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Measurement of Fast Biochemical Reactions

Flow and relaxation methods are being used to study chemical processes in biological macromolecules.

Alan N. Schechter

Biochemists are now attempting to describe the flow of energy and information in the cell in terms of more primary chemical processes in biological molecules (*1*). For macromolecules these processes include conformational isomerizations; helix-coil transitions; the formation of secondary, tertiary, and quaternary structure; and interactions with ligands. These processes, in turn, can be described by the processes of formation and disruption of electrostatic, hydrophobic, and hydrogen bonds and of the transfers of electrons and protons mediating changes in covalent structure.

These chemical processes, in general, occur with half-times of considerably less than 1 second and may be classified as "fast" (*2*). Most methods for studying such reactions are relatively new (*3*), and only in the last decade have these methods been extensively applied to biological molecules (*1, 4*). It is interesting, however, that much of the pioneering work in making fast reactions accessible to measurement came from biochemists. The continuous flow apparatus of Hartridge and Roughton (*5*) for study of reactions of hemoglobin with its ligands, and the accelerated and stopped flow instruments of Chance (*6*) for study of enzyme-substrate interactions first made it generally possible to measure chemical reactions with half-times in the millisecond range. Extension into the microsecond and nanosecond ranges did not occur until the work of Eigen and his collaborators (*7*) with chemical relaxation techniques in the 1950's. These relaxation methods were originally applied to the kinetics of chemical bond formation and disruption and resulted in the first accurate estimates of the rates of these processes. Table 1 lists these values for a number of chemical processes as a reference scale for considering reactions of biological macromolecules. Application of relaxation methods to biopolymers originated in Eigen's and Alberty's laboratories, but they are now employed by many other investigators. In addition, there have been continuous improvements in the performance and versatility of flow instruments so that these too are now being applied to a wider range of problems.

In this article it is my purpose to provide a general review of some of the recent applications of flow and relaxation techniques to fast biochemical reactions. Several of the methods, especially stopped flow and temperature jump, are of such general relevance that they are becoming part of the basic equipment of many groups studying proteins and nucleic acids. This change has been facilitated by the availability of several commercial flow and relaxation instruments (*8*) and of improved electronic and hydraulic components from which instruments can be constructed or from which existing equipment can be modified. Several articles and books devoted to the design and principles of these instruments have appeared (*7, 9*).

Flow Techniques

Continuous flow instruments are based on observation of a reaction along the length of a tube through which the mixed reagents are propelled. The elapsed time after the initiation of the reaction is a function of distance along the tube. This method still has certain advantages (*10*), but the frequent need in biochemistry for strict economy with reagents has made the stopped flow technique more generally useful. In stopped flow instruments, small volumes (usually about 0.1 ml of each) of two solutions are mixed together just before they enter a cell, which has observation ports or other sensors. Flow is mechanically

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Table 1. Typical rate constants for chemical processes as determined by relaxation methods [from Havsteen (*9*)].

Process	Bimolecular rate constants	
	Combination rate (M^{-1} sec^{-1})	Dissociation rate (sec^{-1})
Complex formation		
Diffusive encounter	$\sim 10^{10}$–10^{11}	
Proton transfer involving H^+ or OH^-	10^8–10^{11}	$\sim 10^{-2}$–10^7
Acid-base catalysis	10^8–10^{11}	~ 1 –10^7
Hydration and hydrolysis	$\sim 10^{-2}$–10^{12}	$\sim 10^{-2}$–10^7
Charge interactions	10^9–10^{11}	$\sim 10^{-7}$–10^7
Hydrophobic interactions	10^8–10^9	$\sim 10^2$ –10^4
*Electron transfer**		
	10^3–10^8	

* Also determined by flow and a combination of flow and temperature jump methods. See Eigen and DeMaeyer (*7*), Hammes (*87*), and Claesson (*1*) for rates for other reactions and for specific references. Rates are for reactions in solution near room temperature.

stopped, and then measurements may be made with a minimum of turbulence or other artifacts. The transient of the current or voltage of the detector system, as a function of time, is recorded for analysis.

Figure 1 is a diagram of the most widely used stopped flow instrument. This machine, based on the design of Gibson and Milnes (*11*), is available commercially. In addition a number of other instruments have been described (*12*), some of which are also manufactured. Several designs of fluid delivery components that may be used in conjunction with conventional spectrophotometers and fluorometers have been reported (*13*). These could go a long way toward making facilities for the study of fast kinetics routinely available. However, improvements in stability of power supplies and response time of the spectrometer photodetector are also usually necessary, as well as suitable equipment for recording the transients, such as an oscilloscope and camera. The recent introduction of multichannel pulse-height analyzers, and even "dedicated" computers (*14*), markedly facilitates the invariably slow process of preparing kinetic analyses of data from all of these instruments (see Fig. 2).

Despite this large variety of flow instruments, the hydraulic components set a lower limit, generally between about one-half and several milliseconds "dead time," before reactions can be monitored (*15*). It is unlikely that this limit (attained 50 years ago) will be materially improved in the near future because of the many hydrodynamic problems in mixing which arise under conditions of faster propulsion. Indeed, under usual conditions with viscous solutions of macromolecules this dead time may increase significantly.

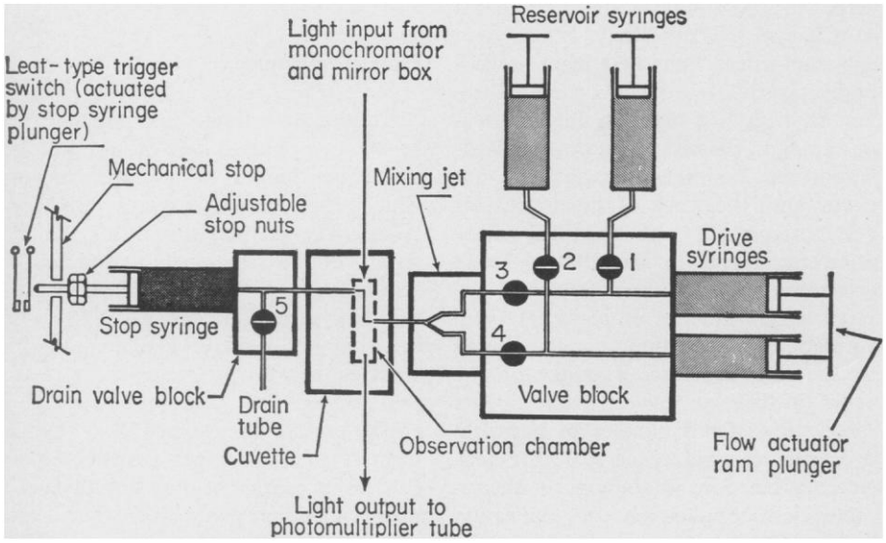

Fig. 1. Diagram of a stopped flow instrument manufactured by Durrum Instrument Co. on the basis of the work of Gibson and Milnes (*11*). For measurements by absorption spectrophotometry, the light input and photomultiplier are at opposite ends of the observation cuvette. Fluorescence can be measured by placing the photomultiplier at right angles to the light input; polarizing optics have been introduced on each side of the cuvette in certain applications.

Important advances have been made, however, in the range of physical measurements that can be accomplished in both continuous flow and stopped flow modes. Absorption spectroscopy remains the most widely used technique, but recent improvements in lamp design and electronic components have allowed access to a larger region of the ultraviolet spectrum. Changes in the intrinsic absorption bands of proteins and nucleic acids can now be measured to complement observations of substrates, cofactors, prosthetic groups, and dyes which absorb in the visible or near ultraviolet regions. Fluorescence can be detected by placing the photomultiplier at right angles to the light source (*16*). Again, electronic advances have made the spectral regions of intrinsic protein fluorescence useful for kinetic measurements. Determination of the polarization of fluorescence is possible (*15*), but there are few published studies with this method.

Since the inception of flow techniques there has been considerable interest in the measurement of optical rotation or circular dichroism. Despite the apparent simplicity of the technique and the description of test reaction measurements (the inversion of sucrose for example) (*17*), there have been few biochemical applications. In the author's experience, pressure-induced cell window birefringence and mixing artifacts severely limit this method in the stopped flow mode.

Among the other physical measurements that have been made on rapidly mixed solutions, one may include calorimetry (*18*), *p*H (*19*), polarography (*20*), and electron paramagnetic resonance (*21*). Although still on a time scale considerably longer than 1 second, continuous flow techniques have been applied to the x-ray crystallography of proteins (*22*), and could be used for Fourier transform nuclear magnetic resonance spectroscopy (*23*).

Chemical Relaxation Techniques

Relaxation kinetics are based on the analysis of the return to equilibrium of a chemical reaction after perturbation. The perturbation may be a step or a periodic function, and it may involve any physical or chemical variable. If the displacement from equilibrium is small, the rate of return may be de-

scribed by a set of linear differential equations.

For a reaction of the form

$$A \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} C$$

the rate expression for a step perturbation is

$$-d\Delta c/dt = \Delta c/\tau$$

where Δc is the deviation of each component from its equilibrium concentration, under the new conditions, and τ is the relaxation time, the time for Δc to be reduced to 1/e of its value at the time of perturbation. It can be shown (*24*) that

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

The relaxation time is the reciprocal of the overall $(k_{12} + k_{21})$ first-order rate constant of the reaction, and is of the same order of magnitude as the half-time of the reaction. If the equilibrium constant of the reaction is known, the individual values of k_{12} and k_{21} can be determined.

For a reaction of the form

$$A + B \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} C$$

$$1/\tau = k_{12}(\bar{c}_A + \bar{c}_B) + k_{21}$$

where $\bar{c}_A$ and $\bar{c}_B$ are the equilibrium concentrations, under the new conditions, of A and B. This dependence of τ on concentrations, for bimolecular and higher-order reactions, may be used to distinguish these from reactions which are first-order in each direction.

Eigen and DeMaeyer (*7*) have published a detailed theoretical treatment of step and periodic perturbations, including mathematical solutions for more complex reactions.

Temperature jump is the most frequently used biochemical perturbation technique. "Joule" heating, by the discharge of a condenser in a suitably conducting reaction mixture, can be used to elevate the temperature by 5° to 10°C in about 1 microsecond (*25*). At least three instruments are commercially available for this technique, and a schematic diagram of the components of such an apparatus is shown in Fig. 3. Flash heating (*26*) has also been employed in a number of systems. Heated or cooled water, circulated around the cell, has been used for temperature perturbations (positive or negative) of relatively slow reactions (*27*). As with flow systems, many modes of physical and chemical observation of a reaction may be employed. Most biochemical work has been done with absorption spectroscopy in the visible region, but ultraviolet absorption, fluorescence emission and polarization, optical rotation, and other parameters have also been measured with temperature jump instruments (*25*).

The other step, or periodic, perturbations used for chemical and biochemical reactions were originally described by Eigen and include pressure jump, ultrasound, and electric field (*28*). A given reaction may be studied with a given intensive physical parameter if there is a net change in the corresponding extensive property of the system during the reaction. Thus, temperature jump perturbation analyses require a change in enthalpy, and pressure or sound perturbation analyses require a change in volume. However, several reactions may be linked in series to generate a measureable change.

Irreversible reactions, or reactions in which equilibrium is very far to one side or the other, are more difficult to study by perturbation methods. Enzyme catalysis frequently falls into this latter class and may best be studied with substrate analogs, for example, which bind but are not covalently changed after combination with the enzyme. Another approach to this problem is to study reactions shortly after they have been initiated, when all reagents are still present in significant amounts. Several groups have published descriptions of combined stopped flow and temperature jump instruments in which the perturbation is applied shortly after the reaction has been initiated by mixing (*29*). This combination should broaden the scope of relaxation techniques in biochemistry.

Figure 4 is a diagram of the approximate time ranges accessible with the major perturbation methods of chemical relaxation. Manual, flow, and other perturbation methods are also indicated for reference.

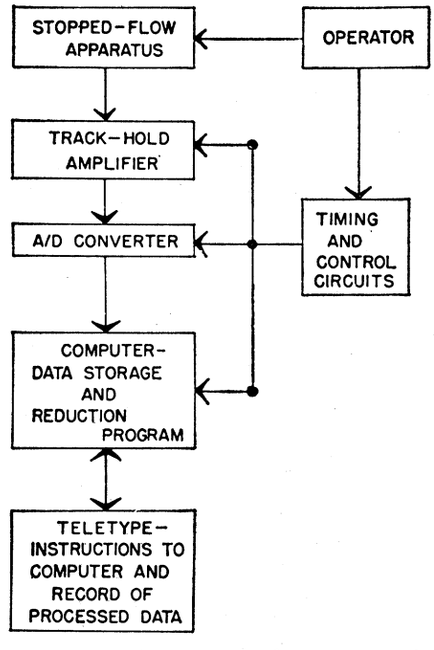

Fig. 2. Block diagram of an automatic data acquisition and processing system for kinetic instruments from DeSa and Gibson (*14*). A "track-hold" amplifier samples the voltage of the photomultiplier at intervals, specified by the timing and control circuits, and the analog to digital (A/D) converter makes these data available in suitable form for storage in the digital computer. The computer is then used with appropriate programs for data reduction and presentation.

Other Techniques

All ways of observing a chemical reaction at equilibrium involve a perturbation and relaxation. This clearly applies to methods closely related to chemical relaxation spectroscopy, such as many applications of flash photolysis in which a photochemical displacement from equilibrium is produced (*30*). Certain applications of flow methods, such as the rapid dilution of a solution by mixing with another volume, are also relaxation measurements. Even absorption, fluorescence, nuclear magnetic resonance, electron spin resonance, and other physical techniques are dependent upon a relaxation process following perturbation with an electromagnetic field. If we know the intrinsic "physical" rates of these processes, it is frequently possible to calculate "chemical" rates from analyses of the spectroscopic parameters, such as line width or polarization.

These physical methods, some of whose time scales are indicated in Fig. 4, are just now being applied to biochemical problems and may markedly expand studies into the nanosecond or shorter time ranges. The development of appropriate pulsing techniques, such as the laser, has facilitated these applications. The difficulty that occurs in these methods, but one that is present to an extent in all kinetic techniques, is the ascertaining of a sufficient number of parameters to characterize adequately the processes of interest in a biochemical interaction involving many thousands of atoms.

Competition between chemical processes may also be used to determine

rates. A number of techniques in biochemistry employ reactions measured in minutes or hours, but give information about underlying fast chemical processes. Hydrogen-deuterium or hydrogen-tritium exchange rates for many atoms in proteins or nucleic acids are measurable by manual techniques, but reflect competition between two chemical processes: the intrinsic hydrogen exchange rates of nitrogen and oxygen atoms with solvent, and the "opening and closing" of the biopolymers so as to expose these atoms to solvent (*31*). Based on hydrogen exchange measurements, the kinetics of the opening and closing of hydrogen bonds in nucleic acids and proteins have been analyzed. Rates in the range of 5×10^4 to 5×10^6 hydrogen atoms exchanged per nucleotide pair per second for DNA have been estimated by these methods (*32*). In this way certain fast reactions can be studied leisurely. However, the limiting factor is usually the derivation of a suitable set of theoretical equations so as to allow calculation of the individual rates of the competing chemical reactions.

Steady state enzyme kinetic analyses are predicated upon the competition between the reaction of binding and dissociation of substrate and the reaction describing the covalent change. Data from the observation of relatively slow changes in substrate level are analyzed by the physical models of Henri-Michaelis-Menten, Briggs-Haldane, or others to investigate presumed intrinsic fast reactions (*33*). As with hydrogen exchange techniques, the analyses are strictly limited by the formal model used.

In general it is probable that chemical kinetics derived from the study of competition between chemical reactions and physical processes are more likely to be reliable than kinetics derived from competing chemical reactions. In all cases, however, "direct" observations of chemical reactions with respect to the number and detailed properties of intermediates are preferable to inferences from extensive analyses of steady state or "slow" kinetics. Flow and chemical relaxation methods allow study of reaction components by multiple physical techniques. It is for this reason that these methods have already made many contributions to the understanding of biochemical systems (*34*).

Several recent investigations in biochemical kinetics will be reviewed to illustrate the extent of interest in the applications of flow and chemical relaxation techniques.

Helix-Coil Transitions

In 1964 Lumry, Legare, and Miller (*35*) used a temperature jump apparatus with polarimeter optics to follow the change in optical rotation of a poly-L-glutamic acid solution in aqueous potassium nitrate. The jump of 8°C, in the middle of the region of the acid-induced helix-coil transition, resulted in a significant change in the rotation of plane polarized light. The transient, however, was always less than the 20-microsecond resolution of the instrument, indicating that the rate constant of the transition was greater than about 5×10^4 sec^{-1}. Hamori and Scheraga (*36*) made use of temperature jump to cause a rapid drop in *p*H in an alkaline solution of poly-L-tyrosine in 0.2*M* sodium chloride and, with the aid of curve fitting, set an upper limit of about 2 μsec for the duration of the resultant optical rotation transient.

Subsequent studies with poly-L-glutamic acid (*37*) and with poly-L-ornithine (*38*) by ultrasonic attenuation have given a value of 1×10^{-8} second for the relaxation time of the volume change accompanying their helix-coil transitions. Dielectric relaxation methods give a comparable value for poly(γ-benzyl-L-glutamate) (*39*). These values agree well with the theoretical predictions of Schwarz (*40*), but are several orders of magnitude smaller than those arrived at from the interpretation of the nuclear magnetic resonance spectra of these and other polypeptides (*41*). The appearance of double α-CH and NH proton peaks in magnetic resonance spectra had been interpreted by several groups as indicating chemical exchange between helical and coiled segments, with a rate constant of about 10^2 sec^{-1}. However, several alternate interpretations of these proton magnetic resonance results have now been offered (*42*) which are more consistent with the chemical relaxation data. It is likely then that the transition from α-helix to disordered coil in these homopolymers is a strongly cooperative process which occurs in nanoseconds.

The renaturation rates of double stranded DNA, measured by decreasing ultraviolet absorption as the temperature is rapidly lowered below that of the melting transition, can be observed by manual methods. Absorption changes occur over minutes and obey overall second-order kinetics (*43*). The results have been interpreted, however, in terms of a model with nucleation events and subsequent rapid propagation processes. In this model, the individual propagation step of forming hydrogen bonds between paired nucleotides has a second-order rate constant of about $5 \times 10^9 M^{-1}$ sec^{-1}, which is near the limit set by the rate of diffusion.

A more direct approach to observing such nucleation and propagation events has been the study, by temperature jump methods, of double helices formed by oligonucleotides of adenine and uridine (*44*). Appropriate studies on oligo-

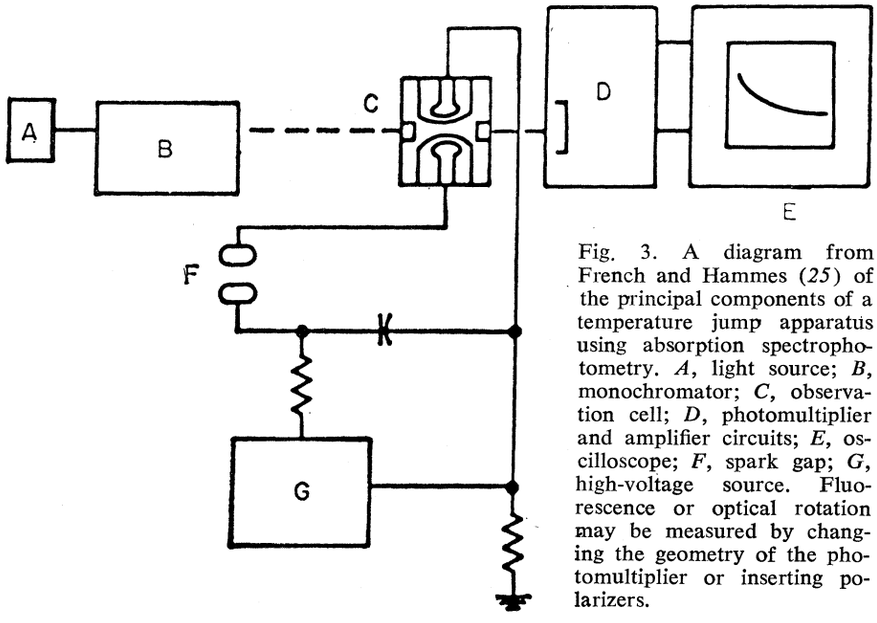

Fig. 3. A diagram from French and Hammes (*25*) of the principal components of a temperature jump apparatus using absorption spectrophotometry. *A*, light source; *B*, monochromator; *C*, observation cell; *D*, photomultiplier and amplifier circuits; *E*, oscilloscope; *F*, spark gap; *G*, high-voltage source. Fluorescence or optical rotation may be measured by changing the geometry of the photomultiplier or inserting polarizers.

nucleotides, of various lengths and at several concentrations, have given evidence for the nucleation reaction (which requires the simultaneous formation of hydrogen bonds between three adjacent base pairs) and the subsequent propagation process at about 10^7 base pairs per second.

Helix-coil transitions of two half molecules of yeast phenylalanine specific transfer RNA have been studied by absorption changes at 260 nm after temperature jump perturbation (*45*). The results also support a rate-limiting nucleation process and subsequent "zippering up." In addition, a fast relaxation event (< 5 μsec) is noted, which may be attributable to the melting of single-stranded stacked nucleotide bases.

Nucleic Acid–Small Molecule Interactions

Temperature jump and flow studies have been particularly useful in elucidating the mechanism of the interaction of nucleic acids with molecules such as proflavine and ethidium bromide, which are thought to intercalate between adjacent base pairs.

Relaxation studies of the proflavine-DNA complex by Li and Crothers (*46*) demonstrate two distinct relaxation processes after a temperature jump. One relaxation is in the millisecond range; the other is faster. Such results are suggestive of a physical model of the following form:

$$\mathrm{P} + \mathrm{DNA} \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} \mathrm{DNA\text{-}P\ (outside)}$$

$$\underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} \mathrm{DNA\text{-}P\ (inside)}$$

where proflavin (P) and DNA first combine in a bimolecular reaction ($k_{12} \sim 1 \times 10^7 M^{-1}$ sec^{-1}), with the dye electrostatically held on the outside of the double helix, followed by a first-order intercalation process ($k_{23} \sim 1.5 \times 10^3$ sec^{-1}).

Temperature jump studies of the ethidium bromide–transfer RNA complex show only one fast relaxation, but flow studies demonstrate several slower first-order processes—again corresponding to intramolecular rearrangements after rapid bimolecular complexing (*47*).

It is likely that these studies will soon be extended to the interactions of nucleic acids with proteins and other ligands.

Protein Conformation Changes

The three-dimensional conformation of globular proteins is generally believed to represent the form with the lowest free energy (*48*). Folding, after biosynthesis, seems to be primarily specified by the amino acid sequence, but little is known of the mechanisms of folding. The study of denaturation and renaturation rates should offer information about such processes.

Many of the values for the rate of denaturation and renaturation in the literature are very slow, with half-times of minutes or hours. However, some of these studies are not on well-defined, reversible transitions. Recently Pohl (*49*) has used temperature perturbation methods to follow the reversible denaturation of chymotrypsin and trypsin in acid. These renaturation rates are first-order with respect to protein concentration and are about 10^1 to 10^2 sec^{-1}.

The denaturation of hemoglobin upon mixing with acid has been studied by stopped flow with absorption optics (*50*). Under certain conditions the reaction can be resolved into three phases. There is an initial fast reaction (with a half-time of about 10 msec) which is manifested as a red spectral shift and seems to correspond to a proton-induced change in the environment of the heme. This is followed by the expulsion of the heme group, a reaction whose half-time varies from 10^{-1} to 10^3 seconds over the *p*H range studied, and is in turn followed by a dimerization of the free heme groups.

Stopped flow spectrophotofluorometry has been used to follow the renaturation, in neutral buffer, of acidified staphylococcal nuclease (*51*). This large conformational change is relatively fast, as compared to such conformational changes in other proteins, and is complete in 1 to 2 seconds. The simplest model to describe the data, however, requires that the change in fluorescence reflects two first-order processes, with rate constants of about 2 and 12 sec^{-1}. The renaturation of collagen as measured by temperature perturbation, with optical rotation optics, is much slower but is also best described by several first-order reactions (*52*).

In a few proteins, then, there is a suggestion of several processes, perhaps including nucleation events, in folding. There is an extensive body of information on the thermodynamics of the denaturation of proteins (*53*), but whether such folding and unfolding reactions are usually cooperative, one-step processes or are usually multistep, is uncertain. Direct examination of these reactions, by flow and chemical relaxation methods in the appropriate time ranges, can demonstrate intermediate states when they exist and possibly correlate these intermediates with known chemical processes.

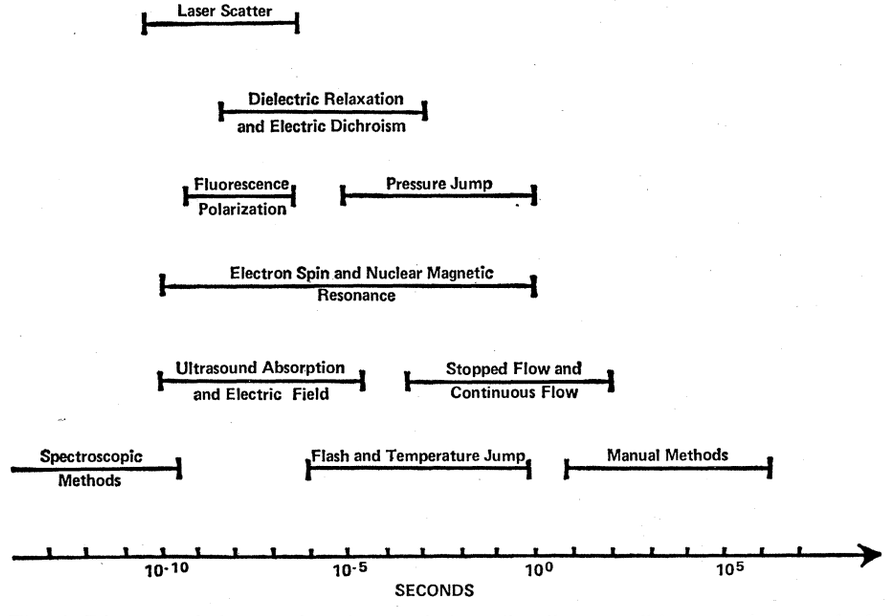

Fig. 4. Diagram of the reaction time periods ordinarily accessible to various methods of measuring fast processes, modified from Eigen and DeMaeyer (*7*) and Havsteen (*9*). The limits are somewhat arbitrary and in some cases can be significantly extended. *Spectroscopic Methods* refers to such processes as light-induced electronic transitions.

Crystalline and Soluble Proteins

Chance, Theorell, and their collaborators have used stopped flow techniques to compare protein-ligand interactions in the soluble and in the crystal phase. This is a problem of immediate interest in biochemistry because of extensive studies by x-ray diffraction of the detailed three-dimensional structures of a number of protein crystals.

Crystalline alcohol dehydrogenase from horse liver combines with nicotinamide adenine dinucleotide at a rate that is 1000 times reduced from that in solution (*54*). The reactions of crystalline ferrimyoglobin and ferrihemoglobin with azide are also significantly slowed in comparison to the reactions in solution (*55*). [In these studies the reactions are followed with double-beam spectrophotometry (*56*) to allow correction for light scattering by the crystals, and appropriate controls are done for the effects of the crystallizing medium.] These reductions in combination rates are more than can be accounted for by diffusion limitation in the crystal. The data, including the temperature dependence of these rates, have been interpreted as indicating that crystal lattice forces may impede such reactions by slowing necessary protein conformational isomerizations. Thus, these studies demonstrate at least one significant difference between crystalline and soluble proteins.

Enzymatic and Hemoprotein Reactions

Fast reaction methods are, in general, necessary in studying enzymatic activity at high concentrations of enzyme and substrate. These levels are approximations to what is thought of as "physiological" cellular concentrations, and allow direct study of the enzyme itself, which is now as much a reagent as a catalyst.

An excellent example of the utility of fast kinetics in helping to distinguish among several possible reaction mechanisms is the study from Eigen's laboratory on the binding of nicotinamide adenine dinucleotide to yeast D-glyceraldehyde-3-phosphate dehydrogenase (*57*). This interaction is cooperative and can be described by both of the major allosteric models (*58*). The models differ, however, in the number of relaxation processes that one would expect after a perturbation. Temperature jump measurements showed three relaxation events, with apparent rate constants of about 7000, 700, and 0.2 sec^{-1}. However, concentration studies indicated that two of these relaxation processes were bimolecular reactions, while the fastest probably reflected an intramolecular isomerization. These findings for the reactions of D-glyceraldehyde-3-phosphate dehydrogenase are consistent with the kinetic predictions of the allosteric model of Monod, Wyman, and Changeux. The generality of this finding for other allosteric enzyme systems, however, is still uncertain.

Hammes' group has used the temperature jump method to study the interaction of bovine pancreatic ribonuclease with substrate analogs, such as uridine 3′-monophosphate and cytidine 3′-monophosphate (*59*). The data best fit an enzyme model with three ionizing groups which affect the binding of substrate analogs. One of these groups is probably involved in a conformational isomerization of the protein, which is seen as a spectroscopic change with a relaxation time of 0.5 to 1.0 msec. These results, including the pK values of the ionizing groups as determined by relaxation measurements as a function of pH, correlate well with information about this enzyme from nuclear magnetic resonance (*60*) and x-ray crystallography (*61*) studies.

Flow and relaxation techniques are now being applied to a great many combinations of enzymes with cofactors, inhibitors, and substrates. Among the enzymes being studied are chymotrypsin (*62*), homoserine dehydrogenase (*63*), L-glutamate dehydrogenase (*64*), lactate dehydrogenase (*65*), alcohol dehydrogenase (*66*), D-amino acid oxidase (*67*), p-hydroxybenzoate hydroxylase (*68*), carbonic anhydrase (*69*), myosin-adenosine triphosphatase (*70*), and others. A detailed review of these experiments is beyond the scope of this article, but it may be noted that in several of these studies there is evidence for one or more intermediates after the original bimolecular reaction (usually at almost diffusion controlled rates) of the enzyme with the ligand. This may be diagrammed as follows:

$$E + L \rightleftarrows (E\text{--}L) \rightleftarrows (EL)' \rightleftarrows (EL)'' \rightleftarrows (EP) \rightleftarrows E + P$$

where enzyme (E) and ligand (L) collide to form an encounter complex (E--L). The complex then undergoes one or more conformational changes to intermediates denoted (EL)′, (EL)″, and the like. When the ligand is a substrate these changes continue until the formation of the enzyme-product complex (EP), and its subsequent dissociation to free enzyme and product (P). The conformational changes generally occur with a relaxation time of about 10^{-3} second. In most of the enzymes with such sequences of events, only one intermediate has been necessary to describe the data. However, in some cases as many as 16 discrete steps have been suggested on the basis of 11 relaxation processes (*71*). It seems likely that these small, fast conformational changes are important in the mode of action of many enzymes. There have been several suggestions that very precise orientation between enzyme and substrate, for electron orbital overlap or directed proton exchange, accounts for part of the extraordinary efficiency of these biological catalysts (*72*). These orientational requirements could be continually obtained during catalysis by the subtle conformational isomerizations.

The extensive studies, with many flow technique innovations by Chance and his collaborators and by other workers on the kinetics of enzyme-catalyzed electron transfer in solution and in biological structures as complex as the mitochondrion have been reviewed (*73*).

The kinetics of the combination of oxygen and other ligands with hemoglobin and other hemoproteins are well characterized. Isolated α and β chains have been shown to combine with oxygen significantly faster than the $\alpha\beta$ combination, but the oxygen dissociation rates are more closely comparable (*74*). Gibson has presented a complete kinetic description of the oxygenation process, based on stopped flow measurements, with calculated rate constants for the four consecutive reversible reactions (*75*). This model, with significant contributions from both dimeric and tetrameric interactions, however, is not a unique interpretation of the data, as he indicates. The reader is referred to other papers (*76*) and reviews (*77*) of this complex problem.

Faster Biochemical Processes

The biochemical studies surveyed illustrate that flow and chemical relaxation techniques have made the millisecond and microsecond time ranges accessible for the investigation of a wide range of questions. Certain of the chemical and physical relaxation methods can now be used for the nanosecond and even shorter time ranges. It is expected that these methods will eventually be useful for studying many

biochemical processes, including those related to the formation and disruption of covalent and noncovalent bonds. So far, these fast techniques have been applied to only a few biochemical problems and, in general, are still quite limited in the amount of information they can supply to delineate a biochemical process. However, for highly cooperative transitions, as illustrated in the ultrasonic studies of the helix-coil transition, this information may be quite important.

The demonstration, by two independent methods, of rotational freedom in antibody molecules is another example of the ability of very fast methods to answer selected biochemical questions. Cathou and O'Konski (*78*) have found that the electric field–induced birefringence of antibody molecules is constant with varying amounts of charged hapten bound to the protein. This finding best fits a previous model of antibody structure which located antigen binding sites on two "arms" of a flexible Y-shaped molecule (*79*). Similarly, Yguerabide, Epstein, and Stryer (*80*) have used nanosecond lamp pulses to measure explicitly the change of fluorescence anisotropy (an index of polarization) of a 5-dimethylaminonaphthalene-1-sulfonamide-ε-lysine hapten bound to an antibody, as a function of time, and find that their data fit a model that requires considerable molecular flexibility. Internal motion or flexibility in DNA has also been detected using nanosecond flash techniques for measurements of polarization (*81*). These nanosecond lamp-pulse methods are an extension of the classical work of Weber (*82*) on the polarization of fluorescence.

The magnetic resonance studies of Cohn and her associates (*83*) on the geometry of the active sites of several metallo-enzymes are further examples of the biological use of the measurement of fast relaxation processes. Electric field–induced dichroism measurements have been used to study the orientations of the backbone and side chains of several polypeptides (*84*). Very recently the angular correlations of gamma radiation from nuclides bound to proteins (*85*), and the spectrum of laser light scattered from nucleic acids (*86*), have been used to measure rotational and translational diffusion processes in these macromolecules.

Although the range of biochemical problems already studied with fast reaction methods is great, it is apparent that only a few systems have been examined in great depth. For certain problems, techniques may not yet be sufficient to answer some important biochemical questions. However, the extent of current methods to measure reactions, over a range of more than 15 orders of magnitude in duration, makes it likely that many more biochemical phenomena will be described in terms of those processes that are fast in comparison to our usual perception of time.

Summary

In the 4 years since Hammes (*87*) reviewed, in this journal, the application of flow and relaxation techniques to the study of primary processes in chemical reactions these methods have been applied to a wide range of biochemical problems. These applications have been facilitated by advances in stopped flow and temperature jump instrumentation, especially with respect to the range of physical properties that may be observed during a reaction, and the availability of instruments.

Information about rates and mechanisms of conformational changes in polypeptides, polynucleotides, proteins, and nucleic acids have been obtained. Structural changes, from small isomerizations to major transitions such as denaturation-renaturation and helix-coil reactions, have been studied. The significance of the spectrum of conformational change for many biochemical processes is just beginning to be understood. The continued use of these and newer relaxation techniques should further merge the biological and chemical descriptions of these processes.

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Semiconductor Radiation Detectors

Basic principles and some uses of a recent tool that has revolutionized nuclear physics are described.

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Just as the theory of atomic structure grew from spectroscopic observations of transitions in the atomic shell, so nuclear theory has developed from studies of the radiations emitted in nuclear transitions. But while the light produced or absorbed in atomic transitions is easy for us to see and measure, we have no simple way of observing nuclear radiations, which may be charged particles (electrons, positrons, protons, and heavier nuclear constituents), or electromagnetic radiations of very short wavelength (gamma rays). Necessarily then, the progress of nuclear physics has closely followed the development of new means of detecting and measuring nuclear radiations.

In the past decade, we have witnessed a major revolution in nuclear experiments as a new device, the semiconductor radiation detector, has appeared on the scene, providing relatively simple and accurate methods of measuring the energy of many of the radiations produced in nuclear processes. Knowledge of fission and of the structure of nuclei, detection of new transuranic elements and determination of their properties, and exploration of the nuclear surface all have advanced because of the development of these new detectors.

Even outside the field of nuclear physics, the influence of these detectors is being strongly felt. In archeology, because of semiconductor radiation detectors, specimens can now be analyzed in fine detail by observing the gamma rays emitted from a sample after neutron bombardment in a reactor, or by observing the characteristic fluorescent x-rays produced when a sample is exposed to an x-ray or gamma-ray source. Biology, geology, mining, criminology, and many types of industrial processing have taken advantage of this new analytical tool.

The power of semiconductor detectors in analysis is graphically illustrated by comparing the gamma-ray spectra

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