top); MTR, a reversible motor (RH6P4, Japan Servo Co., Ltd.); PMT, a photomultiplier tube; small letter a, a nylon tubing; b, a stopper; c, a plastic connector between the piston rod of the air cylinder (AC) and the plunger of a drive syringe (d); d, a drive syringe (50, 100 and 250 μl gas-tight syringe, 1000 series Tef-LL, Hamilton Co.); e, the lever of the 3-WV; f, a teflon tubing; g, an extension tubing connecting the 3-WV to a reservoir syringe (h) (X2-50, Top); h, a reservoir syringe (5 or 10 ml); i, a needle adaptor (teflon); j, an injection needle (1/3 mm diameter); k, a rack; l, a pinion; m, a cuvette of 50 mm height and a diameter (5, 6, 8, 10 or 14 mm) (glass); n, a light-tight box (iron); →, a drive or a filling motion of reagent solution; ⇔, a moving direction of the automated injection parts.

monitored by an oscilloscope (CS-1560A, Trio). An analog voltmeter with ultra-high input impedance (A-1509, Nakamura Scientific) watched dark current and excess light for the photomultiplier.

Figure 2 shows the schematic diagram of the automated injection device that permitted repeated, rapid addition of a reagent solution to a sample in a cuvette. A large reservoir syringe (h) provided a drive syringe (d) with the reagent through a plastic 3-way valve (3-WV), the lever (e) of which was moved by a reversible motor (MTR). A solenoid air valve (SV) gave compressed nitrogen from a tank to power an air cylinder (AC) for a rapid forward drive and slow backward filling. The drive syringe was mounted to an aluminum support block and directly attached to the 3-way valve; the reservoir was joined by tube (g), for the drive parts had to be pressure-proof. An injection assembly which consisted of the drive syringe, the 3-way valve, the reservoir syringe, an injection needle (j) and tubes (f and g) could be connected and disconnected to and from the overall apparatus and cleaned easily and quickly. A solenoid valve could not replace the plastic 3-way valve, since its diaphragm caused a droplet of the reagent solution to flow from the tip of the needle when it was energized and de-energized. Addition volumes depended on the drive distance of the plunger and the capacity of the syringe; three stoppers (b) and three syringes allowed them to change in the range from 0.01 to 0.15 ml. Larger volumes can be injected by an air cylinder with a bigger or a longer piston rod, because friction between the barrel of the syringe and the teflon tip of the gas-tight plunger increased with the capacity of the microsyringe and retarded the flow rate. The drive plunger was joined with the air cylinder by a plastic connector (c) and desired efflux was determined accurately by both selection of an adequate length stopper and a location of the connector fixed at the piston rod. The flow rate was managed by the pressure of nitrogen and speed controllers (SPC); the air cylinder usually operated at 4 to 7 atm. A rack (k) and pinion (l) moved up and down the needle and these alternate situations permitted reset of the reaction vessel and the reagent addition (see below). Our injection apparatus ensured a constant flow rate, a constant reagent volume and an unchanged needle position on the injection.

The automated injection device was constructed on an aluminum plate (see o in Fig. 3) with an opening (p) for the injection needle (k) to pass through. It was covered with a black iron box (n) and remotely controlled. A black plastic reaction chamber (a) of the drawer type had a cylindrical hole to accommodate a vessel holder (d) and lid (e). The upper side of the lid was sealed with aluminum foil (f) that would reflect the light emitted from the cuvette (g) toward the detector (j). During the set period, the needle previously adjusted at the center of the holder moved down, passed through the reflecting seal with a small aperture and reached a desired injection point in the cuvette.

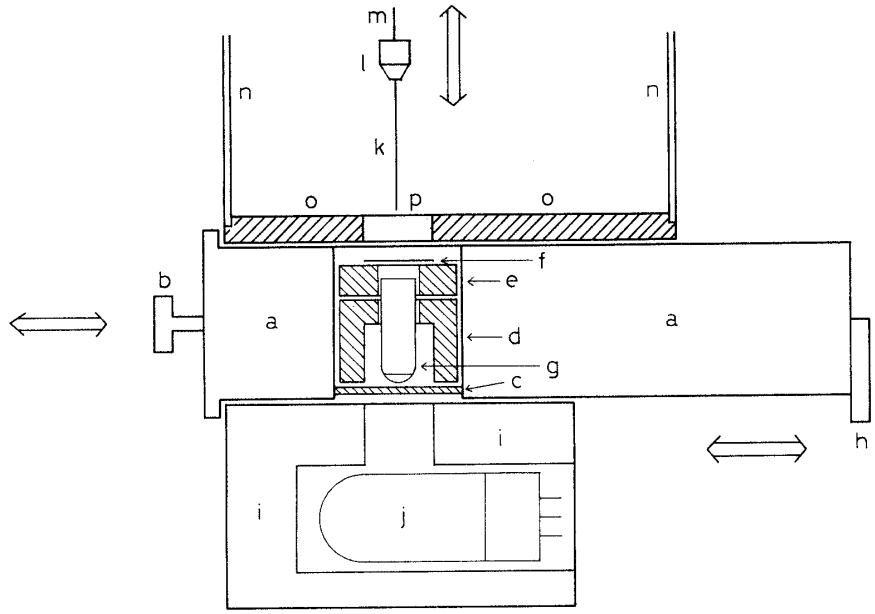

Fig. 3. Schematic Diagram of Reaction and Detector Chambers

Small letter a represents a reaction chamber (black plastic); b, a knob; c, a bottom plate (glass); d, a cylindrical vessel holder (aluminum); e, a cylindrical vessel lid (aluminum); f, aluminum foil; g, a reaction vessel (glass); h, a stopper; i, a detector housing (aluminum); j, a photomultiplier; k, an injection needle, (as for k, l, m and n, see Fig. 2); l, a needle adaptor; m, a teflon tubing; n, a light-tight box; o, an aluminum plate; p, an opening; ⇔, a moving direction of the reaction chamber (a) or the injection needle (k).

After light measurement, the needle moved up in order not to disturb the reset of the reaction vessel, *i.e.* draw-out of the reaction chamber and then the reservoir supplied the drive syringe with the reagent for subsequent operation. Our apparatus utilized several vessels having varied inside diameters of 5, 6, 8, 10 and 14 mm but a fixed height of 50 mm. Of cource, it can use ones of other sizes, if holders and lids are made.

Results

Figure 4 shows the typical data for chemiluminescent reaction of luminol, which are hard copies of CRT (cathode ray tube) graphic area. The light production immediately reaches an emission peak (see b in Fig. 4 A) by 100 ms after the injection of hydrogen peroxide solution (a) and decays slowly to background electric potential in about 2 s. The light-increasing phase probably involves a mixing process, and the decreasing phase mainly consists of a rate process after the completion of the mixing; the latter is usually traced by a stopped-flow spectrophotometer, but not the former process. We analyzed using a personal computer total emission (TE) of the CL phenomenon by dividing it into two summations:

(1) Early emission (EE) is a simple summation of the light output in the form of the product of electric potential and time (volt × second) over the range between a and c in Fig. 4 A.

(2) Late emission (LE) is derived from a definite integral of a first-order reaction formula from time c to infinity with respect to time (see the second term of Formula 1). A semilogarithmic plot, a plot of log $(V_t - V_f)$, gives a straight line (see Fig. 4 C), where V_t is the electric potential at time, t, and V_f the final potential. Thus the late period (between c and d) is regarded as a first-order reaction and has a rate constant of $1.76\,s^{-1}$ ($t_{1/2} = 0.39$ s).
The total emission (TE) is written:

$$TE = EE + LE = \sum_{i=0}^{c/\Delta t} V_{ti} \times \Delta t + \int_{c}^{\infty} V_0 \times \exp[-k(t-c)]dt \qquad \text{(Formula 1)}$$

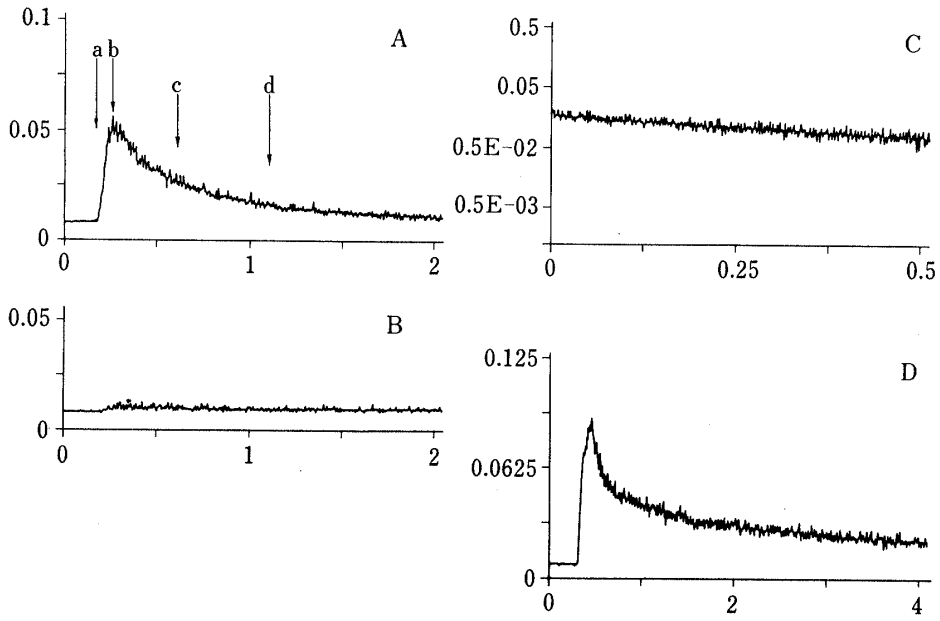

Fig. 4. Printer Output Data for Chemiluminescence

Capital letter A represents emission on luminol decomposition catalyzed by hemin (average data of 6 experiments); B, blank (reaction mixture without luminol, average data of 2 experiments); C, a semilogarithmic plot of the area between c and d in A; D, emission catalyzed by microperoxidase (average data of 4 experiments). Abscissa is time (second); ordinate, electric potential of the DC amplifier output (volt); small letter a in A shows the initiation of the H_2O_2 addition; b, a light peak; c and d, an interval for the semilogarithmic plot. Experiment A was carried out: the automated injection device mixed 0.06 ml of 0.3% H_2O_2 solution with 6.7×10^{-10} M luminol solution containing 10^{-5} M hemin in 50 mM NaOH; the pressure of the air cylinder was 5 atm; the cuvette, 10×50 mm; the needle height from the bottom of the cuvette, 10 mm; the needle diameter, 1/3 mm; the electric potential of the PMT, -650 V; the syringe, 100 μl; temperature, 24.5 °C. Experiment D was coducted: 60 μl of H_2O_2 was added to 100 μl reaction mixture in 75 mM barbital buffer, pH 8.6, made up of 67 μl of 10^{-7} M luminol, 20 μl of 2×10^{-6} M microperoxidase and 13 μl of 250 mM NaOH.[6,7]

where V_{ti} is potential at time, t_i $(=i \times \Delta t)$; Δt, a sampling interval; V_0, voltage at the initial time of LE; k, a rate constant. The personal computer calculated all these values by subtracting average blank data from emission data at each sampling point; V_0 and k were obtained by the least-square fit of the kinetic approximation (see Fig. 4 C). The TE may represent the total quantity of the emission over the luminescent reaction, since its values were consistent with those acquired by measurements over a sufficient time for the completion of the reaction. Consequently this method can save time and effort for experiment.

Figure 5 shows the comparison of rate constants determined by our automated injection device and a stopped-flow apparatus. Measurements ranged from $k = ca.$ 6 to 0.25 s^{-1} ($t_{1/2} = ca.$ 0.1 to 3 s). Apparently the injection unit can observe rapid luminescent reaction ($t_{1/2} =$ 0.1 s) like the stopped-flow system. It is, however, ambiguous that perfect mixing was accomplished in a shorter time than the half-life period of the reaction. The details of the mixing manner can be examined by a physical approach, *e.g.* convolution and deconvolution of the mixing behavior like fluorescence life time measurement.

Figure 6 shows the effects of hemin concentration on emission rate constants, TE and light intensity maxima. The slope for line a (rate constant) indicates that the rate increases with hemin concentration and that it, however, is not directly proportional to. Shevlin and Neufeld put forward a model of complicated dependence of the rate constants of luminol CL on ferricyanide, sodium hydroxide and oxygen.[3b] The influence of the inorganic catalyst seems to resemble that of hemin. The TE has a peak at about 10^{-5} M hemin and decreases in

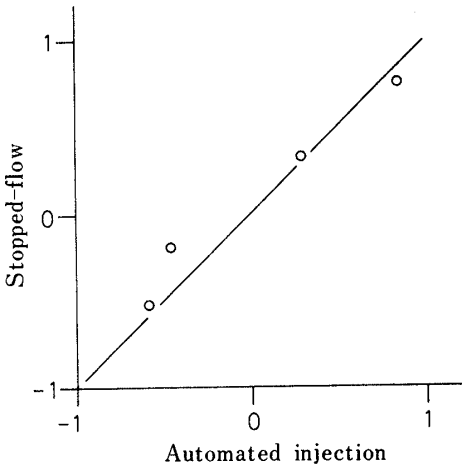

Fig. 5. Correlation between Automated Injection Device and Stopped-Flow Apparatus

The automated injection device added 0.1 ml of 0.03% H_2O_2 to luminol solution involving hemin of diverse concentrations in 50 mM NaOH. The stopped-flow apparatus mixed 0.03% of H_2O_2 with an equal volume of 6.7×10^{-8} M luminol containing hemin of varied concentrations in 50 mM NaOH and measured change in the emitted light. The reaction curves were semilogarithmically plotted and rate constants of first-order were compared. Each circle shows average value of 2 to 4 experiments.

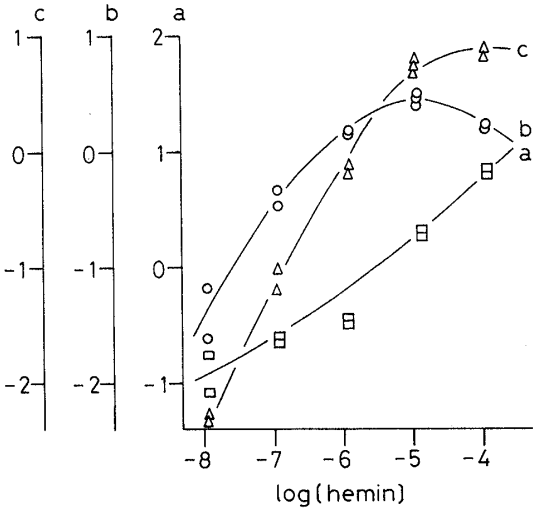

Fig. 6. Dependences of Rate Constant (a), Total Emission (b) and Peak Height Intensity (c) on Hemin

Abscissa is logarithm of hemin concentration (M); ordinate of a, log (rate constant, 1/s); that of b, log (total emission, V × s); that of c, log (peak height, V). Each symbol shows average value of 3 to 5 experiments. Experimental condition was the same as that in Fig. 5.

the more concentrated region. This declination can be attributed to the absorbance of hemin and/or the concentration of sodium hydroxide (see Materials and Methods): 10^{-4} M hemin in 67 mM NaOH transmits only *ca.* 1% light at a wavelength of 420 mm. On the other hand, the light maximum is a little larger even in the darker region. This case appears to result from the higher rate of the light production.

Table I shows the individual values of these quantities and the coefficients of variation (CV). The TE gives the best results: when the experiments were carried out under the condition described in the caption of Fig. 5, its CV's were usually about 1 to 4% for 10^{-5} M hemin (see the CV of the TE in Table I). The CV's for intensity and time of light peak are not good enough owing to white noise: such time-resolved CL output generally involves noisy signal originating primarily from photomultiplier short noise. A digital smoothing we used may be insufficient. It is a slightly modified moving average method[8] and written:

$$y(i) = 1/n \sum_{j=0}^{n-1} x(i+j \times \Delta t) \qquad \text{(Formula 2)}$$

where $x(i)$ is signal intensity (volt) observed at time i; $y(i)$, calculated value at time, i; Δt, interval of sampling. The height of the injection needle tip was ordinarily 10 mm from the bottom of the cuvette (see the caption of Fig. 4). A higher position tended to deteriorate the reproducibilities and the needle diameter also seemed to affect them.

We examined intensities of luminol CL using hemin and microperoxidase as catalysts. Luminol (0.1 ml of 6.7×10^{-10} M) containing 10^{-5} M hemin displays comparable emission maximum to 100-fold concentrated luminol containing 2.7×10^{-7} M microperoxidase (see Fig. 4 A and D). This luminol-microperoxidase system was reported by Schroeder *et al.* as the most sensitive and versatile detection system.[6] Our experimental condition was the same as that written by them except primarily that volumes of the reaction mixture and the hydrogen

TABLE I. Individual Values and Within-Run Variabilities of 6 Experiments

Total emission (Vs):					
2.733	2.798	2.692	2.832	2.775	2.926
MN=2.793;	SD=0.0819;	CV=2.93%			
Early emission (Vs):					
1.749	1.861	1.770	1.903	1.778	1.938
MN=1.834;	SD=0.0791;	CV=4.31%			
Late emission (Vs):					
0.983	0.937	0.921	0.925	0.998	0.988
MN=0.959;	SD=0.0347;	CV=3.62%			
Rate constant (1/s):					
1.692	1.797	1.775	1.952	1.825	1.846
MN=1.814;	SD=0.0860;	CV=4.74%			
Intensity of light peak (V):					
5.469	5.817	5.689	5.708	5.084	6.321
MN=5.691;	SD=0.407;	CV=7.17%			
Time of light peak (s):					
0.324	0.324	0.332	0.34	0.348	0.344
MN=0.335;	SD=0.0103;	CV=3.06%			

MN is mean; SD, standard deviation; CV, coefficient of variation. Observation range of time is 2s; that of electric potential, 10V; n of smoothing, 4. A hundred microliter of 0.03% H_2O_2 was injected into luminol solution containing 10^{-5} M hemin in 50 mM NaOH; diameter of the cuvette was 8 mm; temperature, 24 °C; other conditions were the same as those of A in Fig. 4.

peroxide solution added were 0.1 and 0.06 ml, respectively. Furthermore they diluted hydrogen peroxide with 10 mM Tris-HCl, pH 7.4, whereas we used the reagent diluted with degassed double-distilled water to avoid self-oxidation.[9] As to luminol CL, hydrogen peroxide in water was stable for at least three hours at room temperature. The TE of the microperoxidase solution was about ten times as large as that of the hemin solution. This large quantity in comparison with the emission maximum can be interpreted by the longer emission period of the Schroeder's system.

Discussion

Recently, a number of luminometers have appeared on markets[1] and home-built devices have been reported.[2] However, most of them lack flexibilities and versatilities, when applied to kinetic studies on chemiluminescence reactions (see Introduction).

Our computerized analyzing system improved many of these drawbacks. Our automated injection device can utilize cuvettes of several inside diameters (5, 6, 8, 10 and 14 mm) and addition volumes of 10 to 150 μl; cuvettes of any size can be used and more reagent mingled (see Instrumentation). The shape and the size of reaction vessels together with the mode of reagent injection are improtant design parameters, since adequate rapid mixing is a prerequisite.[1a] Seizt and Neary discussed this problem in some detail.[1d] During repeated observations, a rack and pinion produced an unchanging injection situation. Thus the equipment produced a superior mixing mode (a constant flow rate, volume and efflux position), *e.g.* the CV's of the TE did not exceed 4%. According to our experience, beginners of manual injection with microsyringes could not draw even calibration lines of luminol CL because of their large deviations of data.

All marketed instrument can perform integration of light output in the form of either total pulses or current after a delay time.[1a] Some can be fitted with a microcomputer and any

integration time interval may be selected.[1a] However, the integration over a fixed period is not necessarily proportional to the initial amount of a luminescent material. If the luminescence is first-order reaction and the mixing occurs in an infinitesimal time, the linearity can be derived from the definite integral of the first-order reaction formula. On the other hand, second-order reaction does not give the same result. The TE represented an actual quantity of entire emission and its value may be considered directly proportional to the luminescnet material. Measurment of the TE spends so short time (1 to 2 s) that the method can reduce trivial, routine work and time.

Most commercial units can detect the peak of light intensity and record this value on a digital display or a printer.[1a] One of problems on the peak height determination is that the time-resolved output always involves random noise. Generally the white noise is a permanent component of all measurements and may alter important quantities such as the height, the position and the width of a true curve. Even spurious lines may be created by the noise.[10] We intended to circumvent this problem by digital smoothing, moving average method,[8] but the method showed unsatisfying reproducibilities which were due to incomplete noise reduction. Increase in n of smoothing (see Formula 2) decreases the noise and may improve the CV's, but it will level off the peak and can deteriorate the sensitivity. Since this light peak method requires even shorter observation time than the integration method described above, it may become a useful tool in clinical laboratories that deal with many samples. However, unless a recording unit responds quickly to the signal, it will electrically flatten the peak like the digital filtering. Photomultipliers and silicon photodiodes have good time-responses (ns) and we need not pay attention to these detectors. Our unit has a very short sampling interval and is suited for the present method, but fast Fourier transformation may be necessary for reform.

Concentrated hemin (10^{-5} M) catalyzed luminol decomposition accompanied with rapid intense light production, *e.g.* the emission maximum was about fifty times higher than that with microperoxidase as a catalyst. This strength will render CL measurements easy and cheap, since even detectors without excellent sensitivity suffice the light observation.

To summarise, we made a computerized analyzing system for chemiluminescence that consisted of an automated injection device, a reaction and detector chamber, and a processing unit. This total system is characterized by superior reproducibilities, rapid time-response and various methods of analysis. The principles of the measurement and the analysis are applicable to physical, analytical and clinical chemistry.

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