Review Letter

THE SCOPE OF MODERATE PRESSURE CHANGES FOR KINETIC AND EQUILIBRIUM STUDIES OF BIOCHEMICAL SYSTEMS

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. . . but fertility is the result not of exactness but of seeing new problems where none have been seen before, and of finding new ways of solving them . . .' Karl Popper, Unended Quest (p. 25, Fontana/ Collins Edition)

1. General Introduction

The development of kinetic techniques for the study of biological systems should be directed towards the determination of the number of distinct intermediates, their rates of interconversion and the characterisation of their physical properties. A range of techniques for the initiation and observation of reaction is required to cover the different lifetimes and properties of intermediates. Apart from photochemical reactions, the most widely used methods for the elucidation of reaction intermediates are those involving rapid mixing and rapid temperature changes (for a review see ref. [1]). Magnetic resonance techniques are also being applied to the study of an increasing range of systems.

The use of pressure changes to initiate and characterise biological reactions has a long but not very voluminous history [2,3]. In the present discussion we shall restrict ourselves to the effects of moderate pressures ranging from marginally above atmospheric pressure to 400 atm (40 MPa) which can be used for relaxation experiments. Periodic pressure perturbations through ultra-sound have played an important role in the development of the relaxation method [2]. More recently a method was described which involves repetitive small pressure perturbations and the averaging of a large number of transients [4]. Pressure-jump relaxation techniques with optical detection have been developed in several laboratories [5,6]. In the present article we wish to give a survey of the state of the art with respect to time and signal resolution of relaxations resulting from a step-function pressure change. After the rapid change in pressure the relaxation spectrum of the approach to the new equilibrium can be observed and analysed. In addition we wish to describe a number of examples of the type of biochemical reactions which can be readily investigated with this technique. The theory and practice of the analysis of relaxation spectra is discussed fully elsewhere [1,2]. In a brief description of a pressure-jump device recently [6], Knoche and Wiese pointed out that pressure-perturbation has several advantages over temperature-perturbation as a technique for initiating chemical relaxation phenomena: wider choice of solvent composition, reproducibility with unstable solutions, shorter time intervals between repeats and an extended time range. As will be seen our equipment permits observations after 25 μs and there is no upper time limit, in contrast to temperature-jump by electric discharge heating. The analysis of relaxation spectra is independent of the method of perturbation. However, much larger perturbations can be produced in some systems than can be analysed by the linear algebraic methods used for the interpretation of relaxation spectra (see fig.7).

The effects of pressure changes on biological reactions has three interesting aspects. Living organisms have many pressure-dependent functions and yet they have been able to adapt to live over an approximately 2000-fold range of pressure. Many studies are in progress with the aim of understanding this adaptation

[10]. Secondly, pressure- like temperature-dependence of elementary steps of chemical reactions is used to interpret the structure of transition states [7]. In the present survey we are largely concerned with the third aspect, pressure as a tool to initiate rapid reactions. The identification of intermediates is the prime objective. It is, however, helpful to discusss briefly the cause of the effects of pressure on equilibria, hence on reaction initiation, so that a general idea can be obtained about possible applications. We shall neglect the effects of pressure on rates since we are entirely concerned with methods which relax to one atmosphere, and the rates are thus measured under this standard condition.

A system at equilibrium at an applied pressure, will, when the pressure is released, approach a new equilibrium at which its volume is increased according to

$$\left(\frac{\partial \ln K}{\partial P}\right)_T = \frac{-\Delta V^\circ}{RT}$$

where K, P and ΔV° are the equilibrium constant, pressure and change in standard molar volume. The gas constant is $R = 82$ cm^{3}atm mol^{-1} K^{-1}. For small changes in pressure at 25°C (ΔV in cm^3 and pressure in atmospheres):

$$\frac{\Delta K}{K} = \frac{-\Delta P \Delta V}{24.4 \times 10^3}.$$

As a numerical example one can calculate the effect of a decrease in pressure of 150 atm on a reaction involving a volume change $\Delta V^\circ = 15$ cm^3/mol:

$$\frac{\Delta K}{K} = \frac{150 \times 15}{24.4 \times 10^3} = 0.09$$

which corresponds to a 9% change in equilibrium constant.

We are here largely concerned with reactions involving changes in ionisation and other non-covalent molecular association processes. Some examples of volume changes are listed in table 1 and it will be seen how these effects can be used to study a wide range of processes.

Volume changes during biochemical reactions in aqueous media are largely due to changes in solvent structure due to the interaction with solute molecules. Extensive studies of changes in the volume of water due to electrostriction have been reviewed by Hills [8] and Distèche [9] in a volume [10] which contains good surveys of many other aspects of the effects of pressure on biological processes. The formation or disappearance of charged groups accessible to water result in considerable volume changes and hence processes involving such exposed ionising groups show a marked pressure-dependence. These effects can be due either to conformation changes (including subunit assembly or dissociation) which result in exposure of a charged group or they can be due to a pressure-induced change of the pK of a group linked to a conformational rearrangement. As can be seen in table 1, neutral acids which form two new charge centres for electrostriction are particularly sensitive to pressure changes. In addition, the extent of electrostriction is proportional to the square of the ionic charge, the second ionization of a divalent group thus has a significantly larger $-\Delta V$ than the first. The dissociation of cationic acids results in more variable changes in electrostriction because it is due to the difference in volume of two ionising species

$$R\text{–}NH_3^+ + H_2O \rightleftharpoons NH_2 + H_3^+O$$

and are thus relatively pressure-insensitive. Studies of the effect of pressure over a range of conditions permit one to distinguish between the different causes of electrostriction: ionisation, breaking of salt bridges and exposure of buried charged groups.

A number of authors have reported studies of volume changes during the transfer of apolar groups into water. The consequent changes in solvent structure results in a decrease in volume and entropy of the system [10,11]. The pressure- and temperature-dependence of model systems, studied under ideal conditions, has often been compared with the effects of these intensive parameters on the binding of ligands and assembly processes in macromolecular systems. The assumption that large entropy changes and pressure-dependence go together must be a gross oversimplification. An example of the complex relation between the effects of temperature and pressure on an assembly process has been described recently [12]. The absence of pressure effects on complex processes, such as the binding of an apolar ligand into a hydrophobic crevass

Table 1
Volume changes associated with some representative processes

Ionisation of buffers	(ΔV cm^3 mol^{-1})
Phosphate (pK 2.1)	−15.7
Phosphate (pK 7.0)	−24.0
Acetate	−10.7
Bicarbonate	−25.4
Carbonate	−25.6
Borate	−32.1
Tris	+ 1.0
$MgATP^{2-} \longrightarrow Mg^{2+} + ATP^{4-}$	−23
Hydrophobic effects	
CH_4 in $C_6H_{14} \longrightarrow CH_4$ in H_2O	−22.7
C_2H_6 in $C_6H_{14} \longrightarrow C_2H_6$ in H_2O	−18.2
Hydrogen bonding	
α Helical poly L-glutamate ⟶ random coil	+ 1.0
Poly(A + U) nucleotide helix ⟶ random coil	− 1.0
Protein self-assembly per monomer	
Myosin filament propagation	−280
Tubulin propagation	−26 (35°)
	−50 (15°)
Protein denaturation	
Chymotrypsin	−43
Myoglobin	−100

For references to the above data see [10]

of a protein or subunit dissociation, could be explained by compensating linked processes.

Hydrogen bond transfer from an intrinsic to a water bonded state is generally associated with a small positive and at times a negative volume change. The volume changes encountered appear to depend on the relative packing efficiency of the equilibrium partners. However, the additive effect of these small changes can result in considerable pressure effects on cooperative interactions [13] (see also table 1). The ΔV for de novo hydrogen bond formation is about −3.8 cm^3/mol [14].

The first aim of a relaxation technique is to perturb the equilibrium so that rapid subsequent reactions can be studied. The perturbation can be mediated through an indirect effect such as the rapid change of pH (see table 1) or on the concentration of some other reactant species. The main aim of the present survey is to demonstrate the wide range of reactions which can be studied by such perturbations. Further studies of amplitudes of different relaxations under different conditions should help to elucidate the volume changes and thermodynamic characteristics of the elementary steps. Variety of application rather than the solution of any one problem is emphasised in the remaining sections.

2. Experimental technique

A schematic illustration of the main components of the pressure-jump apparatus used for the experiments described is shown in fig.1. In addition to improving the overall ease of operation, thought was given to obtaining higher operating pressures and shorter pressure-release times, whilst at the same time keeping cavitation and cell resonances at an accept-

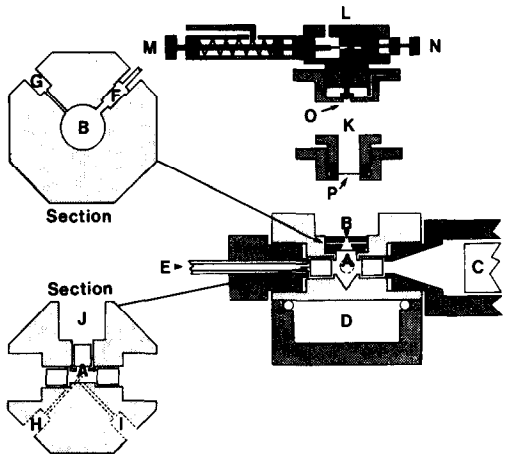

Fig.1. Schematic representation of the pressure-jump apparatus. The instrument is composed of the following components: A, observation cell; B, hydraulic chamber; C, absorbancy photomultiplier; D, thermostatted base; E, quartz fibre optic from light source; F, quartz pressure transducer for the triggering of data collection; G, hydraulic pressure line; H and I, observation cell filling and emptying ports; J, fluorescence emission window; K, bursting disc pressure-release valve; L, mechanical pressure-release valve; M, trigger mechanism; N, reset mechanism; O, valve seat; P, phosphorbronze bursting disc.

able minimum. A facility for using a variety of optical measuring techniques, based on modular light sources, was also incorporated.

The observation cell (A) is situated between thermostatted base (D) and the Teflon disc separating its contents from the oil in the hydraulic chamber (B) above. Inlet and outlet points are provided, one at the apex of the conical base of the cell and the other immediately below the Teflon-disc. The complete instrument is tilted at an angle of 30° to the vertical to facilitate filling. Three windows are provided, two in line and one at 90°. Light is introduced via a flexible quartz light guide. Photomultipliers, with a facility for the placement of optical filters, are used for the detection of transmitted and emitted light.

The hydraulic chamber (B) allows communication between the observation cell, the hydraulic pressure-line, the pressure-transducer for triggering the data-capture system and the various pressure-release devices.

Two different pressure-release devices can be fitted to the cell. One is a bursting disc valve (K) [5] (fig.1). This device can relax the pressure in the observation cell to atmospheric pressure in 25 μs (see fig.2a). The alternative device, a mechanical valve (L) illustrated in fig.1 is based on the design of Dr R. Vanhorebeek of the laboratory of Chemical and Biological Dynamics, University of Leuven, Belgium. Pressure-release is somewhat slower, of the order of 80 μs (fig.2b). The reproducibility of consecutive jumps using this device allows simple computer averaging techniques to be used for noise reduction. It should be noted that

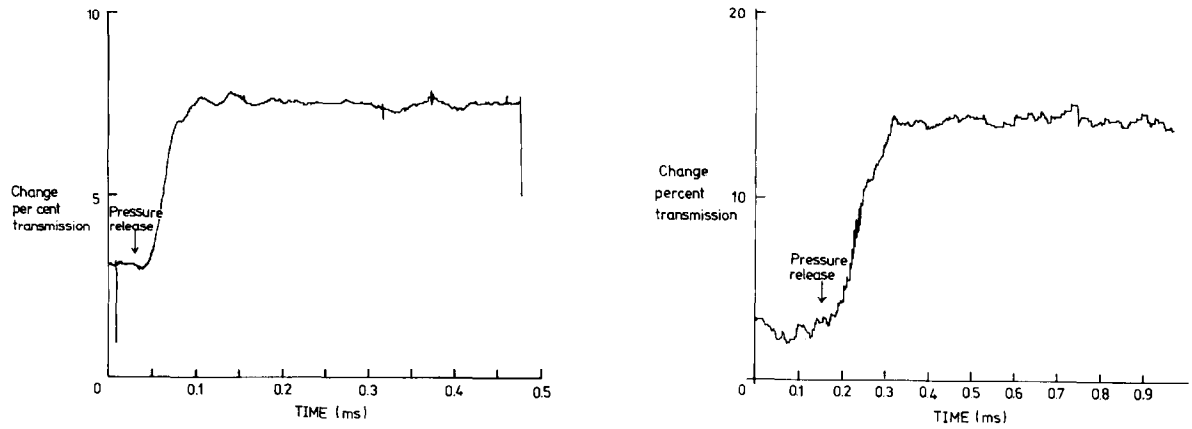

Fig.2a. Rate of pressure-release using the bursting disc device. Pressure-release was followed using a 22 μM phenol red, 0.05 M Tris–HCl buffer, pH 7.9. The pressure change was 150 atm. The data was plotted in analogue form from the transient recorder, no time constants were used. Fig.2b. Rate of pressure-release using the mechanical release device. Pressure release was followed using a 22 μM phenol red, 0.05 M Tris–HCl buffer, pH 7.9. The pressure change was 150 atm. The data was plotted in analogue form from the transient recorder.

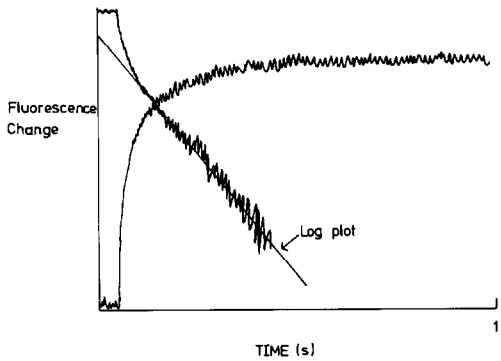

Fig.3. Protein fluorescence change with bovine serum albumin during 70 atm pressure-relaxation, glycine buffer, pH 3.5, protein 1 mg/ml.

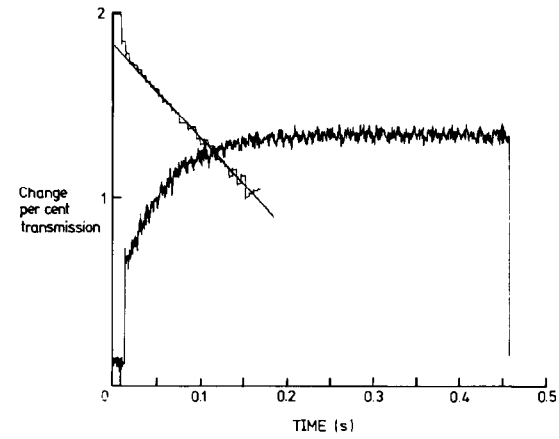

Fig.4. Absorbancy change of the bovine serum albumin–benzyl orange system during 100 atm pressure-relaxation, glycine buffer, pH 3.0, protein 1 mg/ml.

somewhat faster pressure-release is obtained at lower release pressures. The release profile is almost linear rather than exponential.

A number of optical detection techniques were incorporated in the design to enable as large a variety of signals as possible to be used for the physical characterization of kinetically distinct intermediates. Changes in turbidity (fig.6 and 7), light absorption (fig.4 and 8) and fluorescence emission (fig.3 and 9) can be measured over the visible and ultraviolet ranges.

The thermodynamic parameters of a system can be obtained from static experiments in which either the temperature or pressure within the cell are varied. The study of the dependence of equilibria on moderate pressure changes is well illustrated by the data collected on the myosin filament assembly system (fig.3).

3. Examples of application

3.1. *Conformation changes of serum albumin*

The molecular isomerizations of bovine serum albumin at acid and alkaline pH have long served as a pressure-jump test system. In the past these relaxations have been followed by both optical [5] and conductometric [3] monitoring techniques. In the present paper changes in intrinsic protein fluorescence and absorbancy changes due to a spectral shift in the benzyl orange dye spectrum are used to follow the isomerizations at acid pH (fig.3 and 4). These experiments in part illustrate the type of experiment one can perform to follow protein isomerization and also to show the way in which relaxation data is recorded. In the protein fluorescence experiments the conditions are such that two relaxations, separated by an order of magnitude in rate, are obtained. Under the conditions used for the dye binding experiment, however, a single relaxation is observed for the process (fig.4), the initial rapid change is due to the response of the dye alone. In both cases the data are plotted from the computer store together with a log plot and the least square line from which the rate constant was calculated.

3.2. *Myosin assembly*

An example of the way in which protein assembly reactions can be studied is given by experiments on myosin filament-assembly. A detailed account of these and other experiments on myosin filament-assembly is to be published separately. The filaments used for these experiments assemble in vitro under specific conditions of salt and pH. The filaments are bipolar in structure and have a narrow length distribution. They assemble from approximately 100 monomer subunits and are structurally similar, but shorter than those encountered in vertebrate skeletal muscle itself. The marked pressure-sensitivity of the reversible monomer filament equilibrium was shown to exist by Harrington and his co-workers during ultracentrifuge studies [15]. The suitability of an assembly system

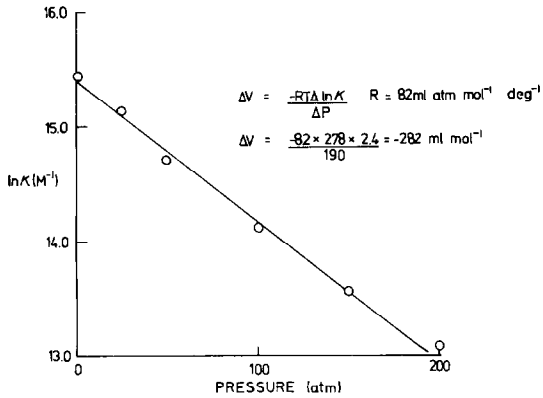

Fig.5. ΔV determination for the propagation stage of myosin filament assembly. The ΔV of -280 ml mol^{-1} was obtained from the pressure-dependence of the propagation equilibrium constant. The data was gathered over a pressure range of 1–200 atm for myosin in 0.160 M KCl, 2 mM $MgCl_2$, 5 mM Tris–HCl buffer, pH 8.3, at 5°C.

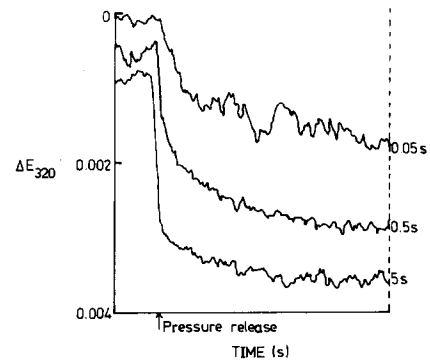

Fig.6. Small perturbation-relaxation experiments on the myosin filament assembly equilibrium. The same relaxation spectrum, recorded at different speeds is shown, the time given by each line being the length of the whole sweep. The pressure-jump was 4 atm. The equilibrium mixture contained 3 mg/ml myosin, 0.140 M KCl, 2 mM $MgCl_2$, 2 mM veronal, pH 8.3, at 5°C.

for investigation by pressure-jump can often be assessed from anomalous sedimentation equilibrium behaviour in response to the hydrostatic pressure gradient in the ultracentrifuge cell [15].

The pressure-jump equilibrium data on myosin assembly (fig.5) can be analysed by two different approaches. In one, the pressure-dependence of the overall equilibrium $K_{overall} = [F]/[M]100$ can be used to calculate a ΔV [15]. In the other, an assumption is made that, under conditions of partial filament dissociation, monomer addition and dissociation occurs at the filament ends [16]. Filament molarity thus remains constant. The pressure-dependence of propagation equilibrium constant $K_{prop} = 1/[M]$ can thus be assessed. Both approaches, despite different mechanistic bases, yield similar ΔV values for the assembly of myosin monomer into filament. This follows from the fact that, for all practical purposes, the value of both association constants depend on monomer concentration alone.

The large ΔV of -280 ml . mol^{-1} monomer for myosin assembly, allows for a choice as to the type of relaxation experiment one can perform. Relaxation following a small shift in equilibrium concentrations can be analysed by relaxation kinetic theory. Relaxation experiments of this type were performed on the myosin system with small 4 atm jumps (fig.6). The pressure change used resulted in a 0.5% change in myosin filament concentration. The temporal width of the relaxation spectrum, unfortunately, precluded analysis by the relaxation kinetic techniques developed for polymerizing systems [17]. Experiments in which a large shift in concentration occurred, relative to the condition at 1 atm, were analysed by standard kinetic techniques. Figure 7 illustrates such a large perturbation-relaxation from 200 atm. Kinetic analysis, in terms of a rate controlling second-order step at some point in the assembly process, appears to fit the data.

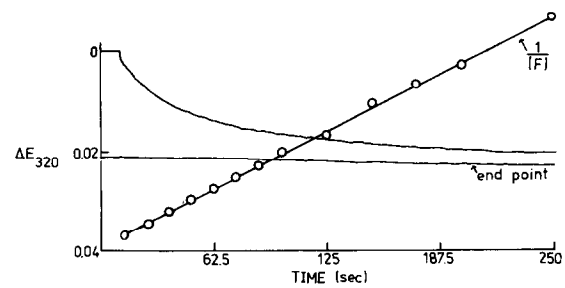

Fig.7. Large perturbation relaxation experiments on the myosin filament-assembly equilibrium. The relaxation, together with a second-order plot of the data analysed in terms of the mechanism $2A \rightleftharpoons A_2$ is shown. The pressure-jump was 200 atm. The equilibrium mixture contained 2 mg/ml myosin, 0.190 M KCl, 2 mM $MgCl_2$, 2 mM veronal, pH 8.3, at 5°C.

3.3. *'On enzyme equilibria'*

It was shown by Haldane [18] that the equilibrium between enzyme-bound substrates and products can be quite different from the overall equilibrium of reactants at catalytic enzyme concentrations. The realisation that many enzymes and other functional proteins occur locally at relatively high concentration and the development of techniques for the observation of short-lived intermediates focused attention on the equilibria between protein-bound reactants [19]. NAD^+-linked dehydrogenases and ATPases with different functions are of particular interest in this connection and a wide range of signals is available for their study [20]. The phenomenon of rapid equilibration of enzyme-bound substrates and products, prior to rate-limiting product dissociation, has resulted in some misinterpretations in terms of 'half of the sites' reactivity [20,21].

The possible applications of pressure-jump investigations to the study of such equilibria are illustrated by the following experiments. The equilibrium concentrations of the reactants in the system:

$$\text{Lactate} + NAD^+ \rightleftharpoons \text{Pyruvate} + NADH + H^+$$

can be changed by a sudden change in pH due to pressure-perturbation of the ionisation of the buffer medium. With phosphate buffer a pressure change of 30 atm causes a pH change of about 0.1. When the above reaction is catalysed by pig heart lactate dehydrogenase the use of different fluorescent and transmission signals makes it possible to distinguish between different forms of bound and free NADH [21]. Total NADH (bound and free) is monitored by change in transmission at 340 nm. The sum of all forms of bound NADH are monitored by measurements of protein fluorescence quenching and the ternary complex enzyme–NADH–pyruvate can be distinguished from the binary complex E–NADH by the considerably enhanced nucleotide fluorescence of the latter species.

Figure 8 shows two pressure-relaxation experiments with the system of reactants equilibrated in the presence of lactate dehydrogenase in phosphate and Tris buffer respectively. The much greater amplitude in phosphate buffer shows that the pressure-dependence of the equilibrium is principally due to the change in hydrogen ion concentration in the presence of phosphate buffer, which has a large volume change on ionisation. In these experiments total NADH (measured by extinction at 340 nm) increased as the hydrogen ion concentration decreased on pressure-release.

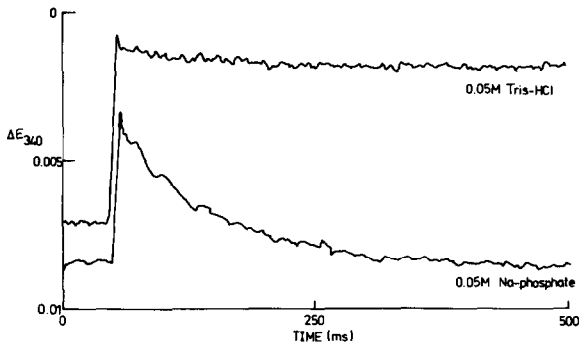

Fig.8. Pressure-jump relaxations of the overall lactate dehydrogenase equilibrium in Tris and phosphate buffers. The ΔP was 150 atm. The two solutions contained 56 μM pig heart LDH subunits, 10 mM NAD^+, 2 mM Li lactate, 0.1 M NaCl and either 0.05 M Tris–HCl or Na-phosphate buffer, pH 7.8 at 5°C, to start.

Protein fluorescence, which is recorded in fig.9 serves as a signal for the fraction of enzyme sites occupied by NADH and is insensitive to free NADH.

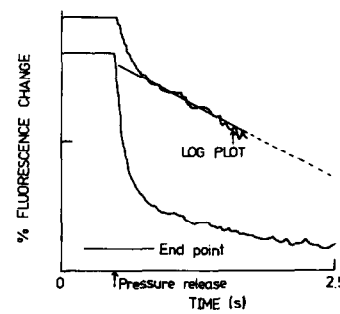

Fig.9. Pressure-jump relaxations of the overall lactate dehydrogenase equilibrium monitored by protein fluorescence quenching. The ΔP was 200 atm. The biphasic computer log plot together with a straight line drawn to the computer calculated reciprocal relaxation time of 1.8 s^{-1} for the slow phase. The solution contained: 1 μM pig heart LDH subunits, 20 μM NAD^+; 2 mM Li lactate; 0.1 M NaCl and 0.05 M phosphate buffer, pH 7.8 at 5°C, to start.

Two relaxations are observed under the conditions of these experiments. Preliminary studies of the substrate concentration-dependence on the relaxation times were compared with computer simulations (Hardman and Gutfreund, unpublished) based on the mechanism for lactate dehydrogenase recently proposed [20]. Both for measurements and simulation, lactate and varying concentration of NAD^+ are mixed with enzyme, left to reach equilibrium and then perturbed by change in pH. All together three relaxation times have been observed from protein fluorescence and extinction at 340 nm. It remains to be seen whether these can be used to characterise the steps associated with the interconversion of enzyme intermediates. One of the observed relaxation times is proportional to initial NAD^+ concentration. Simulation predicts such behaviour for the observation of the ternary complex NADH–*E*–pyruvate. Of the other two observed relaxation times one is independent of and the other inversely proportional to the initial concentration of NAD^+. The range of signals available for intermediates of this reaction makes this a promising system for study by the pressure-relaxation technique. Similarly reactions of myosin and other ATPases have the two characteristics (range of signals and intermediates in equilibrium) which promise useful information from such studies.

The use of our equipment for monitoring extinction and fluorescence at different fixed-pressures at equilibrium (see fig.5) should provide information about the pressure-dependence of individual steps with and without pH-perturbation.

4. Summary of application

In summary we should like to draw attention to the many different applications of the pressure-relaxation technique in the study of functional macromolecules.

The assembly of the quaternary structure of proteins is of interest both because it can give information about the structure and forces involved in their stability and because there are a number of systems in which association–dissociation phenomena can control function. The potential of pressure-relaxation for the study of the assembly of myosin polymers has been demonstrated. In vertebrate muscle the degree of association of subunits is likely to remain constant during its biological activity, however, some similar proteins like tubulin [16] may well assemble as an essential part of their function. Another possible application is the study of interactions related to physiological function of different protein components of the myofibril.

In many enzyme systems, subunit association is linked to ligand binding and control. There is not nearly enough information available about the rates of protein–protein interaction to draw conclusions about the relation between rate of association and the rate of change in activity [22]. It is of course best to study association rates with unmodified protein by monitoring light scattering or intrinsic fluorescence changes during association linked to ligand binding. However, the method recently used [23] to study the assembly of malate dehydrogenase by labelling with donor–acceptor dyes is a useful tool for following the rate of association of monomer to dimer. Preliminary pressure-relaxation experiments on this system indicate that, at the low concentration of the enzyme assay, the fully active species can not have associated to dimer.

Relaxation techniques have been used relatively little for the study of overall enzyme equilibria. The ideas outlined above for the study of 'on enzyme equilibria' should find wider applications for the study of elementary steps of enzyme reactions which are too fast for investigation by flow techniques.

Many processes involving conformation changes and phase transitions linked to temperature changes and/or ligand binding have been studied by the temperature-jump method. Further detailed investigation by pressure-relaxation techniques will give complementary information about the mechanisms of these transitions. Obviously the pressure-relaxation technique promises wide application for the study of phenomena controlled by ligand binding to a protein or a more complex system. The effects of pressure changes on anaesthetics and other drugs affecting membrane phenomena and the mitotic apparatus might be used to study rate processes in such systems. Only continuous extension of the range of kinetic tools and physical and chemical identification of intermediates can result in a satisfactory description of the dynamic behaviour of macromolecules during biological function.

Acknowledgements

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