Determination of the Dead Time of a Stopped-Flow Fluorometer[1]

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This investigation was carried out to develop a convenient alternative method for examining the performance and determining the dead time of a stopped-flow fluorometer. We examined the kinetics for the formation of the fluorescent Mg^{2+}–8-hydroxyquinoline chelate in aqueous solutions. The reversible association of the Mg^{2+} ion with 8-hydroxyquinoline is a second-order process whose on and off rate constants are dependent on pH. We estimated that the Mg^{2+} ion chelate has a fluorescence quantum yield of 0.02 in aqueous solutions. Using this reaction we measured the dead time of a stopped-flow fluorometer at different pH values. Measurements of the dead time were found to be reproducible and accurate. The Mg^{2+}–8-hydroxyquinoline reaction fulfills the requirements for a convenient test reaction for dead time measurement of stopped-flow fluorometers. Although the usefulness of the reaction is primarily to determine the dead times of stopped-flow instruments operating in the fluorescence mode, the reaction can also be used for testing an instrument operating in the absorbance mode. © 1989 Academic Press, Inc.

The determination of the dead time of a stopped-flow instrument is critical for the evaluation of the range of biological and chemical phenomena that can be analyzed kinetically. Several test reactions have been developed for measuring the dead times of stopped-flow spectrophotometers (1–5). However, suitable reactions for testing an instrument operating in the fluorescence mode are not as numerous. The binding of 8-anilino-1-naphthalenesulfonic acid (ANS)[3] to bovine serum albumin

(BSA) (6,7) and the reaction between N-acetyltryptophanamide (NATA) and N-bromosuccinamide (NBS) (8) have been used for measuring the performance of stopped-flow fluorometers. Unfortunately the ANS–BSA reaction has its own disadvantages (8) and the utility of the NATA–NBS reaction is slightly reduced due to the instability of NBS in aqueous solution.

Many sensitive fluorometric assays for metal ions involve the formation of metal chelates that exhibit specific fluorescence characteristics (9). The fluorometric determination of the magnesium ion in serum and urine using 8-hydroxyquinoline as a reagent is a standard clinical procedure (10). In this report, a detailed description of the kinetics for the formation of the fluorescent Mg^{2+}–8-hydroxyquinoline chelate is presented along with application of this kinetically characterized system to the reliable measurement of the dead time of a stopped-flow fluorometer.

MATERIALS AND METHODS

Chemicals. 8-Hydroxyquinoline was purchased from Sigma Chemical Co. and quinine sulfate dihydrate from Merck & Co. They were both used without any further purification. Magnesium chloride hexahydrate (analytical reagent) was from Mallinckrodt.

Solutions and buffers. A 5 mM stock solution of 8-hydroxyquinoline in acetonitrile was prepared and stored at 5°C in a dark glass bottle. Its concentration was determined using an extinction coefficient of 2600 M^{-1} cm^{-1} at 306 nm in 0.1 M Tris–Cl, pH 9.0 (11). A 0.1 M stock solution of $MgCl_2$ in distilled water was standardized with a silver nitrate solution using the Mohr method (12). Buffers were 0.1 M Tris titrated to the appropriate pH with HCl. All buffers were filtered.

Static absorbance and fluorescence measurements. Absorbance measurements were made with a Hewlett–Packard diode array spectrophotometer (HP 8452A) and fluorescence spectra were recorded with a ratio spec-

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[3] Abbreviations used: ANS, 8-anilino-1-naphthalenesulfonic acid; BSA, bovine serum albumin; NATA, N-acetyltryptophanamide; NBS, N-bromosuccinamide.

trofluorometer designed and built by one of us (D.P.B.) and Mr. Gordon S. Ford (13).

Measurement of fluorescence quantum efficiency. The relative quantum yield of fluorescence of the Mg^{2+}–8-hydroxyquinoline chelate was measured using the comparative method described by Parker and Rees (14). In this procedure the true fluorescence emission spectrum of a compound is used to obtain its fluorescence efficiency by comparison with a substance of known quantum yield using the equation

$$Q_1/Q_2 = (F_1/F_2)(A_2/A_1), \qquad [1]$$

where the ratios of quantum yields, Q, are related to the total corrected fluorescence emissions, F, and the absorbances at the exciting wavelength of two fluorescent compounds. Quinine sulfate in 0.1 N H_2SO_4 with a quantum yield of 0.70 was used as a reference compound (15).

The corrected fluorescence emission of quinine sulfate and 8-hydroxyquinoline chelate were recorded using an SLM 8000 spectrofluorometer at an exciting wavelength of 360 nm. The areas under the corrected fluorescence emission spectra were determined by cutting out and weighing the recorder paper. This procedure yielded quantum yield values with a precision of ±2%.

Stopped-flow apparatus. Rapid reactions were performed in a stopped-flow apparatus designed for absorbance and fluorescence measurements. The stopped-flow unit was from Kinetic Instruments, Inc. (Ann Arbor, MI) and the detection system was built by one of us (D.P.B.) and Mr. Gordon S. Ford. In the fluorescence mode a Schoeffel 150-W xenon arc lamp (Schoeffel Instrument Corp., Westwood, NJ) with a stabilized power supply provided the excitation source with a Schoeffel monochromator for selection of the exciting wavelength (12 nm bandpass). Fluorescence emission, selected through appropriate filter(s), was collected at right angles to the incident light. A 20-W (6 V) tungsten–halogen lamp (Osram) was used for absorbance measurements and the wavelength of interest was selected with a Bausch & Lomb monochromator (3 nm bandpass). The optical path length of the flow cell in the absorbance mode is 2.5 mm. Reaction signals (absorbance or fluorescence) were detected with a Hamamatsu photomultiplier tube (R928) and processed with a photometer. The kinetic data were recorded with a transient recorder (Physical Data, Inc., Model 514A) and visualized by display on a Type 503 oscilloscope (Tektronix, Inc.) and on an X-Y recorder. Observed reaction rates were determined directly from the kinetic traces with the aid of an analog computer that converted the data into logarithmic form. The analog computer was designed by one of us (D.P.B.) and Mr. Gordon S. Ford at the University of Michigan.

Rapid kinetic experiments. All experiments were carried out at 25°C; the reservoir syringe containing the 8-hydroxyquinoline solution was wrapped in aluminum foil to prevent potential light damage. For fluorescence measurement a solution of 100 μM 8-hydroxyquinoline in buffer was mixed with an equal volume of solutions containing various concentrations of $MgCl_2$. Concentrations of $MgCl_2$ were at least 10 times that of 8-hydroxyquinoline to maintain pseudo-first-order conditions. The excitation monochromator was adjusted to 360 nm and the emitted light filtered through an Esco GG-400 cutoff filter which cuts off light below 400 nm (Esco Products, Inc., Oak Ridge, NJ). The photometer time constant was 0.1 ms. Under the conditions used, the Mg^{2+}–8-hydroxyquinoline chelate has an absorbance of 0.03 at 360 nm so that inner-filter effects can be neglected (16). In the absorbance mode a solution of 500 μM 8-hydroxyquinoline was mixed with solutions of $MgCl_2$ under pseudo-first-order conditions. Formation of the Mg^{2+} chelate was followed at 360 nm.

Data and error analysis. A linear least-squares fit was used to select the best straight line through the experimental data points. A weighting factor was introduced in the computations to account for variation in the individual standard deviations and was calculated according to equations described in (17). The uncertainty in the determination of the parameters, expressed as standard deviation, was also obtained.

RESULTS

Reaction between 8-hydroxyquinoline and magnesium ion. Watanabe *et al.* (16) and Näsänen (18) have made extensive spectrophotometric, potentiometric, and fluorometric studies on the physical properties of 8-hydroxyquinoline metal ion chelates in aqueous solution. Their work demonstrated that in the pH range of 7.2 to 9.0, 8-hydroxyquinoline and Mg^{2+} combine reversibly to form a stable fluorescent chelate (1:1 complex) with dissociation constants greatly dependent on pH. The Mg^{2+}–8-hydroxyquinoline complex fluoresces maximally at 530 nm upon excitation at 360 nm (16). The spectral characteristics of the fluorescent chelate are depicted in Fig. 1. Using the comparative procedure of Parker and Rees (14), we determined the quantum yield of these complexes as 0.02 in 0.1 M Tris–Cl, pH 8.5.

The overall reaction can be described by the equilibrium

$$Mg^{2+} + HQ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MgQ^+ + H^+, \quad K_d = k_2/k_1,$$
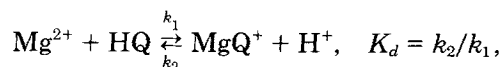
where HQ denotes the protonated form of 8-hydroxyquinoline and k_1 and k_2 are, respectively, the forward and reverse rate constants for the formation of the complex.

The observed rate of formation of the chelate is represented by the equation $k_{obs} = k_1[Mg^{2+}] + k_2$. Under
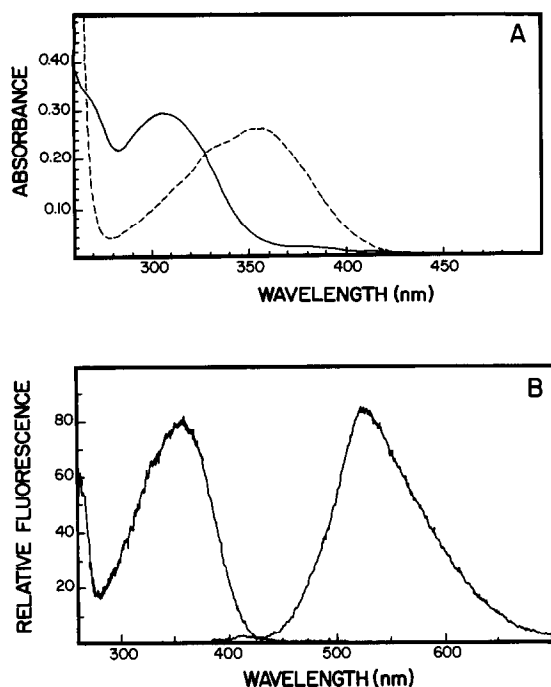
FIG. 1. The spectral properties of the Mg^{2+}-8-hydroxyquinoline complex. (A) The absorption spectrum of a solution of 8-hydroxyquinoline (112 μM) (—) changes dramatically upon addition of a saturating concentration of Mg^{2+} ions (29 mM) (---). The buffer is 0.1 M Tris–Cl, pH 8.5, and measurements were made in a 1-cm cell and at 25°C. (B) The excitation and the apparent fluorescence emission spectra of the Mg^{2+}-8-hydroxyquinoline chelate are shown. Spectra recorded in 0.1 M Tris–Cl, pH 8.5, and at 25°C.

pseudo-first-order conditions k_{obs} is linearly dependent on the magnesium ion concentration and the rate constants, k_1 and k_2, are, respectively, equal to the slope and intercept values of a plot relating k_{obs} to $[Mg^{2+}]$.

The linear relationship of the observed rate of formation of the fluorescent chelate with magnesium ion concentration in Tris buffer at pH 8.5 is shown in Fig. 2. The rate constants, k_1 and k_2, were evaluated as 6.8 ($\pm$0.3) $\times 10^4$ M^{-1} s^{-1} and 40 $\pm$ 7 s^{-1}, respectively. By appropriately selecting the magnesium ion concentration it is possible to cover values of k_{obs} ranging from a limiting value of k_2 to an upper limit of approximately 750 s^{-1} (the upper value being set by instrumental limitations). Using this approach we determined the values of k_1 and k_2 at several other pH values. These kinetic constants are summarized in Table 1.

As complementary experiments we performed a similar kinetic study in the absorbance mode by following formation of the complex at 360 nm. The rate constants evaluated in the absorbance mode are in reasonable agreement with those fluorometrically determined under the same conditions (Table 1).

The dissociation constants of the Mg^{2+}-8-hydroxyquinoline complex calculated from the experimental val-
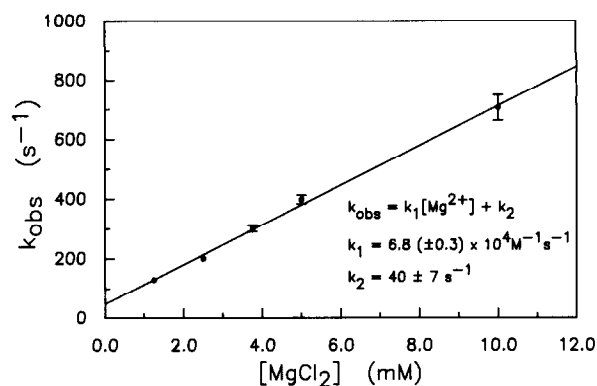

FIG. 2. The effect of magnesium ion concentration on the observed rate of formation of the Mg^{2+}-8-hydroxyquinoline complex. 8-Hydroxyquinoline (100 μM) was reacted with solutions containing various concentrations of $MgCl_2$ and the rate of formation of the chelate was followed fluorometrically in a stopped-flow apparatus as described under Materials and Methods. The experiment was performed in 0.1 M Tris–Cl, pH 8.5, and at 25°C. Each point represents the average value of 10 determinations with its standard deviation.

ues of k_1 and k_2 are in close agreement with those from Fig. 8 of Watanabe et al. (16).

Determination of the dead time of the stopped-flow fluorometer. A measure of the dead time (t_d) of the instrument can be obtained from stopped-flow traces of a first-order reaction of known rate k using the relationship (19)

$$kt_d = 2.303 \log(F_{tot}/F_{obs}), \qquad [2]$$

where F_{tot} is the total change in fluorescence intensity and F_{obs} is the magnitude of the observed change.

TABLE 1

Magnitude of the Rate Constants k_1 and k_2 for the Reversible Association of Mg^{2+} Ion with 8-Hydroxyquinoline and the Calculated Dead Time Values for the Stopped-Flow Instrument

pH[a]	Mode[b]	k_1[c] $(10^{-3} M^{-1} s^{-1})$	k_2 (s^{-1})	Dead time (t_d) (ms)
7.5	F	18 $\pm$ 1	120 $\pm$ 10	1.0 $\pm$ 0.1
	A	15 $\pm$ 2	130 $\pm$ 10	1.4 $\pm$ 0.3
8.0	F	26 $\pm$ 1	72 $\pm$ 4	1.0 $\pm$ 0.1
	A	22 $\pm$ 1	73 $\pm$ 6	1.4 $\pm$ 0.2
8.5	F	67 $\pm$ 4	40 $\pm$ 10	1.2 $\pm$ 0.1
	A	59 $\pm$ 2	40 $\pm$ 10	1.4 $\pm$ 0.2
9.0	F	200 $\pm$ 10	34 $\pm$ 4	1.0 $\pm$ 0.1
	A	170 $\pm$ 10	50 $\pm$ 4	1.2 $\pm$ 0.1

[a] Experiments were carried out in 0.1 M Tris–Cl buffers of appropriate pH and at 25°C.

[b] Detection was by fluorescence (F) or absorbance (A).

[c] Each rate value represents the average determination from two kinetic experiments (such as shown in Fig. 2) with its standard deviation.
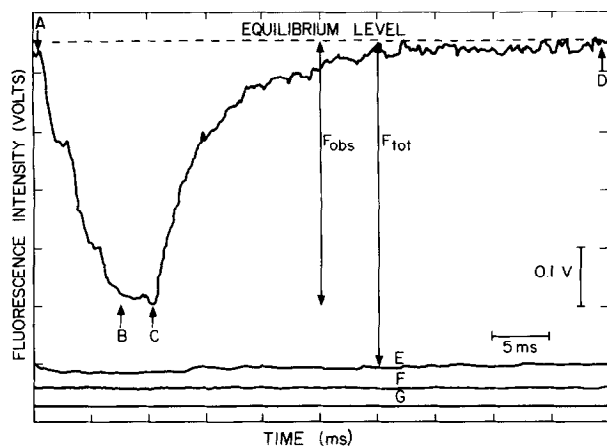
FIG. 3. Time course of the formation of the Mg^{2+}-8-hydroxyquinoline chelate. A solution of 8-hydroxyquinoline (100 μM) was mixed in the stopped-flow apparatus with a 5 mM solution of $MgCl_2$ at 25°C in 0.1 M Tris buffer, pH 8.5. The increase in fluorescence resulting from formation of the complex was recorded. Optimal performance of the stopped-flow instrument is illustrated on the reaction curve by the sequence of letters A to D. Point A is the beginning of flow and point C is where flow stops. The previous cell contents are flushed with fresh reaction mixture (region A–B) and the fluorescence signal reaches a steady-state level (region B–C) before flow stops (point C). It is important to ensure that a sufficient volume of reactants is mixed such that previously reacted reagents are flushed from the cell. This can be verified by the observation of a small interval of steady-state fluorescence as shown in the B–C region of this figure. Region C–D shows the progress of the reaction to equilibrium (point D). The fluorescence baselines denoted by the letters E, F, and G are E, 8-hydroxyquinoline in buffer; F, buffer (0.1 M Tris, pH 8.5); G, dark current (electronic background). The observable fluorescence change (F_{obs}) and the total change in fluorescence (F_{tot}) are also illustrated in the figure.

After rearrangement and substitution of $k_1[Mg^{2+}]$ + k_2 for k, the following equation results:

$$\log F_{obs} = -0.434k_1t_d[Mg^{2+}] + (\log F_{tot} - 0.434k_2t_d). \quad [3]$$

This equation predicts that a value for t_d can be derived from the slope of a plot relating $\log F_{obs}$ to $[Mg^{2+}]$, assuming that the rate constant k_1 has been previously determined.

Experimentally, the magnitude of F_{tot} is obtained by subtracting the fluorescence of 8-hydroxyquinoline in the absence of Mg^{2+} from the final fluorescence signal in the stopped-flow instrument after reaction with Mg^{2+}. The actual change observed in the apparatus, F_{obs}, is measured directly from stopped-flow traces as shown in Fig. 3.

Due to limitations in accurately measuring reaction rates above 750 s^{-1} in the stopped-flow instrument and to the fact that K_d values for the Mg^{2+}-8-hydroxyquinoline complexes are relatively high, complete saturation of the 8-hydroxyquinoline could not be attained in the stopped-flow apparatus for most magnesium ion concentrations employed. This implies that F_{tot} values differ

with changing magnesium ion concentrations. A slight modification to Eq. [3] was necessary to accommodate this problem.

Thus for each magnesium ion concentration, we defined F'_{obs} as the ratio of F_{obs} to F_{tot} and F'_{tot} as being equal to unity. After substitution of these parameters into Eq. [3], the following equation results:

$$\log F'_{obs} = -0.434k_1t_d[Mg^{2+}] - 0.434k_2t_d. \quad [4]$$

If F'_{obs} is measured as a function of $[Mg^{2+}]$, a plot of $\log F'_{obs}$ vs $[Mg^{2+}]$ will have a slope equal to $-0.434k_1t_d$ from which t_d can be extracted. Such an analysis performed on stopped-flow results obtained in 0.1 M Tris–Cl, pH 8.5, and at 25°C is presented in Fig. 4. The value for t_d at pH 8.5 is listed in Table 1 along with t_d determinations at other pH values.

An alternative method of data analysis is to extrapolate the semilogarithmic plots of kinetic traces to times before that of initial observation in the stopped-flow apparatus. Extrapolated plots obtained at various concentrations of the reactant in excess will intersect at true zero time. The difference in time between the extrapolated intersection point and the point of initial observation is the instrumental dead time (2,8,20). This alternate method of analysis yielded comparable dead time values as those presented in Table 1 for the fluorescence and absorbance data at pH 8.5.

DISCUSSION

Our kinetic investigation of the Mg^{2+}-8-hydroxyquinoline chelate along with our ability to reproduce dissociation constants that Watanabe et al. (16) have deter-
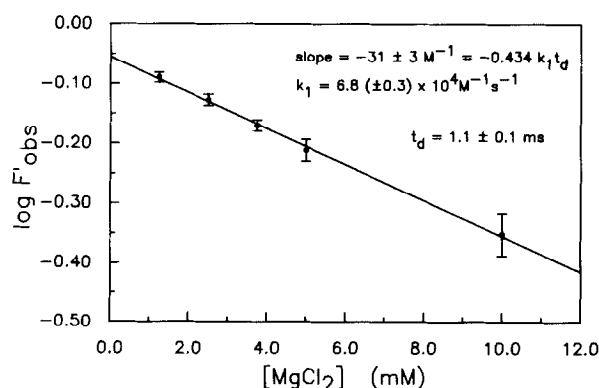

FIG. 4. Graphical determination of the dead time of the stopped-flow fluorometer. By analysis of the kinetic data for the formation of the Mg^{2+}-8-hydroxyquinoline complex, F'_{obs}, which is defined as F_{obs}/F_{tot}, is calculated for each magnesium ion concentration. From the slope of a plot of $\log F'_{obs}$ vs $[Mg^{2+}]$ a value for t_d can be derived. This analysis was performed on experimental stopped-flow data obtained in 0.1 M Tris–Cl, pH 8.5, and at 25°C. Each F'_{obs} value represented is the average from 10 stopped-flow traces with its standard deviation.

mined by fluorometric titration reinforce and confirm that the reversible association of magnesium ion with 8-hydroxyquinoline is a second-order reaction in the pH range of 7.2 to 9.0. Evidence exists that at pH values above 9.5 and at high ligand concentration, higher order complexes of Mg^{2+} and 8-hydroxyquinoline predominate (16,18). The formation of these rather insoluble complexes is negligible under our experimental conditions and consequently did not interfere with our investigation.

The Mg^{2+}-8-hydroxyquinoline reaction provides a reliable method for the fluorometric measurement of dead time. This is illustrated by the fact that although k_1, k_2, and the range of Mg^{2+} ion concentration used differ for each pH, our determined dead time values are very reproducible in a pH range of 7.5 to 9.0. Moreover, these values are the same as determined with this stopped-flow instrument in the absorbance mode (1.1 ± 0.1 ms, data not shown) using the reduction of 2,6-dichlorophenolindophenol by ascorbic acid (1,2).

Discrepancies exist between the fluorescence and the absorbance results (Table 1). First, the value of k_1 in the absorbance mode is approximately 15% smaller in magnitude than that of k_1 in the fluorescence mode. Second, the dead time values in the absorbance mode seem slightly overestimated compared to the fluorometrically determined values. Determinations of dead time in the absorbance mode with the chelation reaction are consequently less reliable. Although these results imply that the reaction may be more complex than the fluorescence data indicate, we are unable to provide a rational explanation for these differences.

We would like to point out that many chelation reactions exist for the fluorometric determinations of inorganic metal ions (9) and that any of these reactions are potential test reactions for determining the dead time of stopped-flow fluorometers. We chose the Mg^{2+}-8-hydroxyquinoline reaction for several reasons: (a) 8-hydroxyquinoline shows a high degree of specificity for Mg^{2+} ions compared to other cations (e.g., Ca^{2+} and Zn^{2+}), (b) the reagents are inexpensive and available commercially, (c) the reactants and the products of the reaction are stable under the conditions used, and (d) the Mg^{2+}-8-hydroxyquinoline chelate has been thoroughly studied in the literature.

The Mg^{2+}-8-hydroxyquinoline reaction is a convenient method for testing the performance of stopped-flow fluorometers. In the pH range studied this reaction is characterized by rates easily accessible by stopped-flow techniques and a range of Mg^{2+} ion concentration convenient to the experimenter. Although the major application of this reaction is to measure the dead time and overall performance of a stopped-flow instrument operating in the fluorescence mode, the same reaction can be used (with limitations discussed above) for testing an apparatus operating in the absorbance mode.

ACKNOWLEDGMENT

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