

6 – EXPERIMENTAL METHODS TO STUDY ENZYMATIC REACTIONS

The study of enzymatic reaction mechanisms involves researching the elementary steps that intervene during the course of a reaction, and determining the rates of appearance and disappearance of reaction intermediates, as well as their relative energetic levels. The majority of intermediates that appear have very short lifetimes and are not generally detectable by classical techniques. Increasingly rapid methods have been progressively introduced for studying enzyme reactions as is the case more generally for all kinetic studies. The discovery of techniques that discern ever lower time constants marked great progress in the approaches taken to study enzymatic reaction mechanisms.

Kinetic studies aim to measure the progress of a reaction over time. As far as the time-scale is concerned, frequently used methods detect times greater than or equal to a second. Between 1 s and 10^{-10} s lies a zone of fast reactions still accessible to kinetic experimentation. Times of less than 10^{-10} s (between 10^{-10} and 10^{-15} s) represent the spectroscopic range, for example, the time taken for an electron to pass from the ground state to the excited state.

Routine methods	Fast methods	Spectroscopic zone
1	10^{-3}	10^{-10}
Seconds		

Kinetic studies of enzyme reactions consist of various aspects. First of all, there is the global reaction for which kinetic parameters are determined, on a time-scale of the order of one second or a minute. Among the methods often employed to follow enzyme reactions, it is useful to distinguish between discontinuous methods (or “point by point”), continuous methods and coupled enzyme assays.

The detection and study of elementary steps require the use of fast kinetic techniques. Two types of method are at our disposal: flow and relaxation, which are based on very different principles. Flow methods permit study at the pre-steady or non-steady state. Relaxation methods apply to reactions at equilibrium or at the steady state.

6.1. DISCONTINUOUS METHODS

Discontinuous methods consist of removing aliquots from the reaction mixture at given time-points and then stopping their reactions, either by changing the pH, or by adding a suitable reagent or an inhibitor specific to the enzyme. Dilute acids are often used, in particular trichloroacetic acid, which not only stop the reaction by lowering the pH, but also precipitate the enzyme. So the aliquot simply requires centrifugation in order to recover the supernatant in which the reaction products remain plus any unused substrate, except if this is a protein. In the sample, we either measure the product that has appeared or the unconsumed substrate. The principal methods of quantification are essentially chemical methods or those involving radioactive isotopes when it is possible to separate the product from the substrate. These “point by point” methods present several disadvantages, however. Sometimes it is difficult to estimate precisely the initial rates; besides, such methods are often laborious. In general, continuous methods are preferable whenever possible.

Let us cite the example of the method used to follow the activity of aspartate trans-carbamylase, an enzyme that catalyses the carbamylation of the amino group of aspartate according to the reaction:



Using ion exchange chromatography the radioactive aspartate [^{14}C], which has not reacted, and the reaction product, carbamyl [^{14}C]-aspartate, can be separated. Firstly, the reaction is stopped by adding acetic acid, which at the same time ensures the protonation of the aspartate's amino group. The reaction mix is then fractionated on a DOWEX 50 column, which is an anionic resin that retains any aspartate that has not reacted and allows the carbamyl aspartate to flow through as it is now devoid of positive charge. This is then directly collected in vials used specially for measuring radioactivity. In practice, the reaction in the aliquot is stopped at the moment of acetic acid addition before being loaded onto the DOWEX column. Thus, a series of samples can be taken, each spaced apart by time intervals of only a few seconds.

6.2. CONTINUOUS METHODS

In continuous methods, the reaction progress is recorded directly as a function of time. If the substrate or the product has characteristic absorption spectra, each shifted relative to the other, the detection can be achieved by **absorbance spectrophotometry** at the wavelength where the difference in absorbance between the substrate and product is the greatest. Figure 6.1 opposite shows the measurement at 373 nm of orthonitrophenol liberated during the hydrolysis of o-nitrophenyl galactoside by β -galactosidase from *E. coli*. The continuous trace obtained is linear for a while and its precision allows v_0 to be well determined.

a peptide bond at neutral pH causes a completely deprotonated COO^- group to appear and an amino group such as:



with the dissociation constant: $K_{\text{diss}} = \frac{(\text{NH}_2)(\text{H}^+)}{(\text{NH}_3^+)} = \frac{\alpha(\text{H}^+)}{(1 - \alpha)}$

giving:
$$\alpha = \frac{1}{\frac{\text{H}^+}{K_{\text{diss}}} + 1}$$

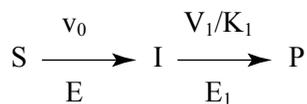
If the pK of the amino group is much lower than the measurement pH, $\alpha \rightarrow 1$; if the pK is equal to the measurement pH, only half of the amine groups liberated by hydrolysis are titrated. Therefore, firstly we have to determine α by titrating at different pH values. Once α is known, the measurement is quantifiable.

Continuous methods are particularly well adapted to the determination of kinetic parameters from graphical plots from the integrated MICHAELIS equation.

6.3. COUPLED ENZYME ASSAYS

When direct continuous methods are not available to record the appearance of product during an enzyme reaction, we can in certain cases and **working in suitable conditions** use a helpful trick. If the reaction product is itself a substrate for an enzyme that catalyses a reaction one of whose products is readily measurable by continuous methods, this second “**coupling**” enzyme can be used to follow the first reaction. We have therefore two consecutive reactions, the kinetics of which have been the subject of theoretical study (e.g. BERGMAYER, 1953; GUTFREUND, 1965; MC LURE, 1969; BARWELL & HESS, 1970; HART, 1970; GOLDMAN & KATCHALSKI, 1971; EASTERBY, 1973; STORER & CORNISH-BOWDEN, 1974).

The simplest example of enzyme coupling (EASTERBY, 1973) can be represented by the following scheme:



S is the substrate of the enzyme E that we wish to study; I, the reaction product, is the substrate for the coupled enzyme E_1 ; it is therefore a simple intermediate of two consecutive reactions. P is the final product, which we measure by a continuous method. The rate of the enzyme reaction under study is given by v_0 ; V_1 and K_1 are respectively the maximal rate and the MICHAELIS constant of the coupling enzyme. **For the enzyme assays to be valid, it is important that the second reac-**

tion is not rate-limiting, therefore V_1 must be much higher than v_0 . The concentration of the intermediate I must be very low and much lower than K_1 . Thus, the appearance rate of P is given by the relation:

$$\frac{dP}{dt} = \frac{V_1}{K_1}(I)$$

in which V_1/K_1 is a *first-order rate constant*.

The reaction rate is given by the following expression:

$$\frac{dI}{dt} + \frac{V_1}{K_1}(I) = v_0$$

with the following conditions: $(I) = 0$ and $(P) = 0$ at time $t = 0$, giving:

$$(I) = v_0 \left(1 - e^{-t/\tau_1}\right)$$

$$(P) = v_0 \left(t + \tau_1 e^{-t/\tau_1} - \tau_1\right)$$

with:

$$\tau_1 = K_1/V_1$$

At steady state:

$$(I)_{ss} = v_0 \tau_1 = v_0 \frac{K_1}{V_1}$$

Figure 6.2 shows the effect of the ratio V_1/K_1 on the time needed to reach the steady state.

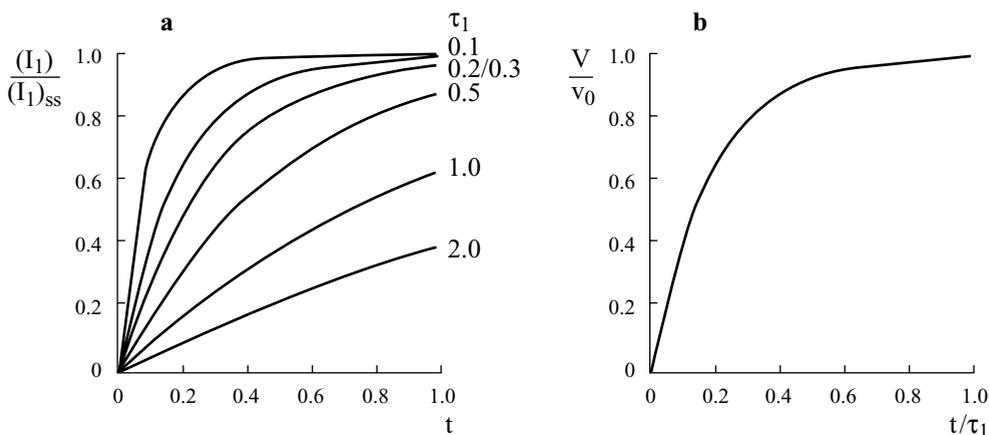


Fig. 6.2 Effect of the transient time $\tau_1 = K_1/V_1$ on the time needed to reach the steady state

(a) relative concentration of the intermediate $(I)/(I)_{ss}$ – (b) rate normalised with respect to v_0 as a function of t/τ_1 . (Reprinted from *Biochim. Biophys. Acta*, **293**, EASTERBY J.S., Coupled enzyme assays: a general expression for the transient, 552. © (1973) with permission from Elsevier)

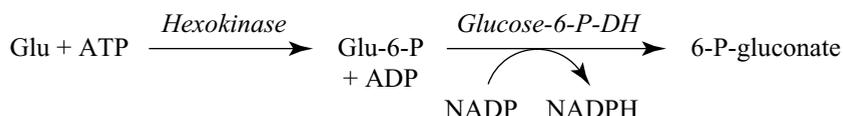
When time becomes sufficiently long, the exponential term becomes negligible in the expression for P, which takes on a simplified form:

$$(P) \rightarrow \infty = v_0(t - \tau_1)$$

giving:
$$\frac{dP}{dt} = v_0$$

The appearance rate of P represents well the rate of the reaction catalysed by the first enzyme.

By way of example, let us consider the coupled enzyme assays used to follow the reaction catalysed by yeast hexokinase. It utilises glucose-6-phosphate dehydrogenase as the coupling enzyme, which requires NADP^+ . We have the following chain of reactions:



The change in absorbance due to the formation of NADPH is recorded at 340 nm. Figure 6.3 shows product appearance over time. The pre-steady state phase appears very clearly; it is followed by the steady state and corresponds to the linear part of the graph. This straight line may be extrapolated to a value equal to τ_1 on the horizontal axis and on the vertical axis, equal to I_{ss} or $v_0\tau_1$, from the expression for the appearance rate of P.

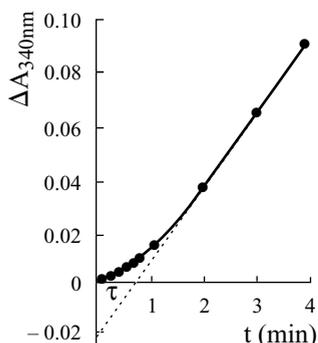
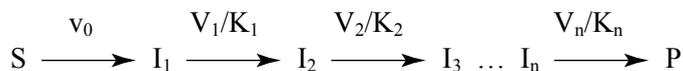


Fig. 6.3 Enzyme assay with hexokinase
Kinetics of NADH appearance over time

It is sometimes necessary to use several coupled reactions. The kinetics of several consecutive coupled reactions was developed by BARWELL and HESS (1970) and by EASTERBY (1973). The reaction sequence can be written:



with $V_1, V_2 \dots V_n$ and $K_1, K_2 \dots K_n$ being, respectively, the values for the maximal rates and the MICHAELIS constants of these consecutive enzymatic reactions. **It is important to work in conditions in which every coupled reaction is first order.**

The system can be described by the following series of differential equations:

$$\frac{dI_i}{dt} + \frac{I_i}{i} = \frac{I_{i-1}}{i-1} \quad i = 2, 3 \dots n$$

with the initial conditions $I_i = 0$ at $t = 0$.

The solution to this equation for I_1 has already been given; for the successive intermediates, the solution is obtained by progressive substitution into the equation. The general solution is:

$$I_i = v_0 \tau_i \left(1 - \sum_{j=1}^i A_j e^{-t/\tau_j} \right) \quad i = 1, 2, 3 \dots n$$

with:

$$A_j = \tau_j^{i-1} \prod_{k=j}^i \frac{1}{\tau_i - \tau_k}$$

The concentration of the intermediate I_i at steady state is given by the relation:

$$I_{i,ss} = v_0 - \tau_i \quad \text{with } \tau_i = K_i/V_i$$

The final product concentration measured is:

$$\frac{dP}{dt} = \frac{V_n}{K_n} I_n$$

Taking into account the initial conditions: $P = 0$ at $t = 0$, we have:

$$P = v_0 \left(t + \sum_{j=1}^n C_j e^{-t/\tau_j} - \sum_{i=1}^n \tau_i \right)$$

with:

$$C_i = \tau_i^k \prod_{j=1}^n \frac{1}{\tau_i - \tau_j} \quad j \neq i$$

At steady state:

$$P_{t \rightarrow \infty} = v_0 \left(t - \sum_{i=1}^n \tau_i \right)$$

the corresponding curve, analogous to that in the preceding figure, intersects the horizontal axis at the point:

$$t = \sum_{i=1}^n \tau_i$$

and the vertical axis at:

$$-v_0 = \sum_{i=1}^n \tau_i$$

which is the sum (bearing a minus sign) of the concentrations of intermediate species at steady state.

Thus, when two coupled enzymes are used, the concentration of P is given by the expression:

$$P = v_0 \left(t + \frac{\tau_1^2}{\tau_1 + \tau_2} e^{-t/\tau_1} + \frac{\tau_2^2}{\tau_1 + \tau_2} e^{-t/\tau_2} - \tau_1 - \tau_2 \right)$$

For example, the coupled enzymatic assay used for yeast hexokinase is not applicable to animal hexokinases because an accumulation of 6-phosphogluconate leads

The constant of integration, C , is determined by the initial conditions $v_2 = 0$ at $t = 0$. Thus:

$$t = \frac{V_2 K_2}{(V_2 - v_1)^2} \ln \frac{v_1(V_2 - v_2)}{V_2(v_1 - v_2)} - \frac{K_2 v_2}{(V_2 - v_2)(V_2 - v_1)} \quad [8]$$

Equation [2] shows that at steady state $v_1 = v_2$ since $d(I)/dt = 0$. The appearance rate of P measured experimentally is equal to the appearance rate of (I) . Figure 6.4 shows the correspondence between the theoretical and experimental curves for the reaction kinetics with glucokinase as followed by assay and using glucose-6-phosphate dehydrogenase as the coupling enzyme, for different values of its concentration. The difficulty resides in evaluating the rate's limit. It is therefore preferable to calculate the time necessary for v_2 to reach 0.99 v_1 rather than relying on the apparent linearity of the experimental curves. The calculation method thus involves at least a partial estimation of v_1 .

With a first-order approximation for the coupling enzyme, the kinetic constants V_2 and K_2 are not separated, but the ratio V_2/K_2 represents the first-order constant. Also, it is not possible to separate the factors that alter each of the apparent values of these constants. On the contrary, in the general rate expression represented by equation [8], it is possible to separate them; the time needed for v_2 to reach a given percentage of v_1 is proportional to K_2 . Consequently, the presence of a competitive inhibitor of the coupling enzyme diminishes the method's efficiency by delaying the time necessary to reach the steady state. Thus, during the assay of glucokinase by glucose-6-phosphate dehydrogenase, the latency period in the presence of ATP, a competitive inhibitor of the coupling enzyme, is notably extended.

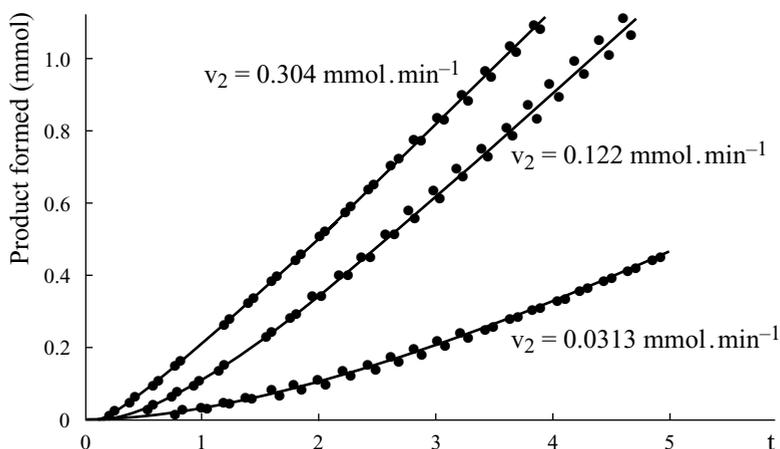


Fig. 6.4 Comparison of experimental and theoretical curves for the enzyme assay of glucokinase with glucose-6-phosphate dehydrogenase as the coupling enzyme

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A coupled enzyme assay requires sufficient coupling enzyme, but this must not exceed the necessary concentration, not merely in the interests of economy, rather because excessively high concentrations of coupling enzyme are sometimes the source of artefacts. For example, glucose-6-phosphate dehydrogenase can use glucose instead of glucose-6-phosphate, however, this parasitic reaction is negligible at the coupling enzyme concentrations necessary for the assay. The use of a coupled enzyme assay applies equally when the product formed is either unstable or difficult to detect. Now, carbamyl phosphate synthetase catalyses the formation of carbamyl phosphate from glutamine, bicarbonate and ATP. Being an acid anhydride, carbamyl phosphate is unstable. We must therefore use the coupling enzyme, aspartate transcarbamylase, which, in the presence of saturating aspartate concentrations, immediately converts the carbamyl phosphate into stable carbamyl aspartate that is assayed by the method described above.

6.4. FLOW METHODS

Flow methods are applicable to the study of enzymatic reactions **in pre-steady state conditions or more generally at the steady state**. They require an experimental device enabling the determinations to be carried out in very short time periods, of the order of a few milliseconds. The problems encountered in fast kinetic studies are the rapidity of mixing the reactants and the detection of modifications appearing in the mixture. The principle of the method consists of quickly injecting the two reactants, e.g. the enzyme and substrate, into a mixing chamber or an observation tube.

6.4.1. GENERAL PRINCIPLE OF FLOW METHODS

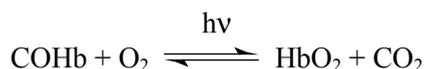
Whatever the flow type, the apparatus contains a device in which the solutions of the two reactants R_1 and R_2 are introduced by forced mixing at the entrance of an observation tube. Beyond this point, when the two solutions are instantaneously combined to homogeneity, the destiny of the reaction mixture differs depending on whether or not there is a continuous flow.

6.4.2. CONTINUOUS-FLOW APPARATUS

In a continuous-flow apparatus (Fig. 6.5 opposite), the reaction mix progresses towards the interior of the observation tube at a constant rate v , such that in any section of the tube S_d , situated at a distance d from the point of mixing, a time t (equal to the ratio d/v) can be unequivocally associated to a well-defined point in the reaction. When the mixture travels a certain length of the tube at a distance d , it is in the same state that it would be in at time t if it remained immobile. While the rate, v , remains constant, a steady distribution of the reactants, products and possibly the

intermediates participating in the reaction studied is established all along the observation tube. If there is a physical property known to correspond to the changes in concentrations of the chemical species present in the reaction medium, for example spectral properties, then by placing an appropriate detector along the length of the observation tube we can obtain an electrical response that is a function of the distance d . Then it suffices to convert these experimental data into concentration and time, respectively.

The first apparatus of this type was constructed in 1922 by HARTRIDGE and ROUGHTON. It comprised an observation tube 5 mm in diameter and consumed 3–6 l of reactant per experiment! It was first used to study the combination of oxygen with carboxyhaemoglobin under the influence of flash photolysis:



The authors succeeded in measuring the rate of the reverse reaction which was carried out in the dark. This reaction is fast and could not be followed at temperatures higher than 15°C. With the device it was possible to measure at 37°C the rate of combination of oxygen with haemoglobin, which occurs in 0.01 s.

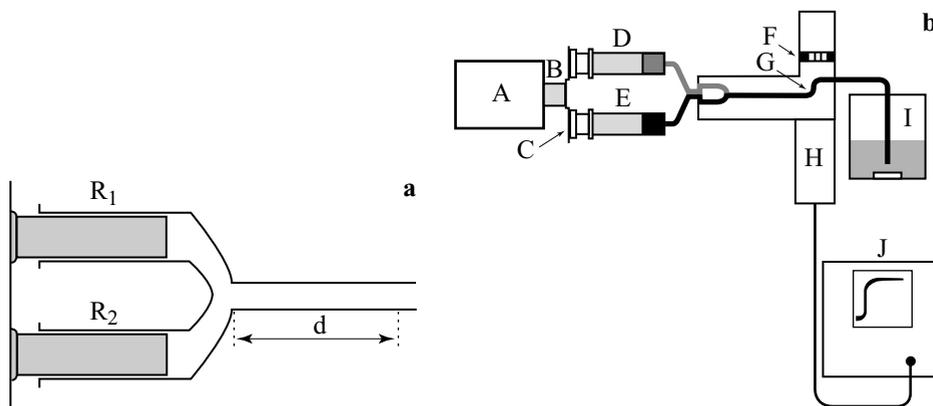


Fig. 6.5 Continuous-flow apparatus

(a) scheme showing the principle of the method – (b) layout of a continuous-flow apparatus. A: constant rate motor; B: drive piston; C: end-stops for the syringe plungers; D and E: syringes; F: filter or monochromator; G: observation point; H: photomultiplier; I: mixing chamber; J: recorder

The advantage with these continuous-flow apparatus is that systems for the detection of relatively long global response times can be adapted to them. This imposes a minimum duration with each stoppage of the detector at a given point in the observation tube and affects the total flow time and consequently the consumption of the reactants. However, in 1935 ROUGHTON and MILLIKAN constructed an improved apparatus consuming less reactant.

A continuous-flow apparatus with a resolution time of 100 μs was developed by REGENFUSS and co-workers in 1985. The system consisted of mixing two solutions by passing them through a tiny aperture of 10 μm . The resulting turbulence provoked a rapid mixing by diffusion in the tiny whirlpools created.

6.4.3. STOPPED-FLOW APPARATUS

The stopped-flow system is designed to provide fast and completely homogenous mixing of two reactants and this mixture, as quickly as possible, must fill the observation tube that is placed in the path of the detector. In this type of apparatus, the detector must be positioned as closely as possible to the point of mixing. Its response time as well as that of its associated circuits must also be as short as possible. Stopping the flow may be passive or induced. It was passive in the first apparatus used by CHANCE when, after the impulse given to the pistons, the system came to a halt by inertia. Thus, with these instruments the flow rate reaches a maximum value and then decreases, tending to zero in a time-interval that can be of the order of the preliminary period of flow. In the apparatus developed by GIBSON (Fig. 6.6), the stoppage is induced and this time it is infinitesimally small. The flow is blocked abruptly at the moment that the rate reaches its maximum. After having passed through the observation tube, the reaction mixture is ejected into a third syringe whose piston may be halted by an adjustable stop.

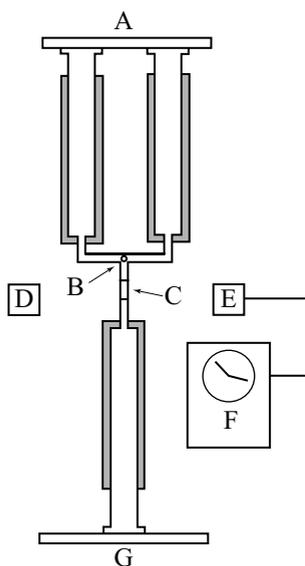


Fig. 6.6 Stopped-flow apparatus based on the GIBSON principle

*A: drive pistons in the mixing syringes;
B: mixing; C: observation chamber;
D: monochromatic light source;
E: detector; F: recorder;
G: stop block for the stopper syringe.*

The resolution time of these apparatus is generally of the order of several milliseconds (4–5 ms); in the best cases, they have resolution times of one millisecond and even half millisecond times have been reported. For this, a certain number of difficulties, which we shall soon address, have to be overcome. The detection system

most often used with stopped-flow equipment is absorption or emission spectrophotometry. It must be sensitive enough to permit detection of minor variations.

6.4.4. QUENCHED-FLOW APPARATUS

When no detection method is readily usable for measuring reaction rates, we can make use of fast mixing methods and then stop the reaction. The function of these multi-mixing or quenched-flow apparatus, whose diagrams are given in Fig. 6.7, relies on the following principles. The reactants are mixed quickly by means of a two-syringe system and left to incubate for the desired time; next, the reaction mixture is again rapidly mixed with a reagent to stop the reaction and the amount of product formed can be determined. The time during which the reaction is left to evolve is fixed according to the length of the incubation tube between the first and second mixer. The resolution time of these apparatus is of the order of a few tens of milliseconds (typically 20–30 ms).

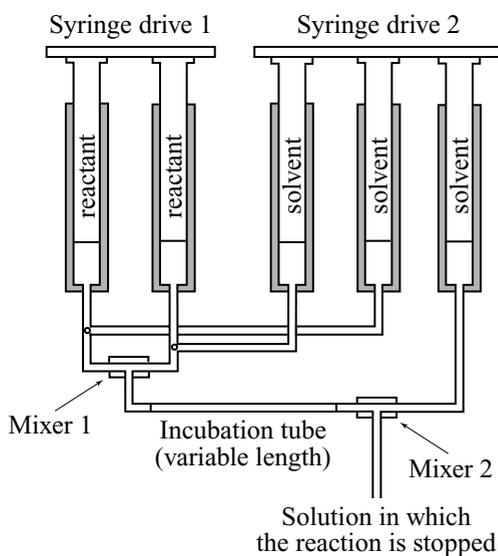


Fig. 6.7 *Quenched-flow apparatus*

6.4.5. CRITERION FOR HOMOGENEITY OF THE MIXTURE

Obviously, it is of the greatest importance that the time required to obtain an homogeneous mix of the two reactants is short relative to the half-life of the reaction studied. From the very beginning of the development of flow methods, the mixing efficiency was investigated using either chemical or physical tests. The chemical tests consisted of measuring the progress of bimolecular reactions that were known to terminate in a time shorter than that measurable by the system. In this way, the mixing efficiency and the minimum response of the apparatus can be evaluated.

The most commonly used reaction is acid neutralisation by a base in the presence of a coloured indicator; the heat of neutralisation can also be measured. The physical tests include, for example, mixing two substances having different optical properties and then using an optical criterion as a means to test the homogeneity of the mixture. This principle was adopted by DUBOIS and TROWSE in the method of SCHLIJEREN.

6.4.6. SOME TECHNICAL PROBLEMS

The resolution time of these apparatus depends on the mixing rate of the reactant solutions. However, this rate cannot pass certain limits. To obtain quickly good mixing by using only minimal quantities of reactants, it is important that the form of the mixing chamber is suitably adapted. In the first systems used, the chamber contained eight jets that delivered the liquid at a tangent, creating a rotational movement in the flow. CZERLINSKI developed a system of mixers with infinite jets involving two thin concentric layers facing one another at the top of a cylindrical observation tube. Other systems have been described in which the many thin layers of liquid come into contact with each other. One such system comprised 25 layers and the flow rate was able to reach $10 \text{ m} \cdot \text{s}^{-1}$. However, it is not beneficial to use too high a flow rate. Indeed, above a certain threshold value slightly higher than $10 \text{ m} \cdot \text{s}^{-1}$, although this depends on the geometry of the mixing chamber, cavitation phenomena may occur. We note also that among the technical difficulties encountered while using this type of equipment were interfering vibrations and liquid leaks.

The methods for the titration of enzyme active sites using the burst technique that we presented in Section 5.4.6 often require the use of the stopped-flow technique. Similarly, the example given concerning the identification of an intermediate in reactions catalysed by β -galactosidase from a mutant *E. coli* strain by the appearance of a paranitrophenol burst was realised by means of a stopped-flow apparatus.

6.5. RELAXATION METHODS

Flow techniques enable rate constants between milliseconds and seconds to be achieved. However, they are not fast enough to allow the detection of certain reaction intermediates during enzymatic reactions. In particular, the formation of the first enzyme-substrate complex is far too fast to be detected by even the best stopped-flow apparatus. The relaxation methods introduced by EIGEN and DE MAEYER covered time-scales one order of magnitude smaller than flow methods. The time-scales associated with the different methods are displayed on page 193. Relaxation methods cover a time-scale of between seconds to 10^{-10} s .

6.5.1. PRINCIPLE OF RELAXATION METHODS

Relaxation methods are applicable to systems at equilibrium or in conditions of steady state. In physics, the perturbation of a system at equilibrium leads to its *relaxation* towards a new equilibrium state. Such is the case, for example, for the reorientation of dipoles after a change in the electric field. The analogy between these processes and those arising in chemical systems led EIGEN and DE MAEYER to use the term *chemical relaxation*. We understand by chemical relaxation, any readjustment of a chemical equilibrium affected by a perturbation of the reactant concentrations, either following a change in the parameters related to that state, or affected by an external force influencing the equilibrium. The method consists of applying extremely rapidly to the system at equilibrium an external perturbation, such as an abrupt change in temperature, pressure or an electrical impulse, which can shift the equilibrium position. This definition also includes the processes studied by special flash techniques, where the equilibrium in the fundamental state is disturbed after transformation of the excited molecules, which become disactivated after reacting. If the perturbation is applied much more quickly than the time needed for readjustment of the chemical equilibrium, the concentrations of the different constituents will reach the new equilibrium position at a rate that is solely dependent on the reaction mechanism i.e. on the individual concentrations and the specific rate constants of each elementary step.

The technology has developed to such a degree that it is possible in practice to change variables such as the temperature or density of the electric field in times as short as 10^{-8} to 10^{-6} s. The discontinuity of the change relative to the rate of relaxation is such that the suffix *jump* is employed to designate this type of perturbation, as in for example the “temperature jump” method, often shortened to **T-jump**.

Two types of perturbation can be used depending on the reaction type to be studied: either a transient perturbation, or an alternative perturbation.

6.5.1.1. TRANSIENT PERTURBATION

A transient perturbation is applied rapidly to the system, which then attains its new state of equilibrium in a relatively long time compared to the perturbation time. Figure 6.8 below illustrates this sort of response for a simple equilibrium:



In the thermal relaxation method, for example, a temperature rise of 10°C can be obtained in less than a microsecond by an impulse of 10^4 – 10^5 v through the solution. The rate equation is a linear differential equation and the relaxation curve is given by:

$$\Delta c = \Delta c_0 e^{-t/\tau}$$

ΔC_0 and ΔC are the changes in concentration at time 0 and t , respectively; τ represents the relaxation time. In the case of simple equilibrium, it is related in a simple way to the specific rate constants for the reaction as shown further on (§ 6.5.3.2). If the reaction comprises several steps, there is a complete spectrum of relaxation times and we obtain quite a complex expression.

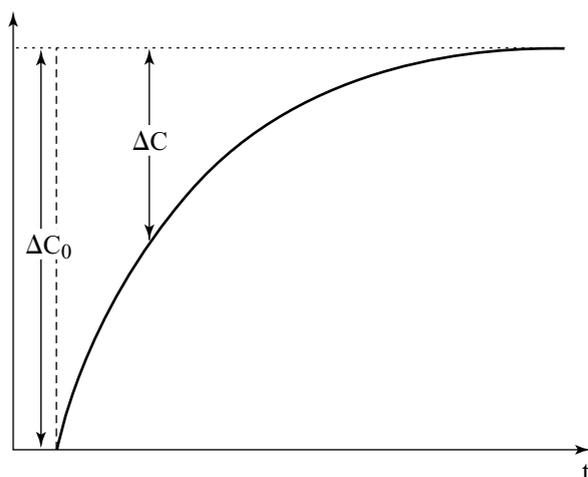


Fig. 6.8 Relaxation of a system subjected to a transient perturbation
The transient perturbation is shown as a dashed line; the relaxation of the system, an unbroken line

6.5.1.2. ALTERNATIVE PERTURBATION

- In the alternative perturbation method, a periodic perturbation is applied to the system at equilibrium with a period of the order of time necessary to restore equilibrium. Due to the finite rate of chemical reactions, the response is periodic, but remains shorter than the perturbation frequency. The phase difference is a measure of the chemical reaction rate. The attenuation of the perturbation represents the energy dissipated by the system and can be measured. It is related to the phase difference by a FOURIER transform. Ultrasonic waves and electric fields are used to generate alternative perturbations. For a periodic perturbation provoked, for example, by an ultrasonic wave, we have the equation:

$$\overline{\Delta C} = a e^{i\omega t}$$

a being a constant, ω the perturbation frequency and $i^2 = -1$. The concentration varies as a function of time according to the expression:

$$\Delta C = a e^{i\omega t} / (1 + i\omega\tau)$$

Thus ΔC oscillates with the same frequency as $\overline{\Delta C}$, but out of phase. Energy absorption per wavelength due to relaxation depends on the frequency, according to the following relation:

$$B\omega\tau / (1 + \omega^2\tau^2)$$

This function has a maximum when $\omega = 1/\tau$. The time limit of each direct method is given by the finite propagation rate of the signal through the system. Such methods were developed and used successfully for the perturbation of systems involving dipolar molecules in an alternative electric field. ▲

6.5.2. PRINCIPAL RELAXATION METHODS

Different relaxation methods exist that differ in the nature of the perturbation and the detection method. Their use depends on the type of reaction under study.

6.5.2.1. THERMAL RELAXATION

Thermal relaxation (*temperature jump* or *T-jump*) is the appropriate method for studying transient phenomena in biochemical reactions such as the formation of enzyme-metal complexes, antibody-hapten reactions, enzymatic reactions and conformational changes in proteins and nucleic acids. It has been used with success in each of these different cases. Of course, its usage requires an obvious thermodynamic condition, namely, that **the reaction is accompanied by a non-zero enthalpy change**, ΔH . Indeed, the thermal dependence of an equilibrium constant is given by the classic equation of VAN T'HOFF:

$$\frac{d \ln K_{\text{eq}}}{dT} = \frac{\Delta H}{RT^2}$$

When ΔH is nil, the equilibrium constant does not change with temperature and consequently nor do the concentrations, therefore the equilibrium cannot be relaxed.

Thermal relaxation allows a time-scale to be covered that ranges from a second to about 10^{-7} s. In this method the perturbation is provoked by a heat shock generated by the discharge from a condenser at high voltage (100 kV), the capacity of the condenser being 0.02 μf . This technique produces a temperature jump of 6°C in less than 10^{-7} s in a volume of 1 mL, and it is homogenous throughout the solution. Such apparatus, schematised in Fig. 6.9 below, can be equipped for detection by absorption (single or double beam) or emission spectrophotometry, or even polarimetry, although this third detection method is generally too slow. The homogeneity of the temperature in the part of the cuvette in which the measurement takes place results from the particularly elaborate form of the cuvette, which, in principle, should also avoid any electrolysis caused by the discharge.

Thermal relaxation methods were very widespread a few years ago. Their use has led to the discovery of important intermediates in enzymatic reactions and helped to define the mechanisms for the functions of biological reactions. The most recent technological advances have given rise to the development of non-conventional T-jump apparatus. The temperature jump is provoked by a very fast impulse. This has permitted ever shorter resolution times to be achieved. Thus, in the apparatus constructed by PHILLIPS et al. (1995), the solutions are heated by a laser pulse, the rays are then absorbed by the dye molecules homogeneously dispersed in the solution. A 10°C rise in temperature is obtained in 70 ps. With such methods, it is important to check that the dye does not interfere with the reaction being studied.

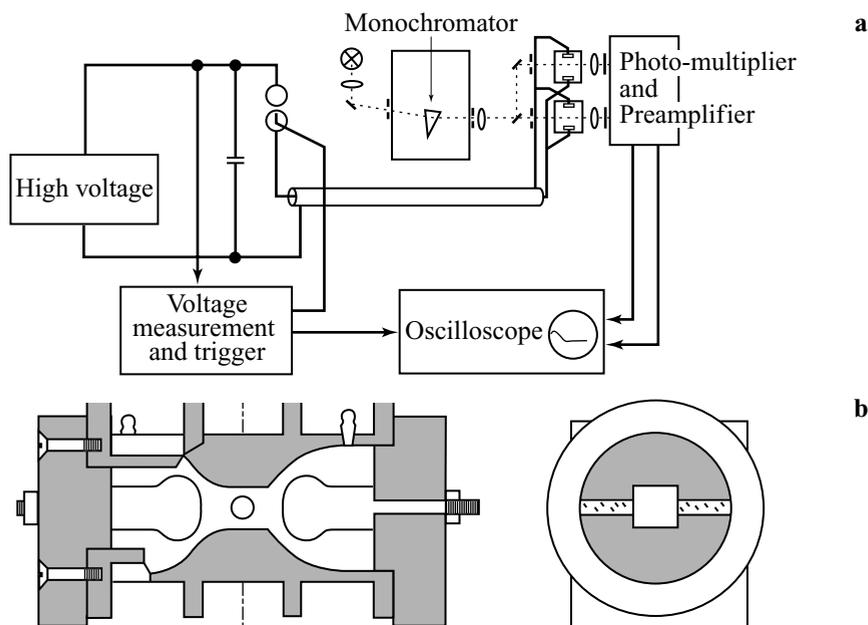


Fig. 6.9 Thermal relaxation apparatus
(a) general scheme – (b) cell

6.5.2.2. PRESSURE SHOCK

In the method of pressure shock (or **P-shock**), the perturbation is provoked by an abrupt change – either positive or negative – in pressure. The detection methods used are conductimetry or spectrophotometry. This technique gives time constants ranging from 10 to 5×10^{-5} s.

6.5.2.3. OTHER RELAXATION METHODS

► The impulse from an electric field also provides a method of perturbation. In general, conductimetry is the detection technique used. These methods allow time-scales to be covered that range from 10^{-4} to 10^{-9} s and even lower.

The dielectric loss in intense electric fields constitutes another relaxation method which can achieve time constants of between 10^{-5} and 10^{-8} s.

The absorption and dispersion of sound can be used for studying certain systems. The time constants in these methods vary between 10^{-5} and 5×10^{-10} s. The detection methods make use of either the phenomenon of resonance, the reverberation of sound, or the diffraction of light at the sound gate. ◀

6.5.3. ANALYSIS OF KINETIC DATA

If we consider the chemical processes, the previously described methods allow analysis of the kinetic data.

6.5.3.1. REACTIONS WITH N CONSECUTIVE STEPS

Let us first consider a reaction with n consecutive steps, which comprises n independent rate equations:



The rate equations can be linearised to give equations of the form:

$$\frac{-\Delta C_i}{dt} = \sum_{j=1}^n a_{ij} \Delta C_j$$

in which the a_{ij} terms are functions of the specific constants for rate and concentration at equilibrium.

These coupled rate equations can be converted into a series of linear first-order equations having the form:

$$\frac{d\Delta y_i}{dt} = \frac{\Delta y_i}{\tau_i}$$

Δy_i being a linear combination of ΔC_i ; the τ_i terms are relaxation times, functions of a_{ij} . The law for the change in concentration of individual species is written:

$$\Delta C_i = \sum_{j=1}^n A_{ij} e^{-t/\tau_i}$$

The n relaxation times are solutions of the determinant:

$$\begin{vmatrix} a_{11} - 1/\tau & a_{12} & \dots & a_{1n} \\ a_{21} & a_{22} - 1/\tau & \dots & a_{2n} \\ \dots & \dots & \dots & \dots \\ a_{n1} & a_{n2} & \dots & a_{nn} - 1/\tau \end{vmatrix}$$

Thus the changes in concentration that result from the perturbation of equilibrium can be represented as the sum of the exponential terms:

$$\Delta C = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + \dots \quad 6$$

The set of relaxation times and their corresponding amplitudes form the relaxation spectrum. These values depend in a known way on the real concentrations and rate constants that determine the dynamics of the system. In principle, the equations could be derived for each kinetic mechanism. Conversely, the measurement of $1/\tau$ as a function of the concentration enables the determination of the individual rate constants and consequently leads to the elucidation of the mechanisms. The relaxation amplitudes are functions of the constants associated with the detection methods, for example, the molar extinction coefficients when absorption spectrophotometry is used for detection or the quantum yields if the detection method is fluorescence. It is not always straightforward to obtain values for the associated relaxation times and amplitudes from relaxation spectra. This is only simple when they are sufficiently separated in time.

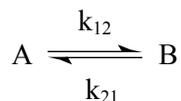
One point needs to be emphasised, after the application of a transient perturbation, **the rate equations can only be solved if the perturbation is very weak** in such a way that the real concentration change introduced by this does not exceed a few percent of the absolute concentration of the reacting species. Although a weak perturbation leads to relatively small effects requiring a greater sensitivity in the detecting equipment, the resulting simplification of the mathematical analysis largely compensates for this disadvantage. In these conditions only, the rate equations corresponding to the kinetic laws for each individual step can be simplified by ignoring the products of the concentration change. This linearisation allows the use of matrix calculations to solve all the linear differential equations simultaneously. Thus for a step having a given order, the rate equation can be represented by the single relationship:

$$\frac{d\Delta C_i}{dt} + \frac{1}{\tau_i} \Delta C_i = \frac{1}{\tau_i} \Delta C_i$$

For single reactions comprising only one elementary step, a single exponential relaxation is obtained. The relationship between $1/\tau$, the specific rate constants and the concentrations can be readily known.

6.5.3.2. FIRST-ORDER REACTION WITH A SINGLE STEP: ISOMERISATION

Let us now consider an isomerisation reaction such as:



The rate equation is: $\frac{dA}{dt} = -k_{12}A + k_{21}B$

When the perturbation of the equilibrium is weak, the concentrations of A and B can be replaced by the final concentrations at the new equilibrium and the shift ΔA and ΔB from this final equilibrium, such that:

$$A = \bar{A} + \Delta A$$

$$B = \bar{B} + \Delta B$$

Thus: $\frac{d(\bar{A} + \Delta A)}{dt} = \frac{d(\Delta A)}{dt} = -k_{12}(\bar{A} + \Delta A) + k_{21}(\bar{B} + \Delta B)$

From the law of mass action we may write:

$$\Delta A = -\Delta B \quad \text{or} \quad \Delta A + \Delta B = 0$$

The equilibrium relationship gives:

$$k_{12}\bar{A} = k_{21}\bar{B}$$

giving:
$$\frac{d(\Delta A)}{dt} = -(k_{12} + k_{21})\Delta A$$

and by integrating:
$$\Delta A_t = \Delta A_0 e^{-(k_{12} + k_{21})t}$$

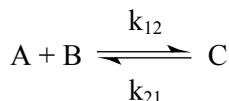
ΔA_t is the change in the concentration of A at time t , ΔA_0 the total change in A between the initial and final equilibrium. Therefore, **the relaxation time:**

$$1/\tau = k_{12} + k_{21}$$

for a monomolecular, single-step process is independent of the concentrations of the species. It is equal to the sum of the individual rate constants of the forward and reverse reactions. The equilibrium constant $K_{eq} = k_{12}/k_{21}$ must be known in order to determine each of them. Consequently, relaxation methods do not dispense with the use of classical methods, they require and complete them.

6.5.3.3. BIMOLECULAR REACTION WITH ONE STEP

Let us consider the following bimolecular equilibrium:



The rate equation is written:
$$\frac{dA}{dt} = -k_{12}AB + k_{21}C$$

As before, we have the relations:

$$A = \bar{A} + \Delta A$$

$$B = \bar{B} + \Delta B$$

$$C = \bar{C} + \Delta C$$

The equations for mass conservation are written:

$$\Delta A = \Delta B = -\Delta C$$

$$\Delta A = \Delta B$$

$$\Delta A + \Delta C = 0$$

which makes the existence of two equations of mass conservation appear more clearly. The equilibrium is written:

$$k_{12}\bar{A} \cdot \bar{B} = k_{21}\bar{C}$$

and by substituting in the rate equation and ignoring the terms in ΔA . ΔB , we obtain the equation:

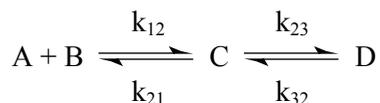
$$\frac{d(\Delta A)}{dt} = -k_{21} + k_{12}(\bar{A} + \bar{B})\Delta A$$

After integrating, this expression becomes:

$$\Delta A_t = \Delta A_0 e^{-t/[k_{21} + k_{12}(\bar{A} + \bar{B})]}$$

The time constant of a simple bimolecular reaction with one step depends on the sum and not the product of the concentrations of the reacting species at the final equilibrium; this results from the weak perturbation and the linearisation of the equation. Thus by plotting $1/\tau$ as a function of the sum of the concentrations $(\bar{A} + \bar{B})$, we obtain k_{21} from the intercept of the linear plot with the vertical axis and k_{12} from its slope. Again the concentrations of A and B at the final equilibrium must be known. Here, yet again, the use of relaxation methods requires having studied the equilibrium beforehand.

6.5.3.4. BIMOLECULAR REACTION FOLLOWED BY ISOMERISATION



This type of reaction is interesting in biochemistry since it represents the first steps of an enzyme reaction, namely, the formation of the MICHAELIS complex followed by its isomerisation. The reacting components could be an enzyme and a poor substrate or a competitive inhibitor.

These two reactions are coupled *via* a common intermediate C; therefore the dependence of the concentrations cannot be given by the previously derived expressions. In principle, two relaxation times could be observed after perturbation of the equilibrium. If the rate constants have the same order of magnitude, the expressions obtained are complex functions of the rate constants and the concentrations. When the two elementary steps have very different time constants, the expression can be simplified.

Let us consider a bimolecular reaction that is much faster than a monomolecular reaction. After the perturbation we observe a relaxation corresponding to the bimolecular step which is not coupled to the subsequent step. The first fast relaxation time is therefore that of a simple bimolecular reaction:

$$1/\tau_1 = k_{21} + k_{12}(\bar{A} + \bar{B})$$

The first reaction has already reached its new state of equilibrium whereas the relaxation of the second has not yet occurred. However, the relaxation of this second phase is coupled to the readjustment of the first equilibrium; the second step therefore corresponds to a change in the concentrations coupled to that of the first step. We have the rate equation:

$$\frac{dD}{dt} = k_{23}C - k_{32}D$$

as well as the relations:

$$\begin{aligned} A &= \bar{A} + \Delta A \\ B &= \bar{B} + \Delta B \\ C &= \bar{C} + \Delta C \\ D &= \bar{D} + \Delta D \end{aligned}$$

and the conservation equation:

$$\Delta A + \Delta C + \Delta D = 0$$

The rate equation may be written:

$$\frac{d(\bar{D} + \Delta D)}{dt} = k_{23}(\bar{C} + \Delta C) - k_{32}(\bar{D} + \Delta D)$$

The equilibrium relationship is: $k_{23}\bar{C} = k_{32}\bar{D}$

The first fast equilibrium is always established during the course of the relaxation of the second. Thus we have:

$$\frac{(A)(B)}{(C)} = \frac{k_{21}}{k_{12}}$$

or:

$$k_{12}(\bar{A} + \Delta A)(\bar{B} + \Delta B) = k_{21}(\bar{C} + \Delta C)$$

By ignoring the product $\Delta A \cdot \Delta B$ and taking into account the mass conservation expression: $\Delta A = \Delta B$, we obtain ΔA :

$$\Delta A = \frac{k_{21}\Delta C}{k_{12}(\bar{A} + \bar{B})}$$

By substituting this expression in the equation for mass conservation, we obtain:

$$\Delta C \left[1 + \frac{k_{21}}{k_{12}(\bar{A} + \bar{B})} \right] + \Delta D = 0$$

giving:

$$\frac{d\Delta D}{dt} = -k_{32}\Delta D - k_{23} \frac{k_{12}(\bar{A} + \bar{B})}{k_{21} + k_{12}(\bar{A} + \bar{B})}$$

by integrating: $\Delta D = \Delta D_0 e^{-t/\tau_2}$

with:

$$\frac{1}{\tau_2} = k_{32} + k_{23} \frac{k_{12}(\bar{A} + \bar{B})}{k_{21} + k_{12}(\bar{A} + \bar{B})}$$

Figure 6.10 below shows the changes in relaxation times with the concentration of the species concerned. It clearly shows that only for high concentrations of $\bar{A} + \bar{B}$ does the relaxation time become constant. From this hyperbolic curve we can obtain the constant k_{32} from its intercept with the vertical axis (extrapolated to zero concentration). The plateau corresponds to the sum of the constants $k_{23} + k_{32}$.

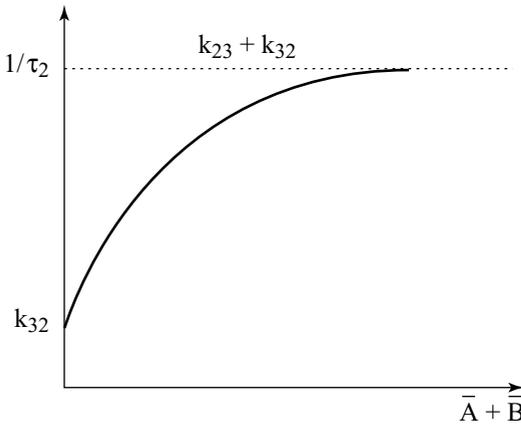
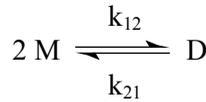


Fig. 6.10 Changes in $1/\tau_2$ as a function of $\bar{A} + \bar{B}$ in a second-order reaction followed by isomerisation

6.5.3.5. DIMERISATION EQUILIBRIUM

Dimerisation equilibrium is another example that applies to proteins, which in certain conditions can self-associate and in others, conversely, undergo dissociation into subunits. Let us consider the following equilibrium:



The rate equation has the form:

$$-\frac{1}{2} \frac{d(M)}{dt} = \frac{d(D)}{dt} = -k_{12}(M)^2 + k_{21}(D)$$

As before, we have the relations:

$$M = \bar{M} + \Delta M$$

$$D = \bar{D} + \Delta D$$

and by substituting into the rate equation:

$$-\frac{1}{2} \frac{d(\bar{M} + \Delta M)}{dt} = -k_{12}(\bar{M} + \Delta M)^2 + k_{21}(\bar{D} + \Delta D)$$

The equation for mass conservation is:

$$\frac{\Delta M}{2} = -\Delta D$$

At equilibrium, we have: $k_{21}\bar{M}^2 = k_{21}\bar{D}$

and:

$$-\frac{1}{2} \frac{d\Delta M}{dt} = -2k_{12}\bar{M} \cdot \Delta M - k_{21} \frac{\Delta M}{2}$$

giving:

$$\frac{d\Delta M}{dt} = -(4k_{12}\bar{M} + k_{21})\Delta M$$

and the relaxation time is given by the relationship:

$$1/\tau = 4k_{12}\bar{M} + k_{21}$$

6.5.3.6. ANALYSIS OF RELAXATION DATA

From these few simple examples, which illustrate well the use of relaxation methods, it is helpful to give some practical guidelines particularly concerning thermal relaxation, which is the most commonly used technique for studying biochemical reactions.

The number of relaxation times observed is a first indication about the chemical reactions studied, in particular about the number of reacting species and their related equilibria. This number is at most equal to the number of reacting species minus the number of conservation equations. Thus in the isomerisation equilibrium, we can count two species and one equation of conservation, giving a single relaxation time. Similarly, in a single-step bimolecular reaction, the number of species is three, with two equations of conservation and therefore a single relaxation time. In the example cited in Sect. 6.5.3.4, there are four species and two equations of conservation, and therefore two relaxation times in theory. However, these two times are only observable if they are sufficiently separated in time.

The determination of relaxation times and the study of their dependence on the concentrations of the system's constituents lead to defining the reaction mechanism itself. Thus it is easy to distinguish between an isomerisation, where $1/\tau$ is independent of the reactant concentrations, and a bimolecular process where this parameter varies linearly with the sum of the concentrations of the two components at equilibrium.

The analysis of the relaxation amplitudes can yield different types of information. Firstly, the change in amplitudes as a function of the concentration provides complementary information about the reaction scheme and its stoichiometry. The relaxation amplitudes are functions of the reaction enthalpy and characteristics related to the observed physical parameter. Thus in a bimolecular reaction:



the total amplitude of the phenomenon is:

$$\Delta P = (\Phi_A A + \Phi_B B + \Phi_C C)e^{-t/\tau}$$

Φ_A , Φ_B and Φ_C being the physical characteristics of the species. If the physical parameter is absorbance, we have:

$$\Delta P = (\epsilon_A A + \epsilon_B B + \epsilon_C C)e^{-t/\tau}$$

ϵ_A , ϵ_B and ϵ_C are the molar absorbances of the species A, B and C, respectively. Thus knowledge of either the physical characteristics or the enthalpy of the reaction enables the determination of the other parameters from the values of the amplitudes.

**6.5.3.7. EXAMPLE OF STUDYING AN ENZYMATIC REACTION
BY MEANS OF THERMAL RELAXATION**

In order to determine if the interaction of trypsin with a substrate, or a competitive inhibitor analogous to a substrate, is followed by an isomerisation step such as a conformational change in the protein, the trypsin-benzamidine interaction was studied. However, this competitive enzyme inhibitor absorbs light in a region of the spectrum poorly measurable by experiment, because the enzyme absorbs at the same wavelengths, making detection of the enzyme-inhibitor complex very difficult. We can however use another trick: proflavin, which is an acridine dye and also a competitive inhibitor of the enzyme, can be used as an indicator. In this study, two types of experiment were carried out. First, the study of the simple trypsin-proflavin equilibrium and then the binding of benzamidine in the presence of the indicator, proflavin.

Binding of proflavin to trypsin

For the equilibrium: $E + P \xrightleftharpoons[k_{21}]{k_{12}} EP$, the reaction occurs in a single step.

We obtain a single relaxation time characteristic of a bimolecular reaction. It is a function of the concentrations of the reacting species:

$$1/\tau_1 = k_{12}(\bar{E} + \bar{P}) + k_{21}$$

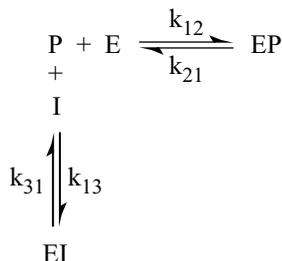
Figure 6.11a indicates the changes in $1/\tau_1$ as a function of $(\bar{E} + \bar{P})$; it shows a satisfactory linearity. We deduce from this the kinetic parameters of the reaction:

$$k_{12} = 7.6 \times 10^7 \text{ mol}^{-1} \cdot \text{s}^{-1} \quad ; \quad k_{21} = 9\,500 \text{ s}^{-1}$$

with $K_p = 1.1 \times 10^{-4} \text{ M}$.

Binding of benzamidine to trypsin

We have the following group of reactions:



If each equilibrium is a simple bimolecular reaction, we should expect two relaxation times. Indeed, there are 5 molecular species and 3 equations of conservation as follows:

$$\Delta P + \Delta EP = 0$$

$$\Delta I + \Delta EI = 0$$

$$\Delta E + \Delta EP + \Delta EI = 0$$

However, as there are two coupled equilibria, we can only detect them if they are adequately separated in time. If the first equilibrium (EP) is fast relative to the second, the first relaxation time will be independent of the second equilibrium. Having independently studied this first equilibrium, it was noticeable that this is indeed the case. Taking into account the fact that the re-equilibration of proflavin and the trypsin-proflavin complex is quite fast so that the first system re-equilibrates independently of the second equilibrium, we have:

$$\frac{1}{\tau_2} = k_{31} + k_{13}(\bar{E} + \alpha\bar{I})$$

with: $\alpha = (\bar{E} + K_p)(\bar{E} + \bar{P} + K_p)$

and: $K_p = k_{21}/k_{12}$

The plot of $1/\tau_2$ as a function of $(\bar{E} + \alpha\bar{I})$ gives a straight line that is satisfactory enough to justify the hypothesis of a simple scheme (Fig. 6.11b) and permits determination of the system's constants:

$$k_{13} = 2.9 \times 10^7 \text{ mol}^{-1} \cdot \text{s}^{-1} ; \quad k_{31} = 600 \text{ s}^{-1} ; \quad K_I = 1.2 \times 10^5 \text{ M}^{-1}$$

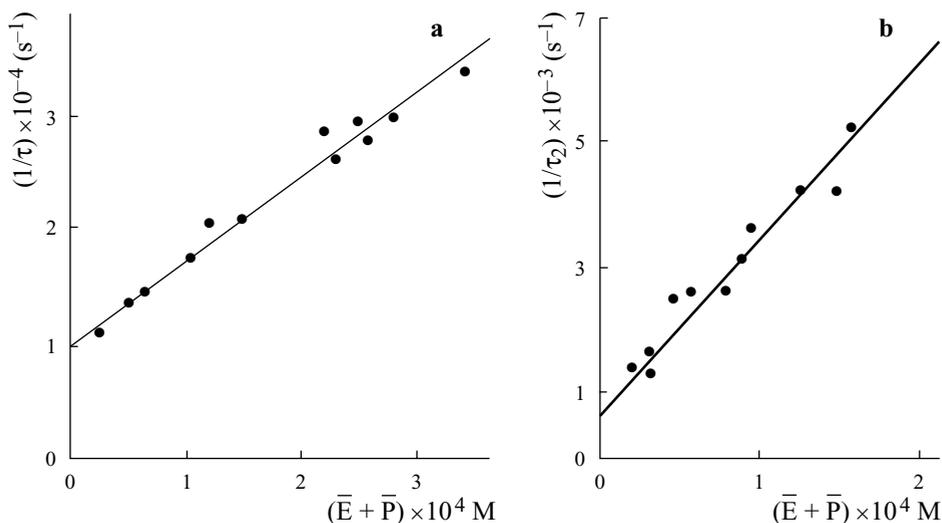


Fig. 6.11 Study of the trypsin-benzamidine interaction with the aid of an indicator, proflavin

(a) changes in the inverse of the relaxation time as a function of the sum of the trypsin and proflavin concentrations at the new equilibrium $(\bar{E} + \bar{P})$, in the absence of benzamidine – (b) change in the inverse of the relaxation time as a function of $(\bar{E} + \bar{P})$ in the presence of benzamidine, with $\alpha = (\bar{E} + K_p)(\bar{E} + \bar{P} + K_p)$. These representations are characteristic of second-order kinetics

6.6. STUDY OF ENZYMATIC REACTIONS

AT LOW TEMPERATURES: CRYOENZYMOLOGY

As the different steps in an enzyme reaction are dependent on temperature, it is conceivable to slow them down to low temperatures in order to facilitate their study. The use of solvent mixtures that lower the freezing point of water has enabled examination of these reactions at temperatures as low as -80°C . These methods have led to the isolation, in stable form, of reaction intermediates that had never been amenable to study until that time, due to their very short lifetimes. While employing techniques at low temperatures, it is important to remember that the addition to the solvent of substances that lower the freezing point (e.g. glycerol, ethylene glycol and ethanol) may cause other effects. In particular, the influence of these substances on the viscosity or the dielectric constant may change considerably the structural and functional properties of the proteins studied (e.g. interactions between subunits, pK of the groups involved in interactions of the protein with its ligands or with itself even, surface exposed to solvent, etc.). In this way it was shown that the presence of such co-solvents stabilised one or other of the conformations involved in the allosteric properties of haemoglobin (BULONE et al., 1983), glycogen phosphorylase (DREYFUS et al., 1978) and of aspartate transcarbamylase (DREYFUS et al., 1984). Several cases of oligomeric enzyme dissociation at low temperature have in fact been reported.

Table 6.1 below indicates the change in dielectric constants of various water-ethylene glycol mixtures at different temperatures. It shows that water-solvent mixtures can be obtained for which the dielectric constant practically reaches 80, i.e. that of pure water at 20°C . The dielectric constant varies with temperature according to the relation:

$$\log D = a - bT$$

a and b are empirical coefficients and T the absolute temperature.

Table 6.1 Dielectric constants of ethylene glycol-water mixtures

(Reprinted from *Cryobiochemistry: an introduction*, DOUZOU P., 1977, Academic Press, London)

Solvent (%)	Temperature ($^{\circ}\text{C}$)													a	b
	+20	+10	0	-10	-20	-30	-40	-50	-60	-70	-80	-90	-100		
0	80.4	84.2	88.1											1.945	2.00
10	77.7	81.4	85.3											1.931	2.02
20	75.1	78.4	82.5	86.9										1.916	2.15
30	72.0	75.7	79.5	84.0										1.912	2.20
40	68.1	72.1	76.3	80.2	84.4									1.880	2.30
50	64.5	68.4	72.4	76.5	80.7	85.0	89.3							1.860	2.35
60	61.1	64.6	67.9	72.0	76.3	80.8	85.3	90.1	95.7					1.832	2.41

Solvent (%)	Temperature (°C)													a b (10^3)	
	+20	+10	0	-10	-20	-30	-40	-50	-60	-70	-80	-90	-100		
70	56.9	60.0	63.4	67.5	71.3	75.3	79.8	84.5	89.5	94.6	100.0	106.1	112.1	1.803	2.47
80	53.0	55.6	58.8	62.3	66.2	70.0	74.2	78.5	83.2	88.1	93.0			1.770	2.50
90	47.5	50.5	53.5	56.8	60.2	63.8	67.8	72.0						1.728	2.52
100	41.9	44.7	47.6	50.3										1.675	2.54

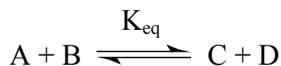
A certain number of systems have thus been studied with success, among which, horseradish peroxidase (DOUZOU et al., 1970), several serine proteases (FINK, 1973, 1974, 1976; FINK & ANGELIDES, 1975), β -galactosidase from *E. coli* (FINK & ANGELIDES, 1975) and several dehydrogenases (FINK & GEEVES, 1979).

6.7. STUDY OF ENZYMATIC REACTIONS UNDER HIGH PRESSURE

6.7.1. PRINCIPLE

During the last few years, numerous works based on the influence of pressure variations on the structure and properties of proteins have been undertaken. Initially destined for the study of structure-function relationships in enzymes from living organisms at atmospheric pressure, these methods today arouse additional interest after the discovery in 1978 of organisms living in deep-sea volcanic environments under pressures of up to 1 000 bars.

The influence of pressure on the structure and the catalytic and regulatory properties of enzymes is linked to LE CHATELIER's law (1888): "Any modification of one of the factors determining the state of a system at equilibrium provokes a shift of this equilibrium in the direction which tends to oppose the change in the factor considered." Thus, if a chemical reaction leads to a decrease in volume, it will be enhanced by an increase in pressure. If the reaction:



is accompanied by a change in volume, the pressure favours the direction of this reaction that leads to a reactant mixture occupying the smallest volume.

The volume change ΔV is linked to the equilibrium constant K_{eq} by the relationship:

$$\frac{d \ln K_{eq}}{dP} = - \frac{\Delta V}{RT}$$

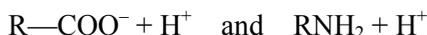
at a constant temperature.

Thus, at a given temperature, by plotting the variation of $2.3 \log K_{\text{eq}}$ as a function of the pressure P , we derive ΔV from the slope of the linear plot obtained. This slope can be positive or negative.

Similarly, for protonation equilibria:

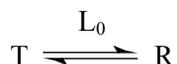


In most cases, the solution of the deprotonated form occupies a smaller volume, which results in particular from the phenomenon of electrostriction. This goes for ionic groups on proteins:



whose ΔV values for the deprotonation are, respectively, 21.3 and 20 $\text{cm}^3 \cdot \text{mol}^{-1}$. Thus, the pressure will tend to diminish the ionisation pK of a protein's amino and carboxylic groups with any consequences that this may entail for its conformation. However, the protonation of the imidazole moiety of histidine residues is characterised by a very small increase in volume ($1.1 \text{ cm}^3 \cdot \text{mol}^{-1}$).

The same considerations apply to the conformational changes in enzymes and more particularly, to allosteric transitions (see Chap. 13):



If the conformations R and T occupy different volumes, the pressure will affect the allosteric constant L_0 and will tend to stabilise the conformation occupying the smallest volume in solution. This has been established in the case of aspartate transcarbamylase (HERVÉ et al., 2004).

6.7.2. ACTIVATION VOLUME

Just as a chemical reaction is characterised by its free energy of activation, ΔG^\ddagger , (see Chap. 1), so it also is by its activation volume, ΔV^\ddagger . Thus, if the conversion of a substrate A into B involves the formation of a transition state A^\ddagger whose formation is governed by the rate constant k_1 such that:



the activation volume ΔV^\ddagger is given by the relationship:

$$\Delta V^\ddagger = V_{\text{A}^\ddagger} - V_{\text{A}}$$

and is related to the rate constant k_1 by the relationship:

$$\frac{d \ln k_1}{dP} = -\frac{\Delta V^\ddagger}{RT} \quad \text{at a constant temperature}$$

Thus, at a given temperature, the graphical plot of $2.3 \log k_1$ as a function of the pressure P provides the value of ΔV^\ddagger .

6.7.3. EQUIPMENT

Figure 6.12 shows a diagram of an apparatus constructed to study enzymatic reactions at steady state. With this apparatus, the reactants can be injected, stirred and samples taken at the desired time intervals, without the pressure of the mixture being modified. It is composed of a cell in which the reaction mixture is subjected to the pressure exerted by piston P. Temperature and pressure sensors allow permanent control of these two parameters.

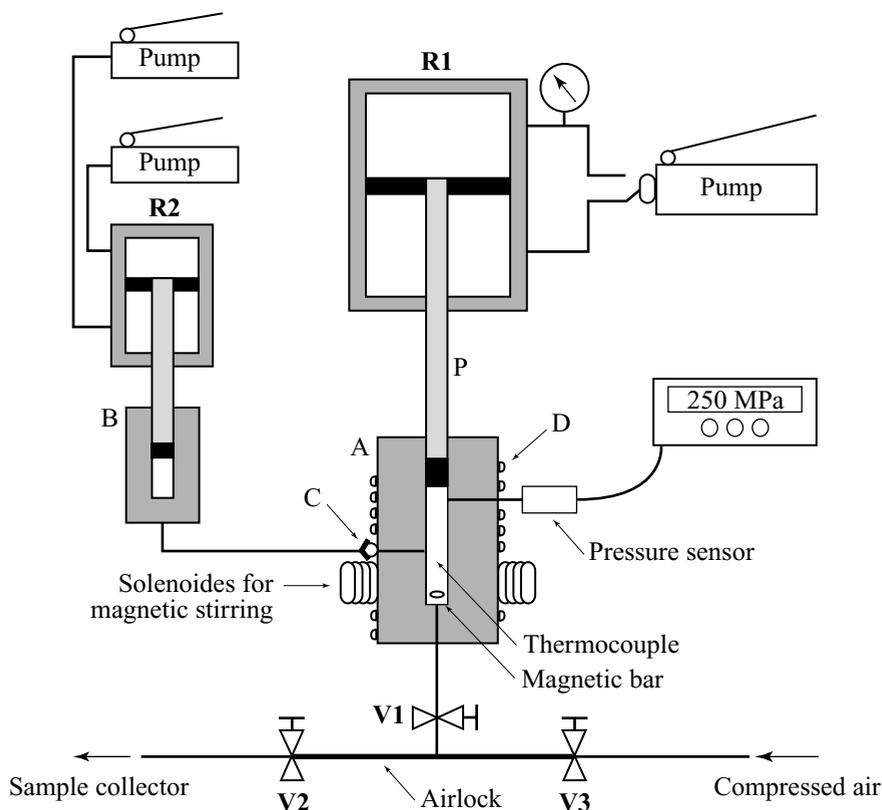


Fig. 6.12 Diagram of the apparatus developed to study enzyme kinetics at high pressure

A: incubation chamber; *B*: injection system; *C*: one-way valve; *D*: system for temperature regulation; *P*: main piston; *R1* and *R2*: hydraulic presses; *V1*, *V2*, *V3*: valves.

(Reprinted from *Anal. Biochem.*, **187**, HUI BON HOA G. *et al.*, A reactor permitting injection and sampling for steady state studies of enzymatic reactions at high pressure: teste with aspartate transcarbamylase, 258. © (1990) with permission from Elsevier)

This cell is constructed in a non-magnetic alloy in such a way as to allow magnetic stirring of the sample. A one-way injection system is used to trigger the reaction. The samples are taken with the help of a small-volume locked chamber which,

contrary to what is schematically represented in the figure, is localised in the body of the cell (HUI BON HOA et al., 1990).

For kinetic studies in the pre-steady state, another type of device, enabling the use of the stopped-flow technique under pressure, was developed by HEREMANS et al. (1980) and BALNY et al. (1984). A diagram of this apparatus is displayed in Fig. 6.13. The stopped-flow module is inserted in the central volume of the high-pressure apparatus. After hermetic closing, the pressure is exerted by a pump and a transmission liquid injected by the inlet PI. When the system has reached the desired pressure, the device DM is used to trigger the stopped-flow system. The incident light beam OB is transmitted into the observation chamber by windows made from sapphire and the signal obtained is then recorded.

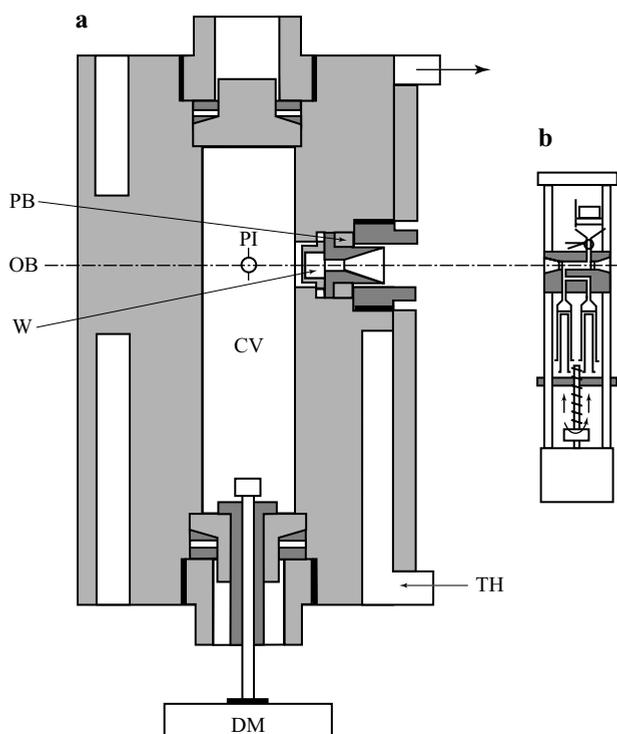


Fig. 6.13 Diagram of a stopped-flow apparatus under high pressure

(a) schematic view of the high pressure cell in the central volume (CV) in which the stop-ped-flow device is inserted – (b) OB: incident light beam; W: sapphire windows; TH: thermoregulation system; PI: inlet for the pressure transmission liquid; DM: trigger for the stopped-flow system. (Reprinted from *Anal. Biochem.*, **139**, BALNY C. et al., High-pressure stopped-flow spectrometry at low temperatures, 182. © (1984) with permission from Elsevier)

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