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AUTOMATED ANALYSIS OF ENZYME INACTIVATION PHENOMENA

APPLICATION TO β-LACTAMASES AND DD-PEPTIDASES

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Abstract—In the presence of a reporter substrate, the progressive inactivation of an enzyme was easily studied by directly transmitting absorbance readings to a microcomputer. Pseudo-first order rate constants as high as 0.3 sec^{-1} were rapidly and accurately measured. When utilization of the reporter substrate did not exceed 10%, the rate of the reaction (v_i) could be considered as proportional to the active enzyme concentration at any time during the analysis and the decrease of v_i was first order with time. This simple method was used to follow the inactivation of β-lactamases (EC 3.5.2.6) by various physical and chemical agents. When a large proportion (30–80%) of reporter substrate was destroyed, a correction was introduced to account for the corresponding decrease of its rate of utilization. This enabled experiments to be performed with a DD-peptidase and a substrate exhibiting a low $\Delta\varepsilon$ upon hydrolysis. For the first time, the inactivation of a penicillin-sensitive enzyme by a β-lactam could be continuously and directly observed. Finally, the method was extended to the study of hysteresis phenomena.

The determination of enzyme catalytic pathways often involves the utilization of specific inactivators. Such studies present an important pharmacological interest, since the affinity of a drug for a given target is a determining factor of its physiological effects. In consequence, it is always useful and often necessary to measure kinetic parameters such as inactivation and reactivation rate constants. Experiments are usually performed, in which the enzyme is pre-incubated with the inactivator. Samples are withdrawn after increasing periods of time and the residual activity determined. This method is time-consuming and, in addition, rate constants larger than $2–3 \times 10^{-3}$ sec^{-1} ($t_{\frac{1}{2}} = 4–6$ min) become rather difficult to measure.

An alternative procedure involves the monitoring of the transformation of a "reporter substrate". Inactivation rate constants are then deduced by analysing the time-dependent variation of product concentration [1] or of the rate of substrate utilization ($v = -d[S]/dt \cong -\Delta[S]/\Delta t$) [2–4]. When the experimental progress curves are analysed manually, it is not easy to determine first-order rate constants larger than 2×10^{-2} sec^{-1} ($t_{\frac{1}{2}} = 35$ sec) and the results are not very accurate. The widespread availability of relatively inexpensive microprocessors, readily amenable to interfacing with spectrophotometers, greatly enhances the possibilities of the "reporter substrate" method.

In this paper, we show how first-order-rate constants as high as 0.3 sec^{-1} can be readily measured using a Beckman DU-8 spectrophotometer interfaced to an Apple II microcomputer. The determination of larger rate constants requires the help of regular stopped-flow equipment.

MODELS AND EQUATIONS

Utilization of a reporter substrate (S)

In the simple methods described before [1, 3, 4], the linearity of the transformation of the reporter substrate was first verified within the limits of the absorbance variations to be observed during the inactivation experiments. It was indeed crucial that the decrease in reaction rate should only be due to the inactivation phenomenon. An important aspect of this contribution resides in the introduction of a simple correction which dissociates the decrease of the rate due to the variation of substrate concentration from that due to the inactivation phenomenon itself.

In the first part of this article, we will however describe the utilization of the simple method where, in the absence of inactivation, the reporter substrate is linearly transformed throughout the experiments.

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Spontaneous inactivation

If the phenomenon is first-order and if the intermediate complexes are denatured at the same rate as the free enzyme, it is clear that

$$\frac{E_t}{E_0} = \frac{v_t}{v_0} = e^{-kt} \quad (1)$$

where E_t and E_0 are enzyme concentrations and v_t and v_0 the rates of transformation of S at times t and 0, respectively. Conversely, if no inactivation of the intermediate complexes occurs, an apparent rate constant k' is obtained and

$$k' = k\left(1 + \frac{[S]}{K_m}\right)^{-1}. \quad (2)$$

If the free enzyme and the intermediate complexes exhibit different sensitivities to the inactivating conditions, the situation becomes more complicated, and it is easier to work at $[S] \ll K_m$ so that only the denaturation of the free enzyme has to be considered. Experiments performed at saturating substrate concentrations yield indications on the rate of denaturation of the intermediate complexes.

Inactivating reagents and reactivation

We assume a competitive interaction between reporter substrate and inactivator. Equations for non-competitive systems have also been derived by Cha [2].

Model 1 represents a simple inactivation pathway

$$E + C \overset{K}{\rightleftharpoons} EC \overset{k_2}{\longrightarrow} EC^* \quad \text{(model 1)}$$

When C_0 is much larger than the total enzyme concentration E_0, the pseudo-first-order rate constant is

$$k_i = \frac{k_2[C]}{[C] + K} \quad (3)$$

In the presence of the reporter substrate with which the steady-state is assumed to be instantaneously established, equation (3) becomes

$$k_i = \frac{k_2[C]}{[C] + K\left(1 + \frac{[S]}{K_{m,S}}\right)} \quad (4)$$

where $K_{m,S}$ is the K_m of the reporter substrate.

In the presence of a high reporter substrate concentration ($[S] \gg K_{m,S}$), the correction factor $1 + [S]/K_{m,S}$ is large and errors on the value of K_m directly affect the value of K. It is thus advisable to keep $[S]$ close to or below $K_{m,S}$.

A further degree of complexity is introduced if EC^* is unstable and can regenerate free enzyme according to model 2

$$E + C \overset{K}{\rightleftharpoons} EC \overset{k_2}{\longrightarrow} EC^* \overset{k_3}{\longrightarrow} E + P' \quad \text{(model 2)}$$

In that case, the contribution of the steady-state must be withdrawn and

$$\frac{v_t - v_{ss}}{v_0 - v_{ss}} = e^{-k_i t} \quad (5)$$

where v_{ss} is the rate of transformation of S at the steady-state and

$$k_i = k_3 + \frac{k_2[C]}{[C] + K\left(1 + \frac{[S]}{K_{m,S}}\right)} \quad (6)$$

The reactivation of the enzyme can be followed in a separate experiment, where the enzyme is, ideally, nearly completely inactivated ($v_{ss} \cong 0$) and the excess of inactivator can be instantaneously eliminated by dilution or destruction. The activity is then monitored in the presence of the reporter substrate until reactivation is complete (v_f). In that case

$$\frac{v_f - v_t}{v_f - v_{ss}} = e^{-k_3 t} \quad (7)$$

Branched pathways, represented by model 3, have been extensively analysed by Waley [5] and Tatsumi *et al.* [6].

$$E + C \overset{K}{\rightleftharpoons} E\Psi \overset{\kappa_x}{\text{—v}} E\Psi \overset{\kappa_x}{\equiv\text{—v}} E \neq \Pi \quad \text{(model 3)}$$

$$\underset{EC^i}{k_4 \downarrow}$$

Our analysis assumes that the steady-state is reached rapidly in the catalytic branch. The characteristic feature of model 3 is that the inactivator is itself partially destroyed. If the initial ratio C_0/E_0 is so high that less than 5% destruction of inactivator occurs, the inactivator concentration can be considered as constant throughout the experiment [4, 7], the decrease of enzyme activity is characterized by a simple exponential (equation 1) and

$$k_i = \frac{(k_i)_{lim}[C_0]}{[C] + K_{m,C}\left(1 + \frac{[S]}{K_{m,S}}\right)} \quad (8)$$

where

$$(k_i)_{lim} = \frac{k_2 k_4}{k_2 + k_3 + k_4} \quad (9)$$

and

$$K_{m,C} = \frac{k_3 K}{k_2 + k_3 + k_4} \quad (10)$$

Equations (4), (6) and (8) can easily be linearized and, if $K_{m,S}$ and k_3 are measured in independent experiments, plots of $[C]/k_i$ vs $[C]$ (models 1 and 3) or $[C]/(k_i - k_3)$ vs $[C]$ (model 2) will yield the values of K and k_2 (models 1 and 2) or $K_{m,C}$ and $(k_i)_{lim}$ (model 3).

When a larger proportion of the inactivator is destroyed, a procedure for determining the parameters has already been described [7]. There is, however, a simpler method by which we may allow for destruction of the inactivator. The inactivation is considered as first-order and at a constant C_0 value, the apparent first-order rate constant is measured for various E_0 values. The reciprocal of that apparent first-order rate constant is then plotted against E_0. It may be shown that the reciprocal of the intercept at $E_0 = 0$ obeys equation (8). This procedure, which has been shown to be adequately robust, corrects for up to 20% destruction of the inactivator. For 20–

50% destruction, a curve, rather than a straight line is obtained, and the extrapolation to $E_0 = 0$ is more uncertain.

Utilization of a large proportion of the reporter substrate

When the variation of absorbance after complete substrate utilization is small, for instance below 0.5 absorbance units, it is not possible to obtain accurate data under conditions where the linearity of the reporter substrate transformation is verified. If a large proportion of the substrate disappears during the experiment, a correction must be introduced to account for the decreased rate of substrate utilization due to decreased substrate concentration. The correction is easily performed by substituting v by V_{max}. Indeed, equation (11) is easily obtained from the simple Henri–Michaelis equation

$$(V_{max})_t = v_t \frac{K_m + [S]}{[S]} = v_t \frac{(K_m,S)_A + |A_\infty - A_t|}{|A_\infty - A_t|} \quad (11)$$

where $(K_m,S)_A$ represents K_m,S expressed in absorbance units and A_∞ the absorbance observed after complete utilization of the substrate. For a given $[S]$, that value must be determined in the absence of inactivator, under which conditions the value of V_{max} should remain constant. This supplies an easy test for the validity of the correction. In the presence of the inactivator, equations (1), (5) or (7) can then be used after substituting v by V_{max}. The decrease of substrate concentration might, however, result in another difficulty. The rate of inactivation depends on the ratio $[S]/K_m,S$ (equations 4, 6, 8), and the "protection" by the substrate decreases with substrate concentration. The experiments are thus better performed under conditions where the protection is minimum, i.e. at $[S] < K_m,S$ or, at least, where the total $\Delta[S]$ in the experiment remains $< K_m,S$.

Hysteresis phenomena

According to Frieden's [8] model, the rate of product formation in most hysteretic systems obeys equation (5).

In that case, no reporter substrate is used, the disappearance of substrate or the appearance of product is directly monitored. The rate constant, k_i, is complex and depends on substrate concentration.

Kinetic parameters for the reporter substrate from complete time-courses

When a variation of absorbance is recorded, the integrated equation, valid for irreversible reactions [9] can be rearranged as in Joris *et al.* [10]. Errors on the estimation of the final absorbance value (A_∞) can have a drastic effect on the values of the parameters [11–14]. Rules have been established which allow a reliable analysis of progress curves [15]. The integrated equation applies in a wide range of circumstances [16, 17]. An alternative method, that of the half-reaction time, relies on the same principle [15]. It is clear that obtaining reliable values requires collection of data at several substrate concentrations. Moreover, utilization of different final concentrations of enzyme also yields indications on a possible progressive enzyme inactivation during the experiment.

MATERIALS AND METHODS

Equipment

Absorbances were measured with the help of a Beckman DU-8 automatic spectrophotometer, which could be used in kinetic or scan (continuous recording) modes. In the kinetic mode, one to five samples could be processed simultaneously and the maximum number of readings per minute varied from three in the first case to one in the second. In the scan mode, the reading frequency was $2\,sec^{-1}$. The spectrophotometer was linked to an Apple II microcomputer via an RS232C interface. The microcomputer was equipped with 48 kbytes of random access memory, a dual disk drive and an Epson RX80 printer. All the measurements were transmitted to the microcomputer as alphanumeric chains of 15 characters, including (a) the cell number, (b) the wavelength, and (c) the absorbance with four decimals. In the kinetic mode, the sample temperature was also transmitted as a chain of five characters.

Programs for collecting and handling the data, including machine language routines, will be described in an independent paper. Alphanumeric data, temporarily saved in a text file, were transformed into the numeric equivalent before definitive saving as numeric sequential files on the disk. For treatment, the numeric data were first transferred from disk to the memory in the Apple operating system. Then the number of readings for a given sample was displayed and the experimenter was given the possibility to group and average them. This was useful in the scan mode when several hundred readings could be accumulated. Then were (i) the number of points per group and the time difference between two successive averages and (ii) the experimental curve constructed with gross or averaged data displayed successively.

The following steps depended upon the type of experiments.

(a) *Inactivation, reactivation, hysteresis.* Equations (1), (5) and (7) imply the measurement of the rate of substrate utilization at a given time. This value expressed in $\Delta A\,sec^{-1}$ was approximated as described by Knott-Hunziker *et al.* [3] and Frère *et al.* [4]. If the time interval between two successive readings (or averages of readings) was r, then

$$v_{t_1+\frac{r}{2}} = \frac{|A_{t_2} - A_{t_1}|}{r} \quad (12)$$

where $t_2 = t_1 + r$. The curve ln v_t vs t was then displayed.

When necessary, the correction for substrate destruction was performed by computing

$$B = A_{t_1+\frac{r}{2}} = \frac{A_{t_2} + A_{t_1}}{2} \quad (13)$$

and equation (12) was replaced by equation (14)

$$(V_m)_{t_1+\frac{r}{2}} = v_{t_1+\frac{r}{2}} \frac{(K_m,S)_A + |A_\infty - B|}{|A_\infty - B|} \quad (14)$$

(A)

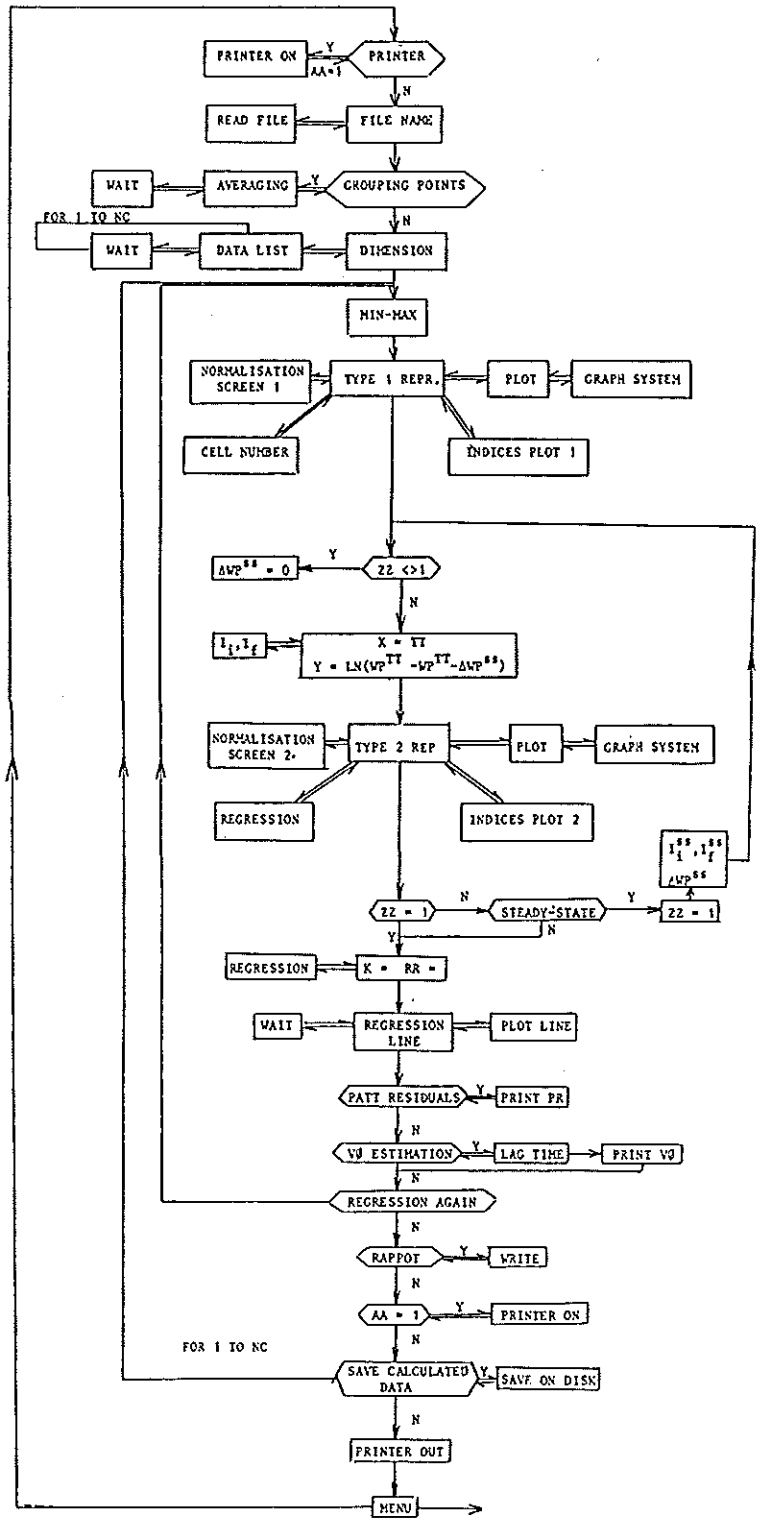
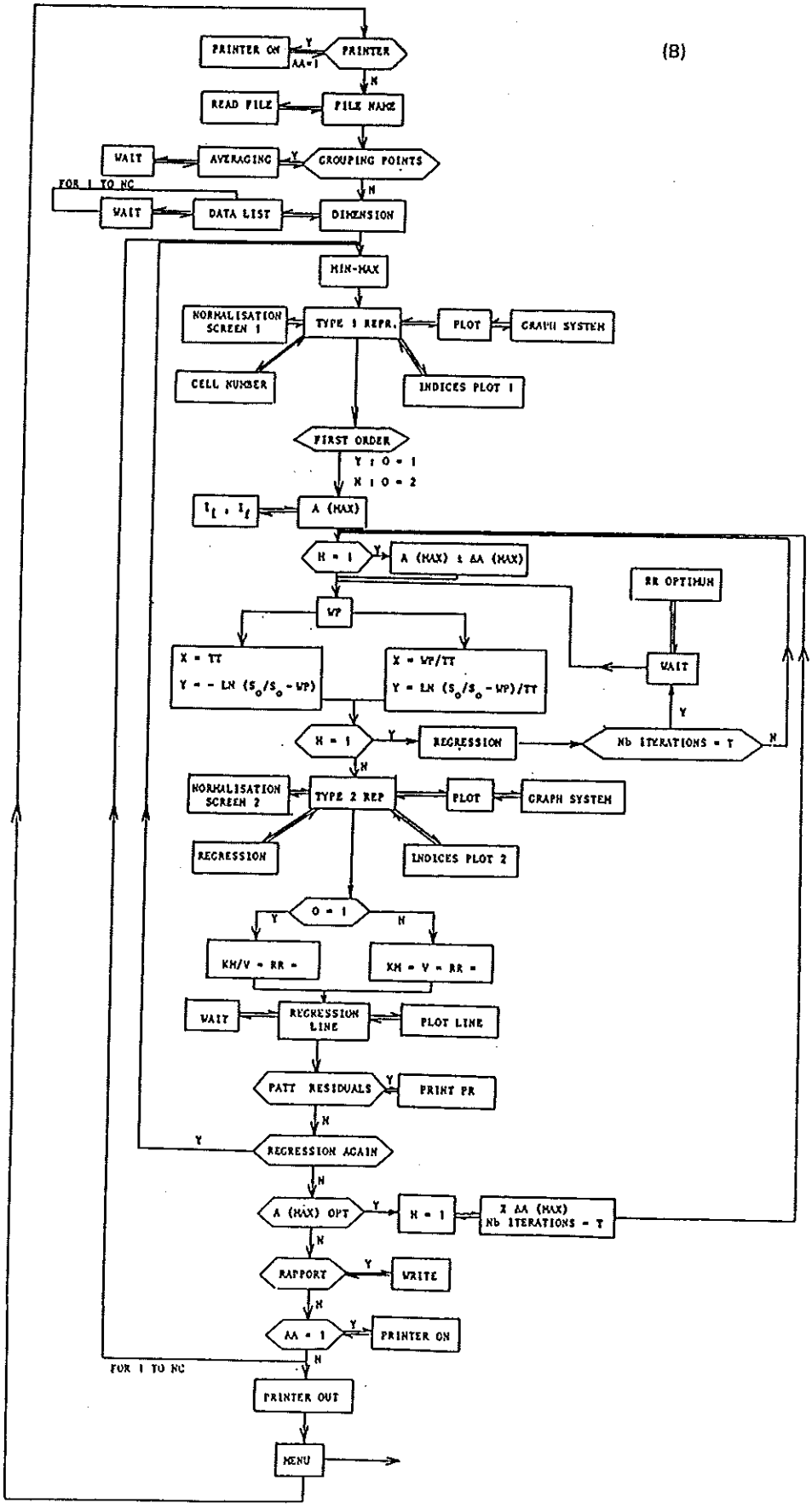


Fig. 1. (A) General scheme of the program for computing inactivation and reactivation rate constants. (B) General scheme of the program for computing K_m and V. The complete program can be obtained from the authors.

(B)


PRINTER ON
PRINTER
Y
AA=1
N
READ FILE
FILE NAME
WAIT
AVERAGING
GROUPING POINTS
FOR I TO NC
WAIT
DATA LIST
DIMENSION
MIN-MAX
NORMALISATION SCREEN 1
TYPE 1 REPR.
PLOT
GRAPH SYSTEM
CELL NUMBER
INDICES PLOT 1
FIRST ORDER
Y : O = 1
N : O = 2
A (MAX)
H = 1
A (MAX) ± ΔA (MAX)
WP
X = TT
Y = - LN (S0/S0 - WP)
X = WP/TT
Y = LN (S0/S0 - WP)/TT
RR OPTIMUM
WAIT
H = 1
REGRESSION
Nb ITERATIONS = T
NORMALISATION SCREEN 2
TYPE 2 REP
PLOT
GRAPH SYSTEM
REGRESSION
INDICES PLOT 2
O = 1
KM/V = RR =
KM = V = RR =
WAIT
REGRESSION LINE
PLOT LINE
PATT RESIDUALS
PRINT PR
REGRESSION AGAIN
A (MAX) OPT
H = 1
± ΔA (MAX)
Nb ITERATIONS = T
RAPPORT
WRITE
AA = 1
PRINTER ON
FOR I TO NC
PRINTER OUT
MENU

The values of V_m and the curve ln $(V_m)_t$ vs t were then successively displayed. In the following paragraphs, v should be replaced by V_m for the cases where the correction was performed.

When no steady-state was reached at the end of the reaction (i.e. inactivation was complete) a least-square regression was performed and the line of best fit displayed with the slope and intercept of the line and the correlation coefficient. It was possible for the experimenter to decide to perform a new regression without the first and/or last few points on which the error could be large.

When a steady-state was reached, the curve ln v_t vs t became parallel to the abscissa for the large values of t. The contribution of the steady-state was removed and a second curve ln $(v_t - v_{ss})$ vs t was displayed. (In reactivation experiments or in hysteresis phenomena when v_f or v_{ss} was larger than v_0, the curve displayed was ln $(v_f - v_t)$ or ln $(v_{ss} - v_t)$ vs t]. The linear regression was performed using those corrected data. Finally, a report was printed with the final numerical values, a graph displaying the data, corrected for the steady-state when necessary, and the best fit line.

The standard deviation on the slopes was computed as described by Mortimer [18]. Several experiments were performed at each inactivator concentration. When the standard deviation on the slope was larger than 2%, the value was rejected. Under these conditions, the average value of k_i was obtained without weighing [19]. Graphs of k_i vs [C] and [C]/k_i vs [C] could be displayed. In the latter case, the parameters k_2 and K or $(k_i)_{lim}$ and K_m were computed by linear regression.

It could also be interesting to extrapolate a value of the initial rate of reporter substrate utilization. Indeed, such data could be used to obtain an independent estimation of K. To do so, the time elapsed between the homogenisation of the sample and the first measurement had to be estimated: values of 10 ± 2 sec or 0.7 ± 0.2 sec were determined when the mixing was performed manually or with the stopped-flow system, respectively. The initial rate was thus extrapolated by taking account of these values for the dead-time. A general scheme of the program is shown in Fig. 1A.

(b) K_m *and* V. As above, readings could be averaged when the experiment had been performed in the scan mode. When the substrate concentration used was below K_m, a line of shallow slope was obtained and it was not easy to determine whether the results were meaningful. An addition was then made to the program which allowed first order kinetics to be tested. Linear regressions were performed on the basis of a simple exponential and the pattern of residuals examined. This allowed the experimenter to decide whether the results could be best interpreted on the basis of a first or intermediate order (see results). The statistical analysis was performed as above.

As outlined above, errors on the value of A_∞ can result in large variations of K_m and V [11–14]. An option was thus added in which an adjustment of that critical value was performed: the initial value of A_∞ was varied by a pre-determined increment and for each new value, the linear regression was performed and a correlation coefficient computed. The value of A_∞ yielding the best correlation was then chosen. The optimization could be performed again using a smaller increment. A general scheme of the program is shown in Fig. 1B.

Enzymes and substrates

β-Lactamase preparations were those described by De Meester *et al.* [20]. The *Streptomyces* R61 extracellular DD-peptidase was purified as described before [21]. Nitrocefin, cephaloridine, β-iodopenicillanic acid and olivanic acid MM13902 were kind gifts from J. B. Ward (Glaxo Research Group, Greenford, Middlesex, U.K.), D. B. Boyd (Eli Lilly, Indianpolis, IN), J. E. G. Kemp (Pfizer Central Research, Sandwich, Kent, U.K.) and G. N. Rolinson (Beecham Pharmaceuticals, Brockham Park, Betchworth, U.K.), respectively. 7-Aminocephalosporanic acid was purchased from Janssen Pharmaceutica, Beerse, Belgium. Hippuryl-DL-phenyllactate was purchased from Sigma (St Louis, MO).

Rapid mixing system

Since the spectrophotometer could transmit two readings per second, it was interesting to develop a cheap and simple mixing system exhibiting a dead time of about 0.5 sec. Two syringes were connected via a T-junction to a flow cell (250 μl, optical path, 1 cm) which was positioned in the cell compartment of the spectrophotometer. All tubing had an inner diameter of 0.8 mm. The exit of the cell was connected to a small suction pump. The flow-rate through the system was 40 ml min^{-1} so that the mixing dead-time was about 0.5 to 1.0 sec. An electrovalve located between the cell and the pump allowed immediate interruption of the flow. Figure 2 shows the result of a series of four shots which were performed over a 40-sec period. The insert shows that the mixing dead time varied from 0.6 to 0.8 sec.

RESULTS

K_m *and* V *from complete time-courses*

In this experiment, it was verified that the values of K_m and V were independent of the final concentration of enzyme (and thus of the total reaction time. The enzyme was the β-lactamase of *B.licheniformis*. The substrate (nitrocefin, 100 μM) was incubated at 30° in 500 μl of 50 mM sodium phosphate pH 7.0 in the presence of 0.02 mg ml^{-1} of bovine serum albumin. The quantity of enzyme was varied from 1.6 to 160 ng. Table 1 shows that the values of K_m and V obtained from the analysis of the complete time-course did not vary with the amount of enzyme utilized and thus with the duration of the experiment. The data were also analysed assuming a first-order disappearance of the substrate. Figure 3 shows the direct recording of the absorbance vs time and the analyses of the curve according to the first-order and the integrated Henri–Michaelis equations. It was clear that the second equation yielded a much better fit. In addition, the analysis according to the first-order equation clearly yielded a non-random pattern of residuals.

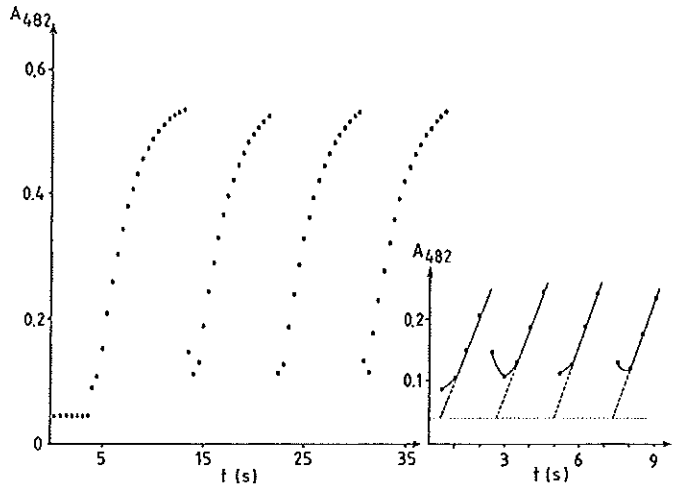


Fig. 2. Determination of the dead time of the stopped-flow system. The experiment was performed in 50 mM sodium phosphate, pH 7.0. The first syringe contained 74 μM nitrocefin, the second 8 μg ml^{-1} of the P99 β-lactamase and 0.1 mg ml^{-1} of bovine serum albumin. The volume of the flow cell was 250 μl (1 cm optical path). The base line was measured by replacing the enzyme solution by buffer in the second syringe. The temperature was 22°. The graph was constructed using the complete print-out of the data by the microcomputer printer. The insert represents an enlargement of the early portions of the curves. Extrapolation of the line drawn through the first points (discontinuous line) to the base line (dotted line) yielded the dead time of the system.

Thermal inactivation of the B. licheniformis *β-lactamase*

The enzyme (20–200 μg, depending upon the temperature) was incubated with 200 μM 7-amino cephalosporanic acid in 500 μl of 50 mM sodium phosphate buffer, pH 7.0. The hydrolysis of the substrate (K_m = 2.2 mM, unpublished) was monitored by recording the decrease of absorbance at 260 nm. The total decrease of absorbance in each experiment did not exceed 0.14 (less than 12% of hydrolysis). The following apparent first-order-rate constants were found: 64°: $k = 2.5 \times 10^{-4}$ sec^{-1}; 66°: $k = 8.7 \times 10^{-4}$ sec^{-1}; 68°: 3.7×10^{-3} sec^{-1}; 70°: 8.6×10^{-3} sec^{-1}. From these data, an approximate activation energy of 600 kJ mole^{-1} was computed for the denaturation of the enzyme. Although this value should be considered with care since the temperature range was quite narrow, it is close to that determined by Arnold and Viswanatha [22] for the thermal inactivation of the closely related β-lactamase I of *B. cereus* (393 kJ mole^{-1}).

Removal of Zn^{2+} *from β-lactamase II of* B. cereus

The reporter substrate was 100 μM cephaloridine (K_m = 400 μM; [23]) and the buffer 10 mM cacodylate pH 6.5 containing 0.5 M NaCl. Hydrolysis of cephaloridine was followed by recording the decrease of absorbance at 260 nm. The enzyme (0.3 μg) was added to a 500-μl solution of substrate containing EDTA concentrations varying from 20 to 500 μM. The inactivation rate constant did not vary significantly with the EDTA concentration. An average value of $7.6 \pm 0.5 \times 10^{-3}$ sec^{-1} (8 experiments) was determined yielding $9.5 \pm 0.8 \times 10^{-3}$ sec^{-1} after correction for the presence of the substrate. This value is about two orders of magnitude lower than that observed by Bicknell *et al.* [24] in a similar experiment performed with the Zn^{2+} β-lactamase of *Pseudomonas maltophilia*. As noted by these authors, the fact that the EDTA concentration did not influence the rate of inactivation suggested that EDTA scavenged the free metal.

Inactivation of the Enterobacter cloacae *P99 β-lactamase by β-iodopenicillanate*

This inactivation occurs according to a simple, unbranched pathway (model 3 with $k_3 = 0$; ref. 10). In a previous study, a maximum inactivator con-

Table 1. Estimations of K_m and V with various amounts of enzyme

Amount of enzyme (ng)	Reaction time* (min)	K_m (μM)	V [μmoles min^{-1} (mg E)$^{-1}$]
1.6	23	43 ± 2	3940 ± 80
3.2	12	46 ± 2	4020 ± 100
6.4	6	44 ± 2	3810 ± 100
16	2.5	43 ± 2	3850 ± 110
32	1.0	40 ± 1	3525 ± 50
64	0.5	40 ± 2	3360 ± 70
160	0.25	46 ± 3	3990 ± 200

* Interval during which the data utilized for the linear regression were collected.

Table 2. Interaction between β-iodopenicillanate (C) and the β-lactamase of *Enterobacter cloacae* P99

Final [C] (mM)	$k_i \times 10^3$ (sec^{-1})	$k_i/[C]$ (M^{-1} sec^{-1})
0.5	10 ± 0.7	20 ± 1.4
1.0	21.5 ± 0.8	21.5 ± 0.8
1.5	31.6 ± 2.0	21.0 ± 1.2
2.0	41.5 ± 3.0	20.8 ± 1.4
2.5	52.2 ± 1.0	20.9 ± 0.4
5	118 ± 3.0	23.6 ± 0.7
7.5	182 ± 8.0	24.2 ± 1.0
14.7	390 ± 40	26.5 ± 2.7

The data were obtained with the simple stopped-flow set-up. Six distinct experiments were performed at each inactivator concentration.

centration of 0.33 mM was used. Under these conditions, k_i remained linear vs [C] and a value of 128 M^{-1} sec^{-1} was computed for k_2/K. Using our simple stopped-flow system, we performed similar experiments and varied the β-iodopenicillanate concentration from 0.5 to 14.7 mM. The enzyme solution (1.2 μg/ml of 50 mM sodium phosphate, pH 7.0 containing 0.1 mg ml^{-1} of bovine serum albumin) was mixed with an equal volume of 500 μM nitrocefin (K_m = 56 μM) in the same buffer. The nitrocefin solution also contained the inactivator. Six experiments were performed at each concentration. Table 2 shows that the observed k_i values remained proportional to the β-iodopenicillanate concentration. The measured half reaction times varied from 69 (k_i = 0.01 sec^{-1}) to 1.8 sec (k_i = 0.39 sec^{-1}). It can be seen that the agreement was excellent for the concentrations below 5 mM and good with those above 5 mM. After correction for the presence of S, a value of 115 M^{-1} sec^{-1} was found for k_2/K, in good agreement with that obtained previously.

Inactivation of the Citrobacter freundii *β-lactamase by β-iodopenicillanate*

The inactivation was studied in 50 mM sodium phosphate buffer pH 7.0 using 100 μM nitrocefin (K_m = 35 μM; ref. 20) as a reporter substrate. The concentration of β-iodopenicillanate varied from 7.5 to 385 μM and the quantity of enzyme from 3 to 10 ng. At β-iodopenicillanate concentrations below 50 μM, the value of k_i remained proportional to [C]. At higher concentrations, a curve was obtained from which the individual values of k_2 and K could be computed after correction for the presence of the reporter substrate. From these data, the following values were calculated: k_2/K = 375 ± 10 M^{-1} sec^{-1} (at low values of [C]), k_2 = 64 ± 2 × 10^{-3} sec^{-1} and K = 153 ± 3 μM (which yields k_2/K = 420 ± 20 M^{-1} sec^{-1}).

Utilization of a large proportion of substrate: inactivation of the Streptomyces *R61 DD-peptidase by cephaloridine and olivanic acid*

Recently, it was found that hippuryl-DL-phenyllactate was a substrate for the penicillin-sensitive DD-peptidase produced by *Streptomyces* R61 [25]. This observation supplied the first substrate whose hydrolysis by that enzyme could be directly and continuously monitored. A complete study of the properties of the new substrate will be presented elsewhere. For our present purpose, it is sufficient to say that only 50% of the racemic (probably the D isomer) was hydrolysed, that the K_m was 3 mM and that $(\Delta\varepsilon)_{260}$ was 500 M^{-1} cm^{-1}.

The enzyme (10 μg, i.e. 0.26 nmole) was incubated at 37° in the presence of 1 mM hippuryl-DL-phenyllactate (i.e. 0.5 mM of the D isomer) in 500 μl of 10 mM Na phosphate buffer pH 7.0 containing cephaloridine concentrations varying from 0 to 50 μM. Absorbance readings (λ = 260 nm) were performed every 66 sec. In the absence of inactivator, 90% of the substrate was destroyed within 25 min. In the presence of the inactivator, up to 60% of the substrate was hydrolysed during the same period of time. Results presented in Table 3 appeared to be quite good (about 10% of standard deviation) and since the values of k_i remained proportional to [C], a value of 115 ± 18 M^{-1} sec^{-1} was computed for the k_2/K ratio (model 1).

With olivanic acid, identical conditions were used with inactivator concentrations ranging between 0 and 20 μM (Table 4). Using those data, a plot of $[C]/k_i$ vs [C] (model 1, equation (4) after linearization) yielded values of 2.3 μM and 1.32 × 10^{-3} sec^{-1} for K and k_2, respectively. It is interesting to note that

Table 4. Inactivation of the R61 DD-peptidase by olivanic acid

[Olivanic acid] (μM)	$k_i \times 10^3$ (sec^{-1})	$[C]/k_i$ (M^{-1} sec^{-1})
4	0.73 ± 0.07	5.5 ± 0.55 × 10^{-3}
8	0.81 ± 0.06	9.9 ± 0.73 × 10^{-3}
12	0.97 ± 0.07	1.24 ± 0.09 × 10^{-2}
16	1.07 ± 0.10	1.50 ± 0.14 × 10^{-2}
20	1.10 ± 0.12	1.82 ± 0.20 × 10^{-2}

Table 3. Inactivation of the R61 DD-peptidase by cephaloridine

[Cephaloridine] (μM)	$k_i \times 10^3$ (sec^{-1})	$k_i/[C]$ (M^{-1} sec^{-1})
10	1.25 ± 0.05	125 ± 5
20	2.1 ± 0.28	105 ± 14
30	2.6 ± 0.25	87 ± 9
40	3.7 ± 0.34	93 ± 9
50	4.5 ± 0.49	90 ± 10

The value of A_∞ was corrected for the contribution of cephaloridine. Three experiments were performed in each case.

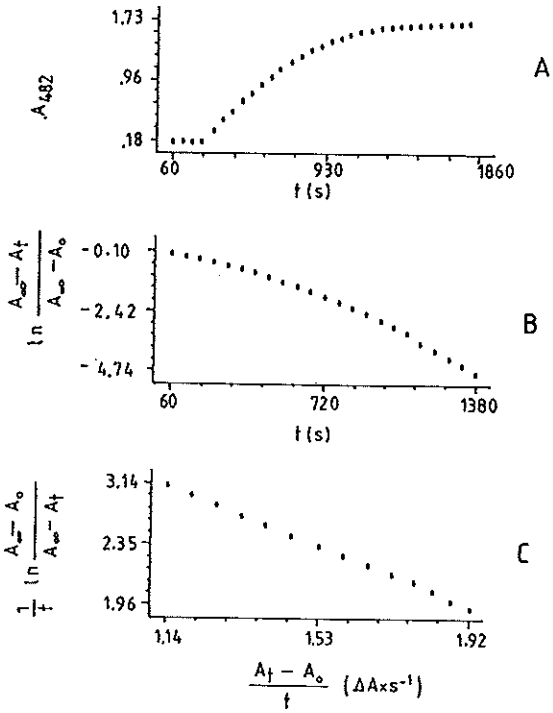


Fig. 3. Determination of the kinetic parameters for the hydrolysis of nitrocefin by the *Bacillus licheniformis* β-lactamase. The quantity of enzyme was 1.6 ng (for other conditions, see text). (A) Direct recording of the absorbance variation (1 reading per min). The first four points represent the absorbance of intact nitrocefin. (B) First-order analysis: plot of $\ln (A_\infty - A_t)/(A_\infty - A_0)$ vs t performed on the first 23 significant readings. (C) Analysis according to the integrated Henri–Michaelis equation: plot of $1/t$ [$\ln (A_\infty - A_0)/(A_\infty - A_t)$] vs $(A_t - A_0)/t$ (15 first significant readings). The following values were found (in ΔA for K_m and $\Delta A\,\text{sec}^{-1}$ for V): $K = 0.61$ and $V = 3.1 \times 10^{-3}$.

in those experiments, the total absorbance variation in the absence of inactivator was only 0.25.

Enzyme hysteresis: interaction between cephaloridine and the β-lactamase of Staphylococcus aureus

Carrey *et al.* [26] have shown that cephaloridine induced reversible inactivation of the *S. aureus* β-lactamase but did not measure accurately the rate of the phenomenon. To 500 μl of 100 μM cephaloridine in 50 mM sodium phosphate pH 7.0, 40 μg of enzyme were added and the decrease of the absorbance was followed at 260 nm. Figure 4 shows: the decrease of absorbance as recorded by the spectrophotometer; the decrease of absorbance after grouping and averaging of the readings by the microcomputer (10 points per group, i.e. one averaged reading/5 sec); the gross graph ln v vs t: the steady-state can be clearly visualized after 30 sec; the corrected graph after subtraction of the contribution of the steady-state.

The slope of the line was $0.14 \pm 0.005\,\text{sec}^{-1}$. At a cephaloridine concentration of 50 μM, the value of the inactivation rate constant was $0.12 \pm 0.005\,\text{sec}^{-1}$.

The reactivation was studied by pre-incubating the enzyme in 100 μM cephaloridine in the same conditions as above. A sample was withdrawn and rapidly diluted 500-fold in 100 μM nitrocefin ($K_m \cong 2.5$ μM) in 50 mM sodium phosphate, pH 7.0. The hydrolysis of nitrocefin was followed at 482 nm. Figure 5 shows the variation of the reaction rate with time and the graph after correction for the steady-state. The slope of the line was $6.3 \pm 10^{-3}\,\text{sec}^{-1}$.

The hydrolysis of cephaloridine by the *S. aureus* β-lactamase has also been studied by Faraci and Pratt [27], who have shown that the substrate-induced inactivation was due to the accumulation of a slowly hydrolysed enzyme-substrate adduct, formed by

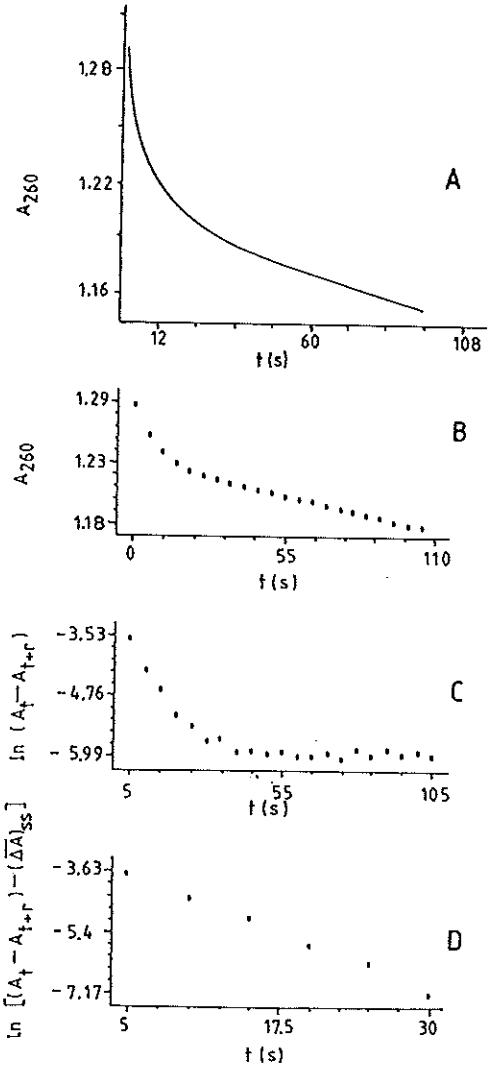


Fig. 4. Substrate-induced inactivation of the *S. aureus* β-lactamase by cephaloridine. For experimental conditions, see text. (A) Direct recording of the absorbance variation. (B) Microcomputer print-out of the absorbance variation after averaging of the readings (1 averaged value per 5 sec). (C) Gross values of ln $(A_t - A_{t+r})$ vs time ($r = 5$ sec). The steady-state was clearly established after 35 sec. (D) First-order graph of the inactivation after correction for the steady-state. $(\overline{\Delta A})_{ss}$, i.e. the average variation of absorbance per 5 sec after establishment of the steady-state was computed using readings obtained between 35 and 105 sec. The graph shows the values of ln $[(A_t - A_{t+r}) - (\overline{\Delta A})_{ss}]$ vs t computed using readings between 5 and 30 sec.

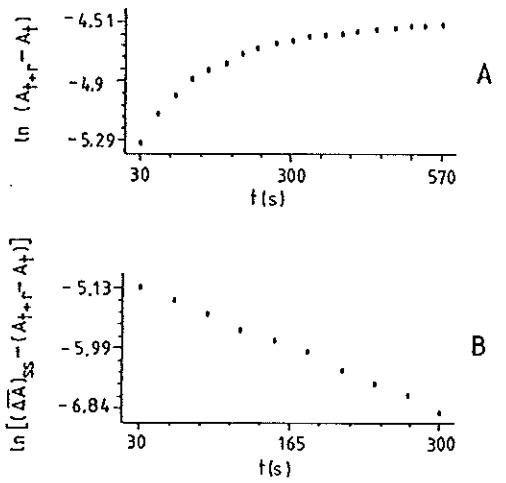


Fig. 5. Reactivation of the cephaloridine-inactivated β-lactamase of *S. aureus* by dilution in a nitrocefin solution. For conditions, see text. (A) Gross values of ln $(A_{t+t'} - A_t)$ vs time. The absorbance values were averages of 60 readings (1 averaged value per 30 sec). (B) First-order graph of the reactivation after correction for the steady-state. $(\overline{\Delta A})_{ss}$ was computed using readings obtained between 450 and 570 sec. The graph shows the values of ln $[(\overline{\Delta A})_{ss} - (A_{t+t'} - A_t)]$ vs time.

expulsion of the substrate 3′ substituent at the level of the acyl enzyme. The value obtained here for the inactivation rate constant (0.12–0.14 sec^{-1}) is in fair agreement with that obtained by those authors (0.055 sec^{-1}). However, we observed a 6-fold larger reactivation rate (6×10^{-3} vs 1×10^{-3} sec^{-1}). Although this might be due to slightly different experimental conditions, that discrepancy will deserve further investigation.

DISCUSSION

Cha [2] and Duggleby *et al.* [1] have used the "reporter substrate" method and measured apparent first-order inactivation rate-constants of 4×10^{-3} and 3×10^{-2} sec^{-1}, respectively. The direct transmission of absorbance readings to a microcomputer resulted in an optimization of the method: constants up to 0.3 sec^{-1} were measured and the collection of a large number of readings (2 sec^{-1}) greatly increased the accuracy of the calculated parameters. To analyse the results, we simply derived an approximate value for $v = \Delta P/\Delta t$ [2–4, 28]. Cha [2, 28] estimated the local rate of the reaction by measuring the slope of the tangent to the curve $[P]$ vs t. As discussed by Orsi and Tipton [29], this can be difficult in many cases. To avoid that problem, Duggleby *et al.* [1] directly analysed the curve $[P]$ vs t with the help of an integrated equation and of a non-linear regression program. In this case, however, the difficulty was to obtain an accurate estimation of the starting time of the reaction. We found that our approximation yielded excellent results and clearly circumvented the difficulties inherent to the two other procedures.

Although we did not examine all the possible cases analysed by Cha [2, 28], it is clear that the method can easily be used to study the dependence of the first-order-rate constants on reporter substrate or inactivator concentrations. In this paper, we studied inactivations by high temperature, by removal of an essential cation and by reaction with mechanism-based inactivators. In all cases, the results could be estimated as excellent. The study of substrate-induced inactivation could also be easily and rapidly performed, as the data in Fig. 4 clearly demonstrated. The correction for the contribution of the steady-state utilization of the substrate was made particularly easy by the direct interaction with the microcomputer. In the measurement of the usual kinetic parameters (K_m and V) by the analysis of the complete time-course of the reaction, short experiments yielded excellent results: even if the reaction was completed within 2–3 min, a large number of significant readings could nevertheless be accumulated (120 per min), the final A_∞ value was reached rapidly and could thus be measured accurately. In the cases where the product might be unstable, it should also be interesting to complete the reaction before such a side-reaction can become significant. When a large proportion of the reporter substrate disappeared during the experiment, a simple correction was introduced which accounted for the decrease of the rate of substrate utilization due to that factor and thus allowed the inactivation phenomenon to be singled out. It is clear that the inexpensive interfacing of a spectrophotometer to a microcomputer, not only greatly facilitated the computation of the kinetic constants, but also extended the range of rate constants values which could be measured accurately by a factor larger than 10.

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