A Simple Procedure for Determination of the Dead Time of a Stopped-Flow Instrument

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Summary The reducing reaction of 2,6-dichlorophenolindophenol by L-ascorbic acid was used to determine the dead time of a stopped-flow instrument. Because this reaction is irreversible, the dead time could be determined by a simple graphical analysis. The dead time values determined by the present method were comparable to those by other methods previously reported.

Key words: dead time, stopped-flow spectrophotometer, 2,6-dichlorophenolindophenol (DCIP).

In the kinetic study of chemical reactions by stopped-flow procedure, it is of great importance to measure the dead time of the instrument. Several test reactions have been used for measuring the dead time in absorbance and fluorescence mode (HIROMI *et al.*, 1968; HOA and DOUZOU, 1973; HIROMI, 1977; TONOMURA, *et al.*, 1978; PETERMAN, 1979; NAKATANI and HIROMI, 1980; BRISSETTE *et al.*, 1989). Recently BRISSETTE *et al.* (1989) have developed a new method for determining the dead time of a stopped-flow fluorometer using the reaction between Mg^{2+} and 8-hydroxyquinoline. In this method, however, some problems remain in terms of convenience. Here we describe the simplification of the method using the absorbance change observed in the reducing reaction of 2,6-dichlorophenolindophenol (DCIP) by L-ascorbic acid instead of a Mg^{2+} -8-hydroxyquinoline system.

A Union Giken stopped-flow spectrophotometer RA-401 equipped with a data processor RA-450 was used. One tenth mM DCIP solution was rapidly mixed with an equal volume of L-ascorbic acid solution in various concentrations and the absorbance change was observed at 524 nm. Usually an observation cell with optical path length of 10 mm was used. Concentrations of L-ascorbic acid were at least 20 times that of DCIP so that the kinetics could be treated as a pseudo-first-order reaction. Experiments were carried out at 25°C, pH 2.0. The pH was adjusted to 2.0 with HCl. Solutions were prepared daily. Absorbance change was displayed on an

oscilloscope, stored in the data processor and afterwards depicted with a *X-Y* recorder. The apparent rate constants were obtained directly from the reaction traces with the data processor.

All the chemicals were of reagent grade and purchased from Nacalai Tesque (Kyoto).

The reaction between L-ascorbic acid (A) and DCIP (D) is represented as follows:

Under the pseudo-first-order condition where the concentration of L-ascorbic acid is in large excess the apparent rate constant (k_{app}) is dependent on ascorbic acid concentration $[A]$ and represented by the following equation:

$$k_{app} = k [A] . \quad (2)$$

A plot of k_{app} vs. $[A]$ will give the slope equal to the second-order rate constant, k . The linear relationship between k_{app} and $[A]$ is shown in Fig. 1.

If a first-order reaction with rate constant k_{app} is observed in an instrument with dead time, t_d , the following relationship results:

$$k_{app} \cdot t_d = 2.303 \log \frac{A_{tot}}{A_{obs}} , \quad (3)$$

where A_{tot} is the total potential absorbance change and A_{obs} is the actual observed absorbance change. Substituting Eq. (2) into Eq. (3), we obtain the following

Fig. 1. The dependence of apparent rate constant (k_{app}) on ascorbic acid concentration ($[A]$). One tenth mM DCIP was mixed with various concentrations of ascorbic acid at 25°C, pH 2.0. Driving pressure was 7 kg/cm².

Fig. 2. Determination of the dead time of the stopped-flow instrument. $\log A_{\text{obs}}$ is linearly dependent on ascorbic acid concentration ($[A]$). The dead time value can be derived from the slope using k value obtained in Fig. 1. Experimental conditions are the same as those in Fig. 1.

Table 1. The dead time values of the stopped-flow instrument at various driving pressures determined by different methods.

Pressure (kg/cm ²)	Dead time (ms)		
	a	b	c
5	2.3 ± 0.1	2.3	2.3 ± 0.2
6	1.9 ± 0.1	2.3	2.0 ± 0.2
7	1.8 ± 0.1	1.7	1.9 ± 0.2
	0.9 ± 0.1*		
8	1.7 ± 0.1	1.8	1.8 ± 0.1

The dead time was determined by: (a) a plot of $\log A_{\text{obs}}$ vs. $[A]$ (the present method); (b) extrapolation of first-order plots; (c) $(2.303/k_{\text{app}}) \cdot \log(A_{\text{tot}}/A_{\text{obs}})$. * Optical path length was 2 mm.

equation:

$$\log A_{\text{obs}} = -(k \cdot t_d / 2.303)[A] + \log A_{\text{tot}} \quad (4)$$

A_{obs} can be directly measured from the reaction traces. Since the reaction between L-ascorbic acid and DCIP is irreversible, by keeping the concentration of DCIP constant, A_{tot} can be kept constant independently of $[A]$. Therefore $\log A_{\text{obs}}$ is linearly dependent on $[A]$ and a plot of $\log A_{\text{obs}}$ vs. $[A]$ will have the slope equal to $-k \cdot t_d / 2.303$ from which t_d can be derived using the k value previously obtained (Fig. 1). Such an analysis is represented in Fig. 2. The t_d values at various driving pressures are summarized in Table 1. A result with a shorter path length

Fig. 3. First-order plots of the reaction traces at various concentrations of ascorbic acid. $(A_t - A_\infty)$ represents the difference between the absorbance at time t and the final level. The concentrations of ascorbic acid were: (a) 2.5 mM; (b) 5 mM; (c) 10 mM. Temperature: 25°C, pH 2.0. Driving pressure: 5 kg/cm².

of cell (2 mm) is also included.

So far two other methods have been employed to measure the dead time of the instrument. First, the dead time was obtained as $(2.303/k_{app}) \cdot \log(A_{tot}/A_{obs})$ using Eq. (3) (HIROMI *et al.*, 1968; HIROMI, 1977). Second, extrapolated first-order plots at various ascorbic acid concentrations will intersect at one point before the time of the first observation. The dead time is represented as the time interval between the point of the intersection and that of the first observation (Fig. 3) (HOA and DOUZOU, 1973; PETERMAN, 1979). It is shown in Table 1 that the dead time values determined by the three methods were fairly comparable to each other. The dead time can be shortened by increasing the driving pressure or by reducing the optical path length of the observation cell.

BRISSETTE *et al.* (1989) have determined the dead time of an instrument using the reaction between Mg^{2+} and 8-hydroxyquinoline. Since this reaction is reversible, A_{tot} varies depending on Mg^{2+} concentration, i.e. a plot of $\log A_{obs}$ vs. $[Mg^{2+}]$ is not linear. Therefore a slight modification is required to solve this problem. Replacing A_{obs} for A'_{obs} defined as the ratio of A_{obs} to A_{tot} in Eq. (4), a plot of $\log A'_{obs}$ vs. $[Mg^{2+}]$ is linear which slope involves t_d . This implies that A_{tot} as well as A_{obs} should be measured at each Mg^{2+} concentration. This point can be much simplified using the reduction of DCIP by L-ascorbic acid instead of the reaction between Mg^{2+} and 8-hydroxyquinoline. Since the reaction between L-ascorbic acid

and DCIP is irreversible, by keeping DCIP concentration constant throughout, A_{tot} remains constant independently of ascorbic acid concentration. And thus measuring A_{tot} is not necessary (cf. Eq. (3)). The reducing reaction of DCIP by L-ascorbic acid is suitable and more convenient for the determination of the dead time of the stopped-flow instruments. During preparation of this paper, we learned that NAKATANI and HIROMI (1980) have already published essentially the same procedure as ours. However, it should be noted that it is not necessary to measure A_{tot} , and the only necessary parameters are k_{app} and A_{obs} in the present method.

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