METHODS OF BIOCHEMICAL ANALYSIS

Edited by DAVID GLICK

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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

METHODS OF BIOCHEMICAL ANALYSIS

Volume 31

CONTENTS

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

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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-

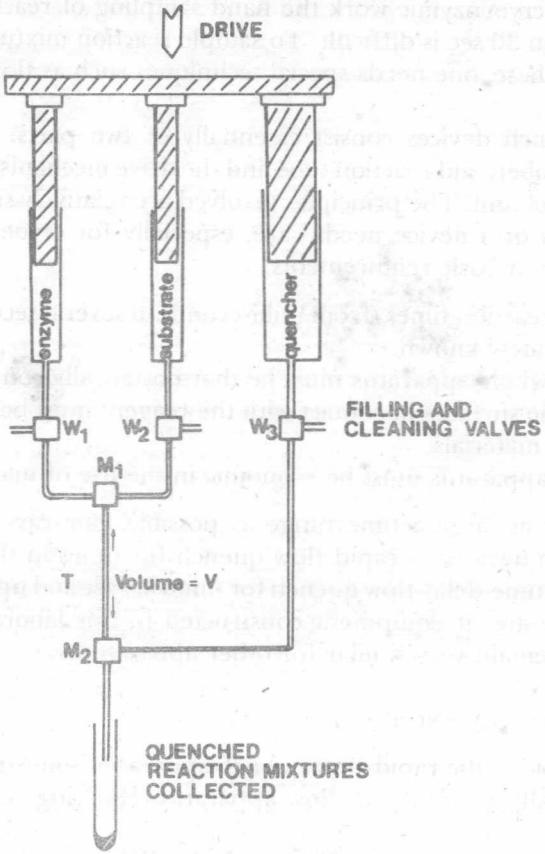

Figure 1. Principle of rapid-flow-quench apparatus. Enzyme and substrate are mixed in M_1, react in the reaction tube T, and the reaction mixture is quenched in M_2.

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

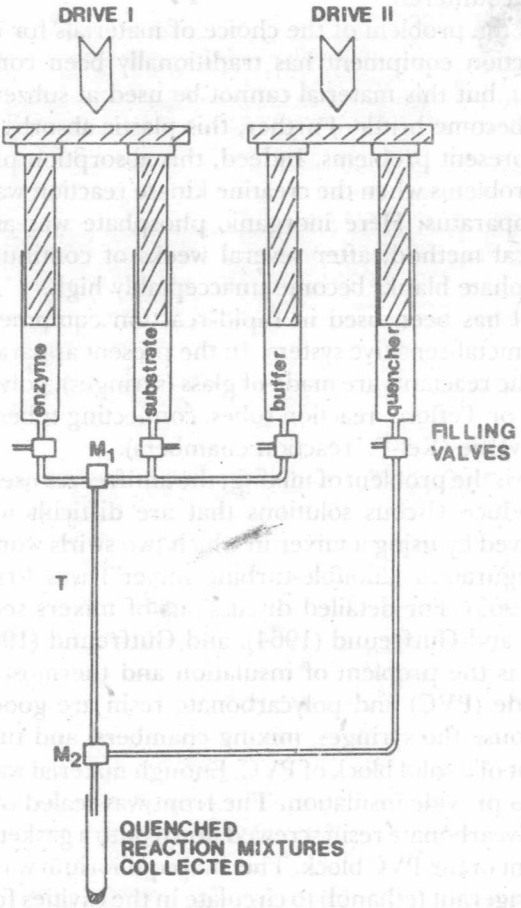

Figure 2. Principle of time-delay flow-quench apparatus. Enzyme and substrate are mixed in M_1, and the reaction mixture incubated in the reaction tube T. The aged reaction mixture is expelled and quenched in M_2.

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).

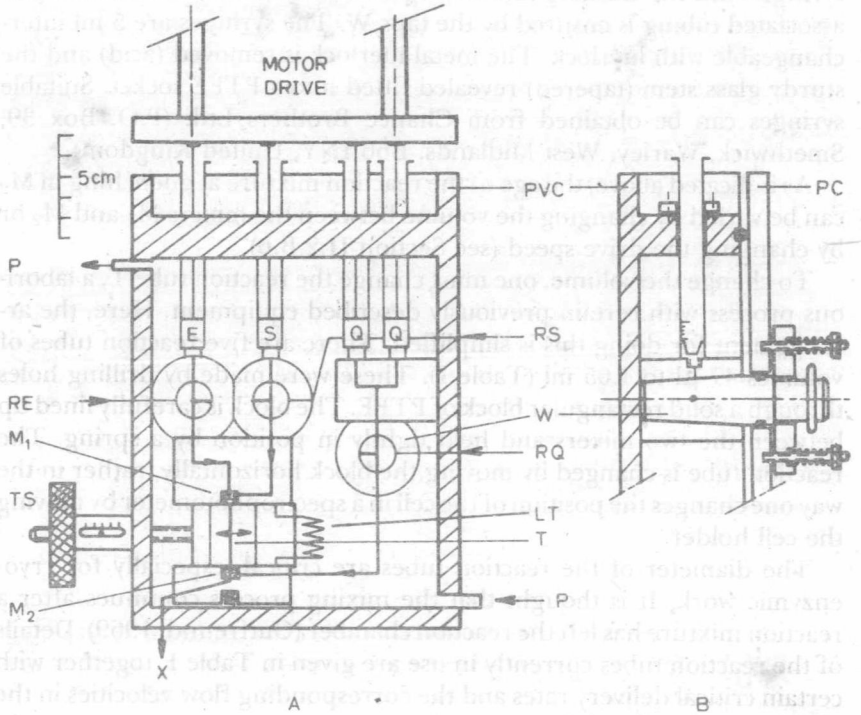

Figure 3. Schematic diagram of the rapid-flow-quench apparatus adapted to subzero conditions. (A) Front sectional view with taps in the experimental position; (B) cross-sectional view showing details of a tap. E, S, and Q represent the syringes containing the enzyme, substrate, and quenching solutions, 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).