METHODS OF BIOCHEMICAL ANALYSIS

Edited by **DAVID GLICK**

Cancer Biology Research Laboratory
Stanford University Medical Center
Stanford, California

VOLUME 31

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PREFACE

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned not only with the results in the developing fields but also with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, applier, the teacher, and the student.

It is particularly important that review services of this nature should include the area of methods and techniques because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore, an emphasis on methodology and instrumentation is a fundamental need in order for material achievement to keep in sight of the advance of useful ideas.

The volumes in this series are designed to try to meet the need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological, and if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, and so on.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate, these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evaluation of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analysis.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, and point of view. It is the Editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the Editor to make this series of volumes as useful as possible and to this end suggestions will be always welcome.

DAVID GLICK

CONTENTS

The Rapid-Flow-Quench Method in the Study of Fast Reactions in Biochemistry: Extension to Subzero Conditions

THOMAS E. BARMAN and FRANCK TRAVERS *Institut National de la Santē et de la Recherche Médicale, U128, CNRS, Montpellier, France*

I. INTRODUCTION

Kinetic methods are a means of elucidating the number of intermediates on an enzyme reaction pathway. They can give information on the interconversions of the intermediates and are a first step toward understanding the mechanism of enzyme catalysis. There is increasing evidence that enzyme mechanisms involve ligand-induced conformational changes. An understanding of these is important; they may be concerned in enzyme specificity (Koshland, 1958). Further, conformational changes are often rate limiting and may serve as sites for the control of activity or the interaction between different enzyme systems (e.g., Engelborghs et al., 1975; Gutfreund, 1975).

Under ambient conditions, most enzyme-catalyzed reactions are rapid with k_{cat} values above 50 sec^{-1}. Thus, the rate-limiting step on the reaction pathway of even a slow enzyme has a half-life of less than 14 msec. Further, not all enzymes have a clearly rate-limiting step (e.g., Knowles, 1976). Enzyme reaction pathway must therefore be studied in the millisecond time range. Also, several enzymes (e.g., certain of the glycolytic enzymes) occur at high concentrations, and if one wants to study these under physiological concentrations, the millisecond reaction time range is again necessary.

There are different ways of studying enzyme reactions. One can carry out experiments under optimum reaction conditions. Here, as indicated above, the time range is in milliseconds and fast reaction equipment is required. On the other hand, one can work under suboptimum conditions, in the second or minute time range, using manual sampling techniques. A third approach is to carry out experiments with a rapid-reaction device under suboptimal conditions.

The first rapid-reaction apparatus, constructed by Hartridge and Roughton in 1923, was a continuous-flow apparatus and is the ancestor of two present-day apparatuses: stopped flow (recently reviewed in this series by Hiromi, 1980) and flow quench (Gutfreund, 1969).

The stopped-flow method is easily the most used rapid-reaction method. Experiments are rapidly performed with the expenditure of little material. Further, several commercial stopped-flow apparatuses are available. However, this method cannot be used with all enzyme systems. With it, the system under study must give some optical signal, and a large number of enzymes do not have the necessary optical properties. Further, even when there is an optical signal, its assignment can be difficult.

In the flow-quench method enzyme and substrate are mixed, the reaction mixture allowed to age and then stopped (quenched) by the addition of a suitable quencher (e.g., acid). The quenched-reaction mixture is then assayed at leisure by any suitable chemical, physical, or enzymic method. Assays can be chosen that are at once highly specific and sensitive. The number of specific chemical reactions available is large, and the use of radioactive substrates can ensure great sensitivity. In special cases reaction mixtures can be frozen and then studied by physical methods such as electron spin resonance (e.g., Bray,1964). The flow-quench method is, therefore, of a more general applicability than stopped flow in that it does not depend on a specific physical signal on ligand binding. It is a chemical sampling technique, and in many cases reaction intermediates can be identified and studied. In such cases their further study can be carried out by the easier stopped-flow method.

Another advantage of the flow-quench method is that opaque, even particulate, systems can be studied, for example, membrane-bound enzymes (sarcoplasmic reticulum ATPases, Froehlich and Taylor, 1975, 1976; Briggs et al., 1978; Sumida et al., 1978; Lowe and Smart, 1977) and intact chloroplasts, (Smith et al., 1976). Further, the method can be scaled up for preparative purposes.

On the other hand, the flow-quench method is a point-by-point method and is laborious. Unlike the stopped-flow method, where a complete kinetic curve is obtained from a single reaction mixture, each experimental point is obtained with a different reaction mixture. For high

accuracy, comparatively large reaction volumes are required. Finally, the flow-quench method suffers from a fundamental weakness in that a quenched reaction mixture is studied rather than the reaction mixture itself.

A further approach to study rapid reactions is to decrease the rapidity by carrying out experiments under suboptimum conditions (see, e.g., Yagi, 1971). A way of doing this is to lower the temperature. This necessitates the addition of an antifreeze (e.g., organic solvent, salt, lipid micelles) and has led to the development of a new technology (subzero enzymology or cryoenzymology; e.g., Douzou, 1974, 1977, 1980; Auld, 1979; Fink and Cartwright, 1981).

Cryoenzymology not only slows down a reaction according to the Arrhenius relationship, but it is also a useful perturbant. First, the antifreeze used may selectively affect certain rate constants. Second, due to differences in the energies of activation of the various rate constants on a reaction pathway, perturbation by temperature may apparently change the pathway. We give examples of the use of cryoenzymology as a perturbant in Section VI.

Adapting a rapid-reaction technique to low-temperature work increases the time resolution and has been done with the stopped-flow method (Allen et al., 1960; Hui Bon Hoa and Douzou, 1973; Auld, 1979; Travers and Barman, 1980; van Wart and Zimmer, 1981; Hooper et al., 1983). Recently a rapid-flow-quench apparatus operating down to at least −20°C was constructed (Barman et al., 1980). With this apparatus the reaction pathways of myosin subfragment-1 ATPase (Barman et al., 1983; Biosca et al., 1983; Biosca et al., 1984b) and creatine kinase (Barman et al., 1980) have been studied in the temperature range +35 to −15°C. The antifreeze used in this work was 40% ethylene glycol.

The rapid-flow-quench method was last reviewed by Gutfreund (1969). In this chapter we discuss some recent developments, in particular the adaptation of the flow-quench method to cryoenzymic conditions. Emphasis is placed on the more practical aspects of the method, especially on the problem of quenching. Quenching is at once the weakness and strength of the method: it is difficult to stop a reaction without perturbing it, yet by a careful choice of different quenching agents important information about different reaction intermediates can be obtained.

II. INSTRUMENTATION

1. Introduction and Principles

The chemical sampling and quenching of reaction mixtures of ages 10 sec or more can be done by hand and is a commonly used method in enzyme

analysis. In cryoenzymic work the hand sampling of reaction mixtures younger than 30 sec is difficult. To sample reaction mixtures at shorter times than these, one needs special techniques such as flow-quench devices.

Flow-quench devices consist essentially of two parts: the syringes, mixing chamber, and reaction tube and the drive mechanism and appropriate control unit. The principles involved are relatively simple, but the construction of a device needs care, especially for cryoenzymic work. There are four basic requirements:

1. The reaction times (from milliseconds to several seconds) must be accurately known.
2. The whole apparatus must be thermostatically controlled.
3. All the surfaces in contact with the reagent must be of chemically inert materials.
4. The apparatus must be economic in the use of materials.

To cover as large a time range as possible, one can construct two flow-quench devices: a rapid flow quench for times in the millisecond range and a time-delay flow quench for times 0.4 sec and up. The outlines given below are of equipment constructed in this laboratory, but the principles remain very similar for other apparatuses.

A. RAPID FLOW QUENCH

The principle of the rapid-flow-quench apparatus is illustrated in Fig. 1. It is essentially a continuous-flow apparatus (Hartridge and Roughton, 1923).

At zero time the tubes are filled with the different solutions up to the taps (W_1, W_2, and W_3), but the remaining spaces are empty (i.e., from the taps through the mixers M_1 and M_2 to the sample-collecting tube).

In an experiment the drive system pushes the plungers of the three syringes at constant speed. Enzyme and substrate are mixed in M_1, and the reaction mixture fills and passes through the reaction tube, T, at a constant speed s. Thus, at any given point along T the age of the reaction mixture is given by $t = V/s$ where V is the volume between M_1 and the point. At this point there is a second mixer, M_2, where the quencher is injected (the volume W_3 to M_2 is smaller than the volume W_1, W_2 to M_2 to ensure that the quencher arrives before the reaction mixture in M_2). The reaction is therefore stopped at time t, collected, and then chemically assayed for the chemical species present at this time t. The apparatus functions for a considerably longer time than t, and one collects a volume of quenched reaction mixture considerably larger than V. The apparatus is washed and dried by the use of the taps W_1, W_2, and W_3 (see Section II.2.B) and the next experiment carried out at a different time by chang-

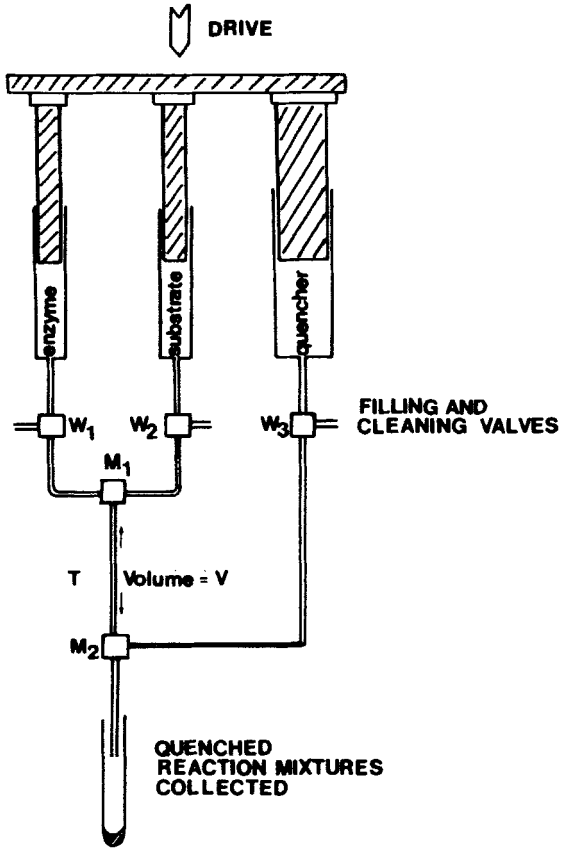

Figure 1. Principle of rapid-flow-quench apparatus. Enzyme and substrate are mixed in M_1, react in the reaction tube T, and the reaction mixture is quenched in M_2.

ing the reaction tube T (i.e., V) or the drive speed (i.e., s). The rapid-flow-quench method is, therefore, a point-by-point method for following a reaction. It is clearly important that the reaction times, t, and the factor by which the reaction mixtures are diluted by the quencher are accurately known.

B. TIME-DELAY FLOW QUENCH

The rapid-flow-quench method is inherently wasteful in materials in that after each experiment the reaction tube remains filled with unused reaction mixture. This method, therefore, is restricted to relatively short

reaction times (up to about 0.5 sec): longer reaction times would require reaction tubes of large volumes (>1 ml) and result in much waste of materials.

A way of overcoming this problem is to use a time-delay flow-quench apparatus (Fersht and Jakes, 1975).

The principle is illustrated in Fig. 2. In an experiment drive I is activated: the enzyme and substrate solution are mixed (M_1) and the flow continues until the reaction tube T is filled with the reaction mixture. The

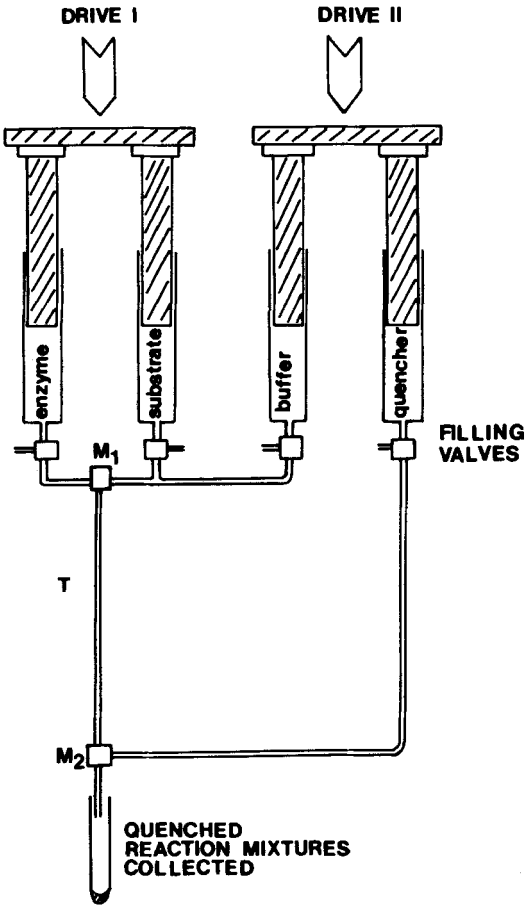

Figure 2. Principle of time-delay flow-quench apparatus. Enzyme and substrate are mixed in M_1, and the reaction mixture incubated in the reaction tube T. The aged reaction mixture is expelled and quenched in M_2.

flow now stops and the reaction mixture incubates for a predetermined reaction time, t. At the end of this time drive II is activated: the reaction mixture is rapidly expelled by buffer via a second reaction chamber (M_2) where the quenching agent is injected. The quenched reaction mixture is collected and analyzed.

2. Flow-Quench Apparatuses for Cryoenzymic Work

A. GENERAL

In the construction of a flow-quench device for subzero work, special problems are encountered.

First, there is the problem of the choice of materials for its construction. Rapid-reaction equipment has traditionally been constructed of Lucite (Perspex), but this material cannot be used at subzero temperatures as it may become brittle. Further, this plastic absorbs small molecules that may present problems. Indeed, this absorption phenomenon caused severe problems when the creatine kinase reaction was studied in an all-Lucite apparatus. Here inorganic phosphate was assayed by a sensitive chemical method; after several weeks of continuous use the apparatus phosphate blanks become unacceptably high.

Stainless steel has been used in rapid-reaction equipment, but it is unsuitable with metal-sensitive system. In the present apparatus all parts in contact with the reactants are made of glass (syringes), polytetrafluoroethylene (PTFE or Teflon: reaction tubes, connecting tubes) or polytrifluorochloroethylene (Kel-F: reaction chambers).

Second, there is the problem of mixing; the antifreezes used in subzero work often produce viscous solutions that are difficult to mix. This problem was solved by using a mixer in which two swirls work in opposition. This configuration ("double-turbine mixer") was first described by Roughton (1963). For detailed discussions of mixers see Roughton (1963), Barman and Gutfreund (1964), and Gutfreund (1969).

Third, there is the problem of insulation and thermostatic control. Polyvinyl chloride (PVC) and polycarbonate resin are good insulating materials. To house the syringes, mixing chambers, and tubes, cavities were scooped out of a solid block of PVC. Enough material was left on the sides and back to provide insulation. The front was sealed off by a thick plate of clear polycarbonate resin screwed down onto a gasket fitted into a grove on the front of the PVC block. Thermal equilibrium was ensured by allowing the refrigerant (ethanol) to circulate in the cavities for at least an hour before any experiment. Temperatures were checked by inserting thin thermocouples into the reaction tubes, mixers, and so on.

Finally, there is the problem of tight taps. When doing experiments over a large temperature range, standard PTFE taps are prone to leaks. This problem was overcome by making the body of the tap of the tough Kel-F plastic and the taper of the softer PTFE, the two being kept tightly together by springs (see Fig. 3).

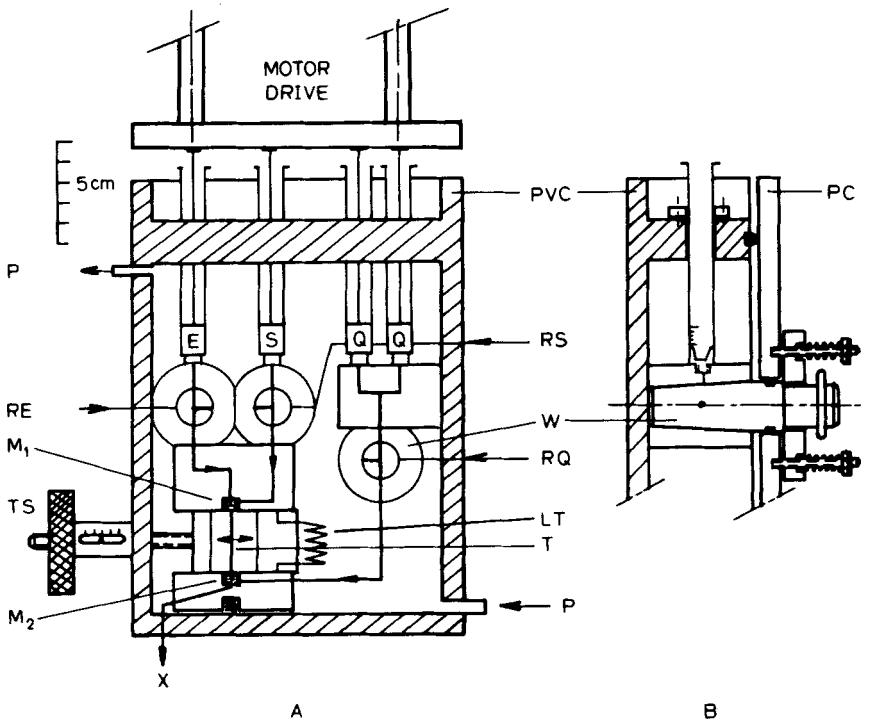

Figure 3. Schematic diagram of the rapid-flow-quench apparatus adapted to subzero conditions. (A) Front sectional view with taps in the experimental position; (B) cross-sectional view showing details of a tap. E, S, and Q represent the syringes containing the enzyme, substrate, and quenching solutions, respectively. M_1 represents the enzyme-substrate mixer, T is a reaction tube, and M_2 is the quenching mixer. Quenched reaction mixtures are collected at X. LT is a long reaction tube, TS is the tube selector, RE, RS and RQ represent the inlet ports from thermostated reservoirs (the reservoirs and wash inlets are not shown), P refrigerant ports and W taps. PVC represents the apparatus housing and PC is the clear polycarbonate resin lid. From Barman et al. (1980).

B. RAPID-FLOW-QUENCH APPARATUS

a. Syringes, Mixing Chambers, and Reaction Tubes. A schematic diagram of the rapid-flow-quench apparatus is given in Fig. 3 and an overall photograph is in Fig. 4.

In order to ensure that equal volumes of enzyme and substrate solutions are mixed in M_1 and that the resulting reaction mixture is mixed with an equal volume of the quenching solution in M_2, the four drive syringes are disposed as in Fig. 3: one each for enzyme and substrate solutions and two for the quenching solution. The refilling of the drive syringes and the washing and drying of the reaction tube, mixers, and associated tubing is ensured by the taps W. The syringes are 5 ml interchangeable with luerlock. The metal luerlock is removed (acid) and the sturdy glass stem (tapered) revealed fitted into a PTFE socket. Suitable syringes can be obtained from Chance Brothers Ltd. (P.O. Box 39, Smethwick, Warley, West Midlands, B66 INY, United Kingdom).

As indicated above, the age of the reaction mixture at quenching in M_2 can be varied by changing the volume between the mixers M_1 and M_2 or by changing the drive speed (see Section II.2.B.b).

To change the volume, one must change the reaction tube T, a laborious process with certain previously described equipment. Here, the arrangement for doing this is simplified. There are five reaction tubes of volumes 47 μl to 1.05 ml (Table I). These were made by drilling holes through a solid rectangular block of PTFE. The block is carefully lined up between the two mixers and held tightly in position by a spring. The reaction tube is changed by moving the block horizontally, rather in the way one changes the position of the cell in a spectrophotometer by moving the cell holder.

The diameter of the reaction tubes are critical, especially for cryoenzymic work. It is thought that the mixing process continues after a reaction mixture has left the reaction chamber (Gutfreund, 1969). Details of the reaction tubes currently in use are given in Table I, together with certain critical delivery rates and the corresponding flow velocities in the different reaction tubes.

With water, the range of delivery rates was 2.8−10.9 ml sec^{-1}, all of the tubes could be used and the flow velocity range was 89−1397 cm sec^{-1}. At lower flow velocities there was poor mixing of enzyme and substrate.

With 40% ethylene glycol at −20°C, the flow velocity range was more limited (see Section III.3). First, the use of tube No. 3 (Table I) led to incomplete mixing of enzyme and substrate at any speed available. This could be because of its relatively large cross-sectional area (0.031 cm^2).

TABLE I

Characteristics of Rapid-Flow-Quench Apparatus for Cryoenzymic Work

Tube No.	Reaction Tube Dimensions			Limits of Flow Velocities and (Reaction Times)[a] in Reaction Tube			
				Water		40% Ethylene Glycol[d]	
	Length	Diameter	Volume[b]	2.8 ml sec^{-1}[c]	10.9 ml sec^{-1}	4.6 ml sec^{-1}	7.3 ml sec^{-1}
	cm	cm	ml	cm sec^{-1} (msec)	cm sec^{-1} (msec)	cm sec^{-1} (msec)	cm sec^{-1} (msec)
1	4	0.1	0.047	359 (16.5)	1397 (4.2)	591 (10.2)	935 (6.4)
2	4	0.15	0.087	158 (30.2)	616 (7.8)	260 (18.4)	411 (11.6)
3	4	0.2	0.14	89 (50)	347 (12.9)	[e]	—
4	18	0.16	0.37	139 (132)	542 (34)	229 (80)	362 (50.7)
5	52	0.16	1.05	139 (373)	542 (97)	229 (228)	362 (144)

[a]Reaction times (in milliseconds) are given in parentheses.
[b]Dead volume (ΔV) of 16 μl included.
[c]Delivery rates.
[d]At −20°C.
[e]Tube 3 could not be used with 40% ethylene glycol at −20°C.

Second, the remaining tubes could only be used within the rather narrow delivery rate range of 4.6–7.3 ml sec^{-1} giving a flow velocity range of 229–935 cm sec^{-1}. Lower flow velocities than 229 cm sec^{-1} resulted in poor mixing; velocities above 935 cm sec^{-1} burst the glass syringes.

The limits imposed by the mixers are further discussed below.

b. Drive Mechanism and Control. The drive unit is a reversible variable-speed motor of constant torque (1.2 N·m is suitable) with the appropriate control unit. Suitable motor drive and control units can be obtained from Clerely (5 rue d'Auteuil, 75016 PARIS, France); from Thorn Automation Ltd. (P.O. Box 4, Rugeley Staffordshire WS 15 IDR, United Kingdom), or Boston Gear Works (Quincy, Mass.). The drive unit is connected to a rotating screw via an electromagnetic clutch-brake unit (e.g., Stone Platt Transmissions, Featherstall road South, Oldham OL 9 9 NA Lancs., United Kingdom). The rotating screw activates a nut fixed to a crossbar to which the pistons of the four syringes (Figs. 3 and 4) are attached by small magnets. With a Clerely motor drive unit and 5-ml Chance Interchangeable syringes, the delivery rates in the reaction tube can be infinitely varied in the range 0–16 ml sec^{-1}, but as explained above, the usable range is smaller than this.

For the economy of the reagents and the accuracy of the experiment, it is important to have reaction mixtures of reproducible volumes. The device used can be seen in Fig. 4. The travel of the crossbar (and thus the pistons) is set by the position of a shutter on a 5-cm steel ruler (fixed to the crossbar) relative to a photoelectric cell (fixed to frame of the apparatus). This photoelectric cell controls the clutch brake unit.

For an experiment, the following sequence of events takes place. The motor is turning at the required speed with brake activated and the clutch deactivated. A microswitch is pressed; this deactivates the brake and activates the clutch, and the pistons travel until the shutter reaches the photoelectric cell. This deactivates the clutch and activates the brake, causing the pistons to stop. Thus, one obtains accurately predetermined volumes of quenched reaction mixtures that are delivered into test tubes and can then be chemically analyzed.

With the present apparatus 1 ml of each of enzyme and substrate solution are required for an experiment. Smaller volumes lead to a diminution of the accuracy (see below).

C. TIME-DELAY FLOW QUENCH

The time-delay flow-quench apparatus is based on that of Fersht and Jakes (1975) and was adapted to subzero conditions as outlined above.

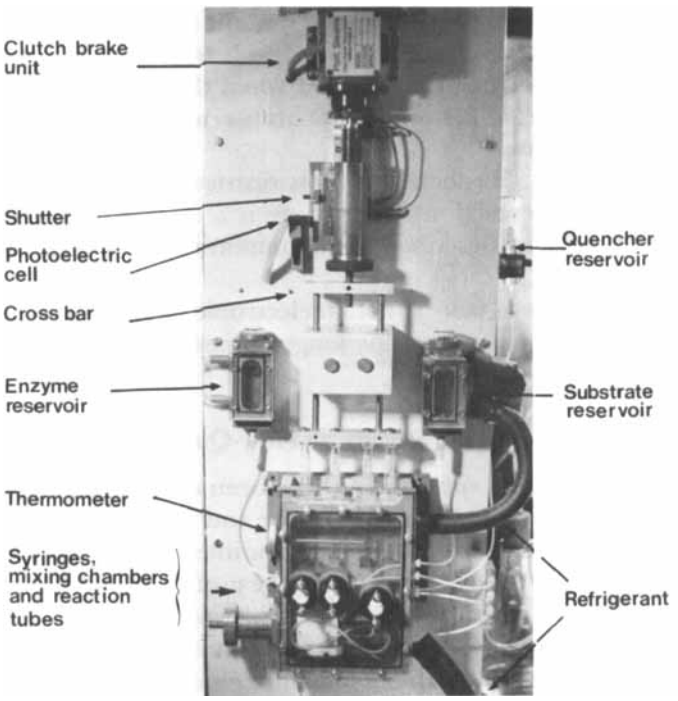

Figure 4. Rapid-flow-quench apparatus adapted to subzero conditions. For details of the disposition of the syringes, mixing chambers, and reaction tubes, see Fig. 3.

As for the rapid-flow-quench apparatus, the reagents (enzyme, substrate, buffer wash, quencher) are contained in 5-ml all-glass Chance syringes. The reaction tube is a thick-walled PTFE tube of internal diameter 0.16 cm and volume 1 ml.

Since the incubation time is independent of the flowrate, a simple pneumatic drive system is used. This consists of two compressed air pistons (Circlair Joucomatic International), one for the enzyme and substrate syringes, the other for the buffer wash and quencher syringes (Fig. 2). The pistons are activated by Skinner electric valves at 9 kg cm^{-2}. There is no advantage in using higher pressures as this does not decrease the filling and emptying times of the reaction tube.

Typically, at 9 kg cm^{-2} it takes about 40 msec to fill the reaction tube with 1 ml of 40% ethylene glycol at −20°C. This provides a flowrate of 12 $msec^{-1}$ down the reaction tube, which ensures good mixing. Under the same conditions the reaction mixture is expelled by 1.2 ml of buffer through the second mixing chamber where 1.2 ml of the quenching agent is injected at the same time. This takes place in about 90 msec.

The apparatus is essentially self-cleaning. At the end of an experiment the first mixer and reaction tube are filled with buffer; any remaining material from the experiment is expelled when the following reaction mixture enters. The cleanliness of the apparatus can be tested for by the use of colored solutions.

The shortest reliable incubation time is restricted by the filling and emptying times of the reaction tube. There is a certain compensation effet; with the apparatus described the minimum incubation time is 0.4 sec.

For incubation times of 0.4–10 sec an electronic timer control system is used to operate the air valves; for longer times manual switching is satisfactory.

3. Selected Literature Survey of Flow-Quench Devices

A number of flow-quench apparatuses have been constructed. Many of these have been designed for special purpose and may have features of interest to the reader. Thus, whereas it is not the intention here to give full details of all of these, some comments might be useful. Table II is a list of recent flow devices with some indications of their performances and any special features. For earlier equipment or variants of those listed see Pinset (1954), Ruby (1955), Barman and Gutfreund (1964), Flohé et al. (1972), Smith et al. (1976), Boyer and Stempel (1979), and Thayer and Hinkle (1979).

The simplest way of stopping a reaction is to inject the reaction mixture into the quenching solution held in a beaker. This method suffers from certain limitations. First, viscous solutions are poorly mixed, even at high injection rates (12 m sec^{-1}). Second, accurate results are critically dependent on a sharp stop of the drive system; a slow deacceleration results in the quenching of excessively aged reaction mixture. Third, it is important that the quencher should be at the same temperature as the reaction mixture, and it may be difficult to thermostatically control a beaker outside the rest of the apparatus. A quencher at a temperature significantly higher than that of the reaction mixture could lead to a speeding up to the reaction immediately before the quenching. This would be particularly troublesome under cryoenzymic conditions. Nevertheless, when precautions are taken, beaker quenching can be used (e.g., Aldridge et al. 1964; Barman and Gutfreund, 1964; 1966a,b; Wålinder et al., 1969; Mårdh, 1975a; Lowe and Smart, 1977; Boyer and Stempel, 1979).

Accurate reaction times are critically dependent on accurate drive speeds. A variety of drive mechanisms have been used, ranging from springs and pneumatic drives to stepping motors. For accurately known

TABLE II

Examples of Recent Flow-Quench Devices

Type of Apparatus	Time Range	Drive System	Special Features[a]	References[b]
Rapid flow	10–500 msec	Rack and pinion, clutch/motor	Additional mixers can be added; assay valve(s)	Lymn and Taylor, 1970[c] (Lymn, Bigson and Hanacek, 1971)
Rapid flow/ time delay	5 msec to 5 sec	Cam drive	Anaerobic conditions; usable flowrates down to 1 $m \cdot sec^{-1}$; T	(Ballou and Palmer, 1974)[d]
Rapid flow	5–150 msec	Pneumatic	Reaction mixture volumes = 0.6 ml	Fersht and Jakes, 1975
Time delay	150 msec to minutes	Pneumatic		Fersht and Jakes, 1975
Rapid flow	2.5–100 msec	Pneumatic and spring	Reaction mixture divider: same reaction mixture quenched in two different quenchers; beaker quenching	Mårdh, 1975a Wålinder et al., 1969 Mårdh and Zetterquist, 1974
Rapid flow	4–400 msec	Stepping motor	Ball mixer; additional mixers can be added; assay valve, T	(Froehlich et al., 1976)[e]
Beaker mixing	0.5 sec to minutes	Pneumatic	Reaction mixture volumes down to 20 μl, T	Eccleston et al., 1980)[f]
Rapid flow	4–300 msec	Screw-clutch/ brake motor	Adapted to subzero temperatures, T	Barman et al., 1980
Time delay	0.4 sec to minutes	Pneumatic		Barman et al., 1980
Rapid flow/ time delay	4 msec to minutes	Pneumatic	T	(Cash and Hess, 1981)

[a]T, thermostatically controlled 0°C and up; unless otherwise stated, reaction mixture volumes 1–3 ml.
[b]Full technical details are given in references in parentheses.
[c]Also, used by Froehlich and Taylor (1975).
[d]Also used by Schray et al. (1973) and Wilkinson and Rose (1979).
[e]Also used by Froehlich and Taylor (1976) and Sumida et al. (1978).
[f]Similar apparatus by Kanazawa et al. (1970).

and reproducible drive speeds motor-driven systems are probably preferable; in addition, these can be made very flexible (e.g., Ballou and Palmer, 1974; Froehlick et al., 1976; Barman et al., 1980). For details of various drive systems used, the reader is referred to the references in Table II.

Useful features are included in certain flow-quench devices. To overcome the problem of acceleration and deacceleration of the drive system assay, valves have been used (e.g., Lymn et al., 1971; Froehlich et al., 1976). These are so arranged that samples are only taken when the velocity of the drive is constant. Another way of overcoming this problem is to have dead volumes before the enzyme substrate mixer and after the quenching mixer (see Section III.4).

Another useful feature is a reaction mixture divider. There are provisions for this in the apparatus of Mårdh (1975a). With this apparatus one could, for example, divide myosin-ATP reaction mixtures (see Section V.3) into two streams. One stream could then be quenched by an ATP chase (giving information on the binding of ATP) and the other by acid (giving information on the chemical step).

With certain apparatuses additional mixers can be added (e.g., Lymn et al., 1971; Froehlich et al., 1976; Mårdh, 1975a). With such systems one could study the interaction of transient intermediates with a second substrate or even enzyme, but as pointed out by Gutfreund (1969) not many of these devices have been tried out in practice.

4. Commercial Flow-Quench Apparatuses

The Durrum D-133 multimixer (Durrum Instruments, Palo Alto, Calif.) has been available for more than a decade and has been used in several laboratories (e.g., Benkovic et al., 1974; Kurzmack et al., 1977; Briggs et al., 1978; Verjovski-Almeida et al., 1978; Raushel and Villafranca, 1979; Meek et al., 1982; Bryant et al., 1983; Gabikov et al., 1983; Grazi et al., 1983). The Aminco-Morrow stopped flow has been modified for flow-quench work with beaker quencher (Lowe and Smart, 1977).

Recently, Update Instruments (Madison, Wisconsin) made available a combined rapid-flow–time-delay quench apparatus (precision syringe Ram). This apparatus has been used by Cross et al. (1982) and Grubmeyer et al. (1982). With this apparatus mixing times of 250 μsec are claimed. Further, with its small reaction volumes (down to 130 μl) it is highly economical of solutions. The drive system consists of a printed circuit motor programmable to provide variable displacements and push-rates.

A quenched-flow apparatus is manufactured by Hi-Tech Scientific Ltd. (Church Fields, Salisbury, Wiltshire, United Kingdom).

III. TESTING AND PRECAUTIONS

1. Introduction

An important application of a flow-quench apparatus is in the investigation of transient phases (see Section V.2). The success of this depends on the answers to certain questions: Are the mixers efficient? What is the true age of the reaction mixture at quenching? Is all the reaction mixture sampled of the same age? Is the ratio of the volumes and the reaction mixture to quencher accurately known? Needless to say, the final result depends on an accurate and specific chemical analysis of the product or intermediate under study.

These questions refer in particular to the rapid-flow-quench technique. With the time-delay flow-quench technique the reactions take place for longer times and at rest and the incubation times are electronically controlled.

The alkali hydrolysis of 2,4-dinitrophenyl acetate is a convenient reaction for testing the performance of rapid-reaction equipment (Gutfreund, 1969). Reaction mixtures are quenched in acid and the 2,4-dinitrophenol produced estimated directly at 320 nm (Barman et al., 1980). In acid the molar extinction of 2,4-dinitrophenol at 320 nm = $5.6 \times 10^3\ M^{-1}\ cm^{-1}$. At 20°C in water the second-order rate constant for the hydrolysis is 56 $M^{-1}\ sec^{-1}$; in 40% ethylene glycol 216 $M^{-1}\ sec^{-1}$. Under pseudo first-order conditions (i.e., concentration alkali $\gg$ ester) a desired rate can be obtained by adjusting the alkali concentration.

2. Time Calibration

It is evident that the time calibration of a rapid-reaction device is of prime importance. There are two types of time error: errors in reaction times and errors in zero time. Errors in reaction times give rise to false kinetic constants. An error in zero time can give rise to false transient phases.

The danger of a potential zero time error is illustrated in Fig. 5, where the initial formation of arginine phosphate by different concentrations of arginine kinase was studied. The extrapolation of the study-state rates to zero time give transient burst phases of product, that is, the amount of the complex enzyme–arginine phosphate at zero time (see Section V). The amplitudes of the burst phases are proportional to concentration of enzyme.

These transient phases were obtained with an apparatus that had been time-calibrated as described below. However, an identical result would have been obtained had there been no transient phase and a zero time error of about 2 msec. If a transient phase is obtained with an uncalibrated

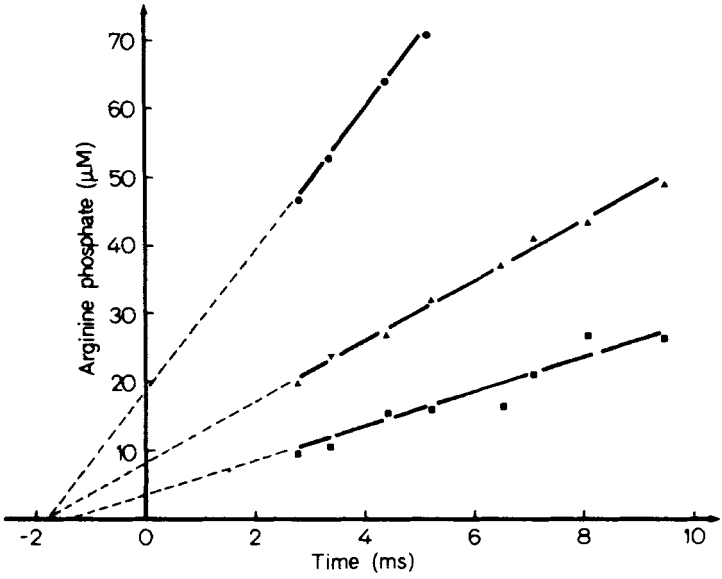

Figure 5. Initial formation of arginine phosphate by different concentrations of arginine kinase at 12°C and pH 8.6. Arginine kinase concentations were 111 (—●—), 56 (—▲—), and 28 μ*M* (—■—). From Barman et al. (1978).

apparatus, this uncertainty is reduced if one observes the *kinetics* of the phase.

The true reaction time, t, is given by $t = V/s$ where V is the volume between the mixers M_1 and M_2 (Fig. 1) and s the delivery rate. Thus, for a precise time t, s and V must be accurately known.

The delivery rates (ml sec^{-1}) are determined by filling the apparatus (syringes, mixers, reaction tube, etc.) with water and collecting weighed and timed samples at different drive speeds. With a motor drive, as here, the drive speed is accurate and reproducible and in our hands has presented few problems. The errors in the delivery rates are less than ±2%.

The volume between the mixers is made up of the reaction tube itself (V_i) and of a dead volume (ΔV), that is, the volumes between the mixers and the reaction tube. The volume of the reaction tube is obtained by weighing (water or mercury), but the dead volume is difficult to measure directly. It can be determined from experiments with the 2,4-dinitrophenyl acetate–NaOH system.

The true reaction time is $t = (V_i + \Delta V)/s$ where the dead volume ΔV, which is independent of the reaction tube, must be measured. The hydro-

lysis of 2,4-dinitrophenyl acetate is followed under pseudo-first-order conditions and can be expressed by

$$\ln(\mathrm{DNP}_\infty - \mathrm{DNP}_t) = \ln \mathrm{DNP}_\infty - \frac{(V_i + \Delta V)}{s} k$$

where DNP_∞ and DNP_t are the product, 2,4-dinitrophenol, at times ∞ and t, respectively. Experiments are carried out at different t by varying s at constant V_i. A plot of $\ln(\mathrm{DNP}_\infty - \mathrm{DNP}_t)$ against $1/s$ is linear with slope, $m = (V_i + \Delta V)k$. When this is done for a number of tubes, a series of lines of different slopes m is obtained. A secondary plot of m against V_i gives a straight line that cuts the abcissa at ΔV.

This procedure was carried out with the apparatus shown in Fig. 3, and a dead volume of 16 μl (±4 μl) was estimated (Fig. 6); $k = 3.6\ \mathrm{sec}^{-1}$, which compares with the 3.9 sec^{-1} obtained from a stopped-flow apparatus under the same conditions.

The dead volume obtained was used to obtain the effective reaction tube volumes available with the apparatus. These, together with the corresponding reaction times using certain flowrates, are summarized in Table I. It is clear that an accurate estimate of the dead volume is of critical importance with reaction tubes of small volumes.

Two further time checks on the apparatus were carried out. First, the hydrolysis of 2,4-dinitrophenyl acetate was studied under zero-order conditions using a single reaction tube at different flowrates. A plot of 2,4-dinitrophenol produced against time was linear with no product at zero time (Fig. 7). The rate constant obtained (27 sec^{-1}) is close to that obtained by stopped flow (28.5 sec^{-1}). Second, the reaction was followed over an extensive time range, that is, by the use of a number of reaction tubes and flowrates. Any unaccounted-for dead volume would give rise to breaks in the data where the reaction tubes are changed; none could be discerned (Fig. 8). Taken together, these results show that the apparatus is accurately time-calibrated.

3. Mixer Efficiency

Poor mixing occurs at low flowrates (laminar flow in reaction tube) or high speed (cavitation). The quality of mixing is determined by the geometry of the mixer, but the mixing process may continue in the reaction tube (Gutfreund, 1969). Each apparatus constructed has, therefore, a particular range of usable flowrates.

Under cryoenzymic conditions the flowrates are limited by the viscosity of the solvent. Here the highest viscosity used is about 20 cP (40% ethylene

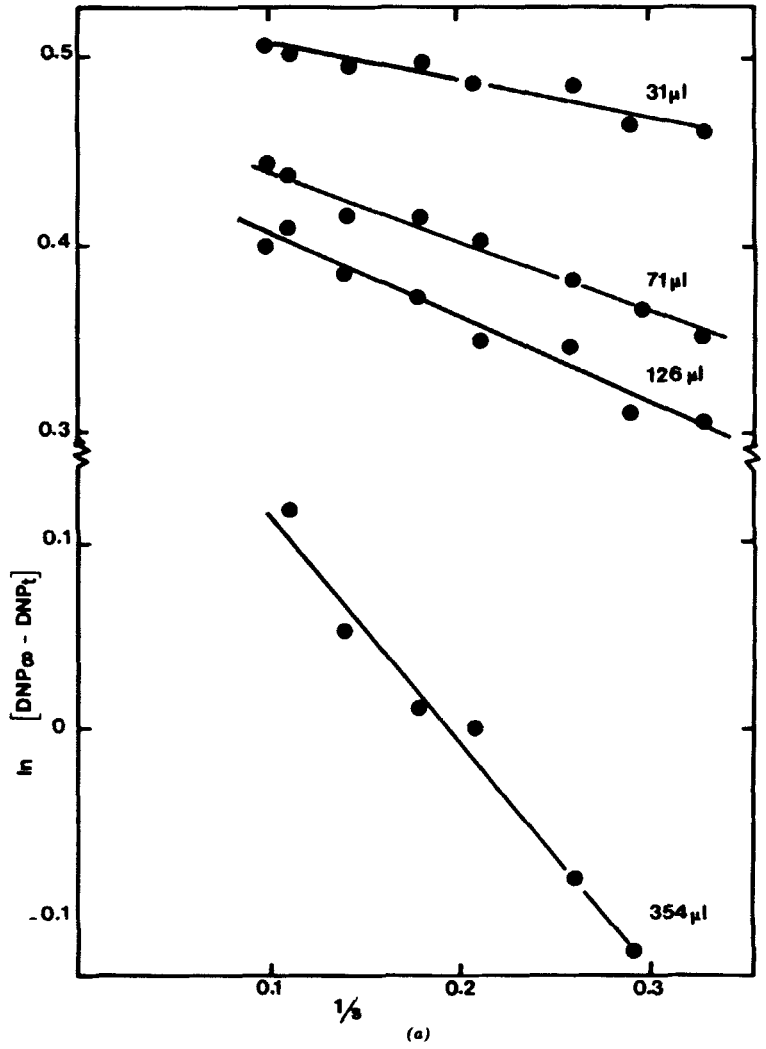

Figure 6. Determination of the dead volume (ΔV) of rapid-flow-quench apparatus using the alkaline hydrolysis of 2,4-dinitrophenyl acetate at −5°C. The syringe concentration of 2,4-dinitrophenyl acetate was 1 m*M* (25 mg dissolved in 2 ml ethanol, to 100 ml in 2 m*M* HCl in 40% ethylene glycol). The reaction mixtures (0.5 m*M* 2,4-dinitrophenyl acetate, 0.125 *M* NaOH) were quenched in 0.5 *M* HCl. Experiments were carried out at different times by varying the delivery rate(s) at constant reaction tube volume (V_i). *(a)* Plot of $\ln(DNP_\infty - DNP_t)$ against 1/s (sec ml^{-1}) with reaction tubes of volume (V_i) 31−354 µl. *(b)* Secondary plot of the slopes (m) from Fig. 6*a* against V_i. Negative intercept on V_i axis gives $\Delta V = 16 (\pm 4)$ µl. DNP_∞ and DNP_t are 2,4-dinitrophenol concentrations at times ∞ and t, respectively.

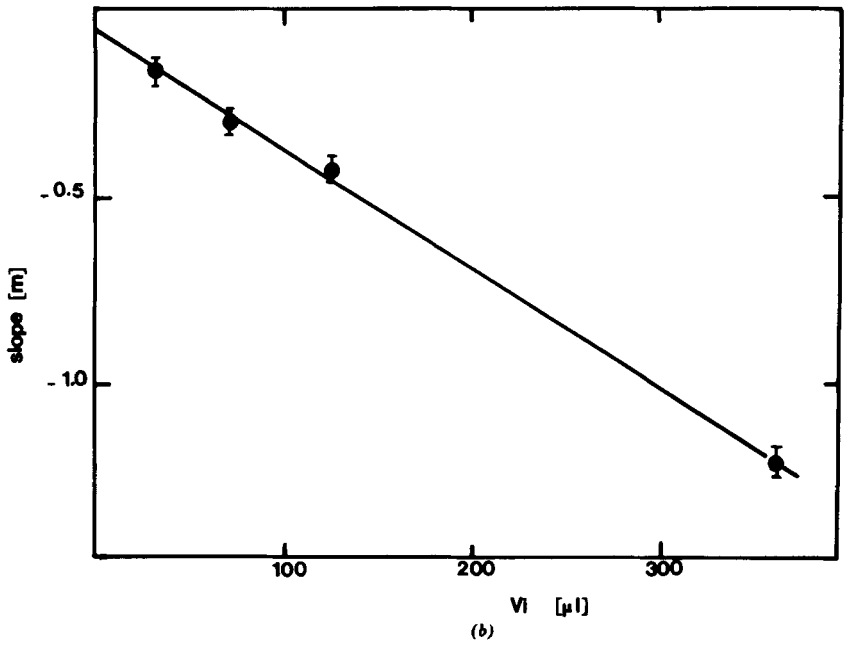

Figure 6. *(Continued)*

glycol at −20°C). With the stopped-flow apparatus of Markley et al. (1981) the upper limit in viscosity is 50 cP.

The present apparatus has two mixers in each of which poor mixing can occur. Poor mixing of enzyme and substrate leads to low product formed, whereas poor quenching results in high product.

At high flowrates mixing problems were not encountered. The limit was set by the solidity of the syringes (especially with 40% ethylene glycol at low temperatures) rather than by cavitation.

At low flowrates, however, there are mixing problems, and under a given set of conditions there is a minimum flowrate for efficient mixing. This problem is illustrated in Fig. 9. The hydrolysis of 2,4-dinitrophenyl acetate was followed at −20°C, and it is clear that below 4.5 ml sec^{-1} the mixing process becomes inefficient. Since the aberrant points are high (i.e., too much 2,4-dinitrophenyl acetate remaining), the first mixer is at fault. With water, the lower limit in the flowrate is 2.5 ml sec^{-1}.

The usable flowrates with our apparatus in water and 40% ethylene glycol are given in Table I.

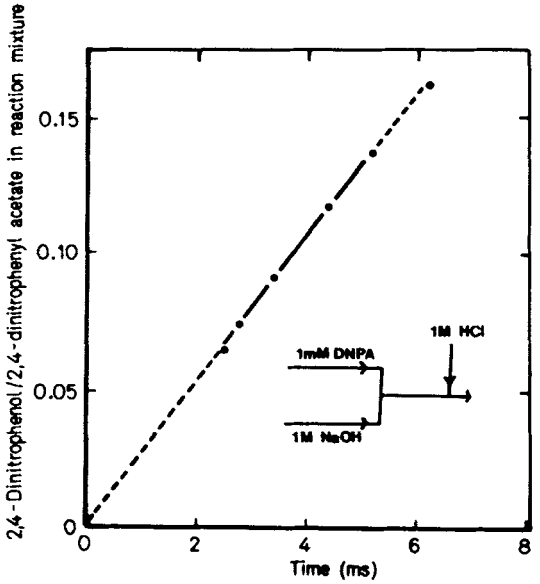

Figure 7. Time calibration of rapid-flow-quench apparatus. The initial hydrolysis of 2,4-dinitrophenyl acetate (DNPA) was followed under near-zero-order conditions at 25°C. From Barman et al. (1978).

It is noteworthy that if there is a mixing problem, it is in the first mixer. In no case, with either the alkaline hydrolysis of 2,4-dinitrophenyl acetate or with several enzyme reactions studied, have we had problems with the quencher mixer. This observation is in accord with Gutfreund (1969).

In conclusion, the apparatus takes samples in the time range 4.2–373 msec with water and 6.4–228 msec with 40% ethylene glycol at −20°C. These time limits are set by the solidity of the syringes at high speeds and the efficiency of mixing in the first mixer at low speeds (Fig. 1). A further limitation with ethylene glycol is that there is a maximum limit of the diameter of the reaction tube. Thus, at −20°C the tube of diameter 0.2 cm (tube 3, Table I) could not be used: with it, 2,4-dinitrophenyl acetate and NaOH were incompletely mixed at any drive speed.

4. Precautions for Accuracy

Because of the inertia of the drive systems and the time responses of the clutch and brake (these are less than 40 msec), there may be an acceleration–deacceleration effect on the reaction mixture. This is to a certain

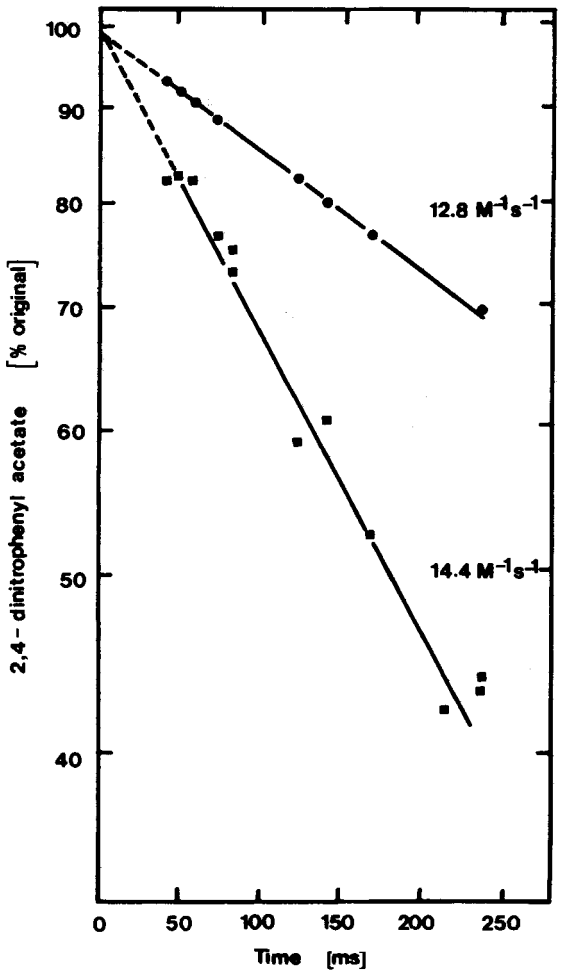

Figure 8. Semilogarithmic plot of the hydrolysis of 2,4-dinitrophenol acetate in NaOH at −20°C using the rapid-flow-quench apparatus. Ethylene glycol (40%) was used as anti-freeze. The reaction mixture concentrations of NaOH were 0.125 (—●—) and 0.25 *M* (—■—). From Barman et al. (1980).

extent minimized by the motor turning at the required speed before an experiment.

One way of overcoming the inertia effect is to collect the quenched reaction mixture only in the middle of the run; this has been done by the use of assay valves (Table II). A simpler way (but more extravagant in

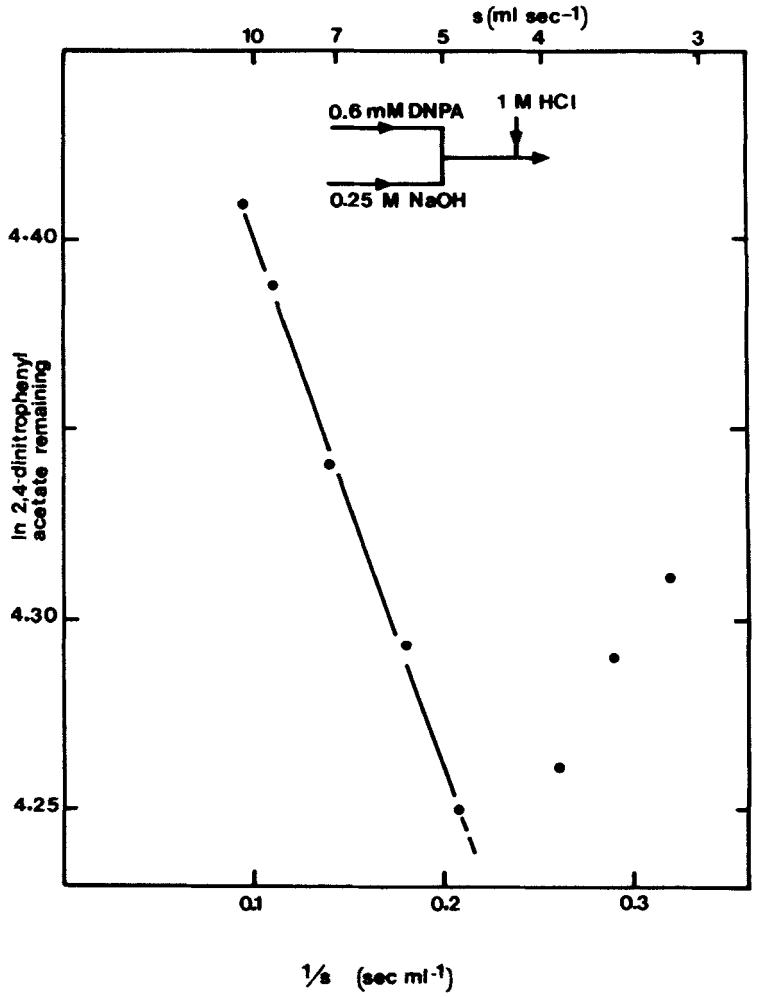

Figure 9. Determination of the lower limit in the flowrate of a rapid-flow-quench apparatus in 40% ethylene glycol at −20°C. The reaction tube (1.05 ml) was kept constant and the flowrate varied. From the straight line a rate constant of 1.6 sec^{-1} was obtained.

materials) is to provide dead volumes before the first mixer and after the second mixer. This method is used in the apparatus presented in Fig. 3. The volumes between the enzyme and substrate syringes and M_1 are each about 0.12 ml. These, which are left empty before an experiment, must be filled before the mixing takes place (M_1), and during this time a steady flow is approached. Similarly, the space between M_2 and the exit (X) is about 0.2 ml and empty. This allows the flow to stop before any excessively aged reaction mixture is quenched and collected.

A means of checking on the velocity of the drive block is to connect it to a linear potentiometer and storage oscilloscope (e.g., Lymn et al., 1971).

After the chemical analysis of the quenched reaction mixtures, concentrations are expressed in terms of the reaction mixture concentrations of enzyme and substrate. For this an accurate value for the ratio of the volume reaction mixture to volume quencher is required. With beaker quenching this ratio can be obtained by using an accurate amount of quencher and then weighing the beaker after the experiment. With a quencher mixer it is technically difficult to arrange for the two volumes to be equal with all the reaction tubes; to determine the ratio, colored solutions can be used. However, it is important to use a gradual increase (or decrease) in drive speed; any sudden change in speed may cause the crossbar–piston assembly to overshoot somewhat, which would modify the ratio of the volume reaction mixture to the volume quencher.

To reduce potential mixing problems, it is important to keep the compositions of the enzyme and substrate solutions as close as possible. For example, the mixing of a solution of high ionic strength with one of a low ionic strength could produce artifacts.

To increase the accuracy of the assay of the quenched reaction mixture, it is important to keep the ratio of the substrate to enzyme concentration as low as possible without affecting the kinetics of the process under study. When the myosin subfragment-1-[γ-^{32}P]ATP system was studied, ratios in the range 4 : 1–10 : 1 were used (Barman et al., 1983).

The apparatuses described work satisfactorily with the 2 : 4 dinitrophenyl–NaOH system down to −20°C, but as a precaution the enzyme studies carried out so far have limited to −15°C (Section VI).

When the above precautions are taken, the rapid-flow quench apparatus yields accurate data. The limiting factor seems to be the accuracy of the chemical analysis rather than the apparatus itself or its manipulation. Examples of the use of flow-quench apparatuses under ambient and subzero conditions are given in Section VI.

5. Overall Performance

With rapid-reaction devices an important consideration is the dead time. This defines the shortest reaction time available with a particular device.

With a rapid-flow-quench apparatus the dead time is determined by the geometry of the mixers (cavitation at high flowrates), by the smallest reaction tube available, and by the mechanical strength of the syringes. Further, the physical properties (e.g., viscosity) of the solutions may effect the dead time. As shown above,the dead time with the present apparatus is 4.2 msec in water and 6.4 msec in 40% ethylene glycol at −20°C. In a previous apparatus constructed in this laboratory (not adapted to cryoenzymic work) the dead time was 2.2 msec.

The importance of dead time is that it defines the fastest reaction that can be studied with a given apparatus. This, if one assumes that the shortest sampling time corresponds to the half-life of the reaction under study, then with a dead time of 4.3 msec first-order reactions with rate constants up to 161 sec^{-1} can be studied with reasonable accuracy. The corresponding figures for dead times of 6.4 and 2.2 msec are 108 $^{-1}$ and 315 sec^{-1}, respectively.

A certain number of experiments were carried out to test the overall performance over a wide temperature range (−20 to 35°C).

Typical semilog plots for the alkali hydrolysis of 2,4-dinitrophenyl acetate at −20°C using the rapid-flow-quench and time-delay flow-quench apparatuses are illustrated in Figs. 8 and 10, respectively. The second-order constants agree well. As the plots extrapolate to 100% remaining ester, neither apparatus has a significant zero time error.

A problem with the conditions used above (40% ethylene glycol, −20°C) is that above 0.25 *M*, NaOH is poorly soluble. A faster reaction is the alkali hydrolysis of *o*-nitrophenyl chloroacetate (Holmquist and Bruice, 1969). This has been used to test stopped-flow equipment under subzero conditions (Auld, 1979).

A critical test for a rapid-reaction device is to determine the temperature dependency of a well-characterized reaction. An Arrhenius plot of the rate constant concerned should be linear; any malfunction (especially mixing problems caused by an increase in viscosity as the temperature is lowered) should manifest itself by a deviation. An Arrhenius plot also tests the apparatus over a large time range. An Arrhenius plot for the second-order rate constant for the hydrolysis of 2,4-dinitrophenyl acetate by NaOH in 40% ethylene glycol is shown in Fig. 11. An activation energy of 38 kJ mol^{-1} was obtained; this compares with 35 kJ mol^{-1} obtained by a stopped-flow apparatus in the same solvent. An activation energy of 37 kJ mol^{-1} was obtained in water (Barman et al., 1980).

6. Special Precautions

In rapid-reaction devices solutions are squirted at high speed through tubes of narrow bores. This may expose the components of a reaction mixture to high shear forces and pressures that may have a deleterious effect on the system under study. One would expect large oligomeric enzyme structures or membrane-bound enzymes to be particularly vulnerable. For example, the fatty acid synthetase system was inactivated in a rapid-flow-quench apparatus (Cognet and Hammes, 1983). The inactivation was reduced by the use of a short (5−17 cm) reaction tube with a large diameter (1.5 mm). The enzyme was further stabilized by including 10% glycerol in the reaction medium.

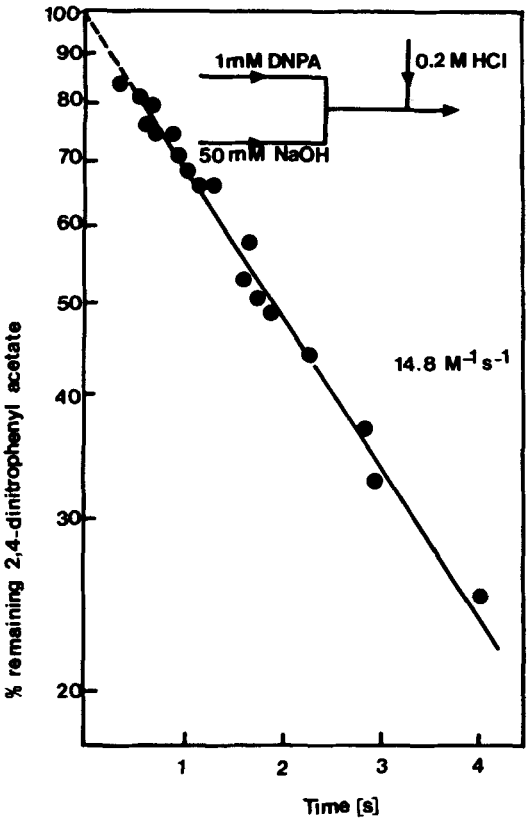

Figure 10. Semilogarithmic plot of the hydrolysis of 2,4-dinitrophenyl acetate in NaOH at −20°C using the time-delay flow-quench apparatus. From Barman et al. (1980).

However, such adverse effects seem to be rare. For example, the 12-subunit enzyme glutamine synthetase *(Escherichia coli)* was studied by the rapid-flow-quench technique without any adverse effect (Meek et al., 1982). Sarcoplasmic reticulum ATPases from a number of sources (Froehlich and Taylor, 1975, 1976; Briggs et al., 1978; Sumida et al., 1978), and even intact chloroplasts (Smith et al., 1976) were apparently not denatured in rapid-flow quench devices. Mårdh (1975b) carried out careful control studies on the bovine brain Na^+-K^+-stimulated ATPase and found no adverse effect even at high flowrates. Cash and Hess (1981) studied the influx of Rb^+ into plasma membrane vesicles in a rapid-flow-quench device and found no evidence for denaturation.

Problems may be encountered when solutions of different viscosities are mixed in a rapid-reaction device—for example, experiments at very

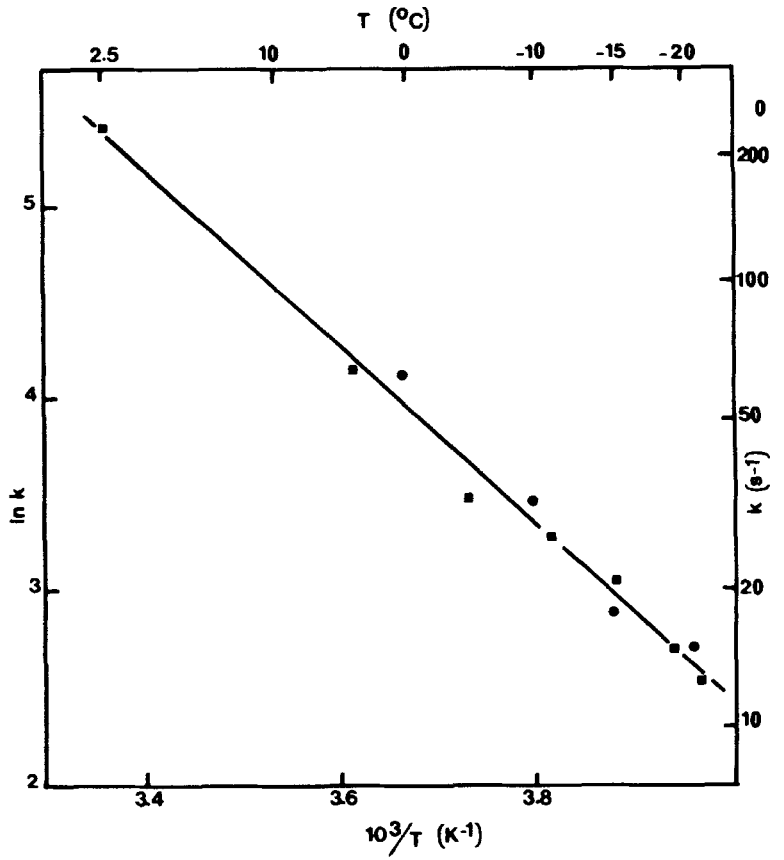

Figure 11. Arrhenius plot for the second-order rate constant for the hydrolysis of 2,4-dinitrophenyl acetate in NaOH. The rapid-flow-quench (—■—) and time-delay flow-quench (—●—) apparatuses were used. From Barman et al. (1980).

high enzyme concentrations or with systems such as actomyosin (e.g., Biosca et al., 1984a). It is therefore important to test the device to be used under conditions where solutions of different viscosities are mixed, and we have modified the alkaline hydrolysis of 2,4-dinitrophenyl acetate for this purpose. With the apparatus described in Section II.2.B, 0.25 *M* NaOH in 68% glycerol (viscosity = 20 cP at 20°C) was mixed with 1 m*M* 2,4-dinitrophenyl acetate in water. The reaction mixture was quenched in

aqueous acid. The rate constant obtained (22 sec^{-1}) is close to that obtained by mixing all of the reagents in 34% glycerol (24 sec^{-1}).

IV. QUENCHING

1. Introduction

The quenching method suffers from one fundamental difficulty, how to "freeze" a reaction without in any way affecting it. The problem is that almost whatever quencher is used, the system under study must in some way be perturbed. This is of course common to most methods of following a reaction; the very act of observation involves some degree of perturbation. Even a "gentle" quenching method such as rapid freezing, which may not denature the enzyme, may shift temperature-sensitive equilibria, which could lead to false estimates of the intermediate under study. A relatively "gentle" method, the "cold"-substrate chase (isotope trapping; Rose, 1980), is discussed in some detail in Section IV.2.

Despite early reservations (e.g., Roughton, 1963), it has been found that stopping a reaction rapidly is probably not a problem (Gutfreund, 1969). With a rapid-flow device with two mixers it appears that the lower limit of the flowrate (i.e., upper limit in reaction time) is set by the quality of the mixing in the enzyme–substrate mixer rather than by that of the stopping mixer (e.g., Gutfreund, 1969; Froehlich et al., 1976; Barman et al., 1980; also see Section III.3). This was found to be so even when reaction mixtures were quenched (in acid) in a beaker (Barman and Gutfreund, 1964). Thus, the quenching time for trypsin plus *N*-benzoyl-L-arginine ethyl ester reaction mixtures was less than 0.5 msec in 0.36 *M* sulfuric acid (Barman and Gutfreund, 1966a). A similarly short quenching time was found for the stopping of the dephosphorylation of phospho-alkaline phosphatase in 7 *M* perchloric acid (Aldridge et al., 1964). With 40% ethylene glycol as solvent, however, beaker mixing is very inefficient, even at high flowrates (Travers and Barman, unpublished work). Beaker quenching is therefore not suitable with viscous systems.

Slow quenching may occur when certain quenching agents are used and are detected by high values for the product being assayed for at low flow speeds. This slowness can often be corrected for by increasing the concentration of the quenching agent or by using a higher flowrate (also see Section III.3).

An important feature of the quenching method is that one can stop

reaction mixtures of the same system in different quenching media. By a careful choice of quenching agents different intermediates can be trapped and determined, and this adds to the flexibility of the method.

2. Cold-Substrate Chase

Enzyme plus radioactive substrate reaction mixtures of known ages are quenched by the addition of a large molar excess of the same, but nonradioactive (cold) substrate dissolved in the same buffer as the reaction mixture. The mixture is incubated for several turnovers, quenched in acid, and radioactive product determined. The successful application of this method depends on enzyme-bound substrate being converted to product (k_{cat}) more rapidly or at least not much slower than it is desorbed (k_{off}). This method was developed by Rose (1980 and references therein).

The importance of cold-substrate chase experiments is that they can lead directly and unambiguously to the kinetic constants describing the formation of a productive enzyme–substrate complex. In favorable cases the desorption of substrate from a productive enzyme–substrate complex is several orders of magnitude slower than product formation in which case cold-substrate chase experiments titrate enzyme-active sites.

We consider in some detail the cold-substrate chase method. Its particularity is that reaction mixtures are quenched by unlabeled substrate in the same solvent as the reaction mixture itself.

A. KINETIC CONDITIONS

A typical enzyme reaction pathway might be

$$E + S \overset{K_1}{\longleftrightarrow} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E^*S \rightleftharpoons X_3 \text{ ---- } X_{n-2} \overset{K_{n-1}}{\rightleftharpoons} X_{n-1} \overset{k_n}{\longrightarrow} X_n \text{ ---- } E + P$$

where X_n is the nth intermediate and the asterisk is a different conformation of the enzyme. K_1 is an association constant that describes a rapid equilibrium for the formation of the collision complex ES, which then isomerizes to E^*S. This isomerization (Gutfreund, 1955), or induced fit (Koshland, 1958), is a key step on several enzyme pathways.

There are two kinetic conditions for the applicability of the cold-substrate chase. First, $k_{-2} \ll k$ ($k = k_{+2}\,[S]\,K_1/(1 + K_1\,[S])$; that is, that the ΔG_0 for step 2 is high. For this condition to hold, there may be a lower limit to the concentration of the substrate S. Second, $k \gg k_{cat} = k_n\,K_{n-1}/(1 + K_{n-1})$ where k_n is the rate-limiting step. Thus, on a time scale $t \sim 1/k$, the enzyme turnover can be ignored since $t \ll 1/k_{cat}$.