A Four-Channel Temperature-controlled Stop-flow Mixing Apparatus Adapted for the Beckman Model DU Spectrophotometer

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(Received 6 July 1955)

Many of the current research problems in the field of enzymology are characterized by a need for considerable precision in measurements of kinetic and equilibrium constants. Frequently this requirement can be met only with suitably designed reactant-mixing devices, such as Chance's rapid stopflow mixer (Chance, 1947). Although the emphasis on the design and construction of such equipment has been in the direction of high-speed resolution, there are numerous instances in which the reaction times observed are of the order of a minute or more. In these circumstances the rigorous engineering requirements of a mixer are not as severe as those in which the time resolutions are of the order of milliseconds, or even microseconds (Chance, 1947). This provides sufficient flexibility in the design of the mixing chamber to permit the use of multiple-channel systems. The advantages of multiple-channel systems are particularly apparent in studies of relatively unstable enzyme preparations, or in situations where a very high degree of accuracy is desired. With multiple channels feeding into one mixer it is possible to obtain frequent controls in conjunction with the experimental studies. This is especially useful in inhibition studies. The ideal arrangement permits the investigator to make measurements of the activity of the uninhibited enzymes a few minutes (or seconds for very unstable enzyme preparations) before and after the inhibitory study is made. A desirable feature of any mixer is an adequate temperature control of the mixing and reaction chambers. This was incorporated in the two-channel mixer of Beers & Sizer (1953), where the mixer and reaction vessel were combined in the quartz cuvette of the spectrophotometer.

The present paper describes the design and construction of a four-channel temperature-controlled stop-flow mixing apparatus adapted to the Beckman model DU spectrophotometer. Although it is being used for studies of the reactions of catalase, it can be used with equal facility in the study of any reaction involving absorption-spectrum changes.

EXPERIMENTAL

The apparatus

The mixer and temperature-controlled cylinder supporting the four syringes, which empty into the four channels leading to the mixer, are constructed as one unit and fastened by means of adjustable screws to a brass block containing the quartz reaction vessel. The quartz vessel is supported within the brass block by means of nylon bushings. The spent reaction mixture drains from the vessel through the base of the brass block. The flow is controlled with a needle valve external to the block. Each

Fig. 1. Temperature-controlled stop-flow mixing apparatus in position. Numbers refer to the parts of the apparatus described in the text.

Fig. 2. Cut-away section of temperature-controlled stop-flow mixing apparatus. Numbers refer to parts of apparatus described in the text.

of these components of the mixing apparatus is described in detail below. The numbers in parentheses refer to numbers in Figs. 1 and 2.

Syringe support and driving mechanism (see Fig. 1). Four 20 ml. Luer syringes (1) of approximately equal bore are supported vertically in a Plexiglas (methacrylate) cylinder (2) 8.5 cm. in diameter and 8 cm. high, with a wall thickness of 0.7 cm. The cylinder is joined at the bottom by means of trichloroethylene to a Plexiglas base plate (3) 10 × 10 cm. Within the cylinder on the base plate is a thin sheet of Bakelite (4), which acts as a light-seal over the mixer. A square cover (5), 14.5 × 14.5 cm., with a recessed ring to receive the top edge of the cylinder, is clamped to the cylinder by means of four nylon screws (6). These

extend into Plexiglas extensions (7) fused to the base plate. A tight seal is obtained with a rubber gasket (8) between the cover and the cylinder. An inlet (9) and an outlet (9) are provided in the wall of the cylinder for circulating the cooling fluid. The barrels of the syringes (1) are inserted through four holes (10) in the cover, the centres of which form a square approximately 3.5 × 3.5 cm. Each hole is lined with a rubber grommet to assure a watertight seal. The ground-glass tips of the syringes are inserted into precision-ground tapered holes (11) in the base plate (3) (see Fig. 2). It is important that the fit of the cylinder through the grommet be tight enough to hold the syringe securely in the tapered joint of the base plate and to prevent leakage of circulating fluid round the barrel of the syringe.

If the fit is too loose the pressure of the circulating fluid will force the syringe out of the tapered joint; if the fit is too tight the barrel of the syringe will be compressed and bind the syringe plunger.

At the four corners of the cover plate (5) are brass tubes (12), which serve as guides for the plunger platform (13) used to force the syringe plungers (14) down simultaneously. The plunger platform is fitted with four brass rods (15), which fit loosely into the plunger guides (12). The heads of the syringes fit into a circular recess (16) in the platform. Two of the plunger guides are fitted with nylon set screws (17) which serve to keep the platform in a fixed position while the syringe plungers are being manipulated. One brass rod is marked with graduations (18) to indicate the volume of material expelled from the syringes. In initial studies the plunger platform was equipped with heavy brass weights to act as the driving force, but this was abandoned in favour of manual pressure because of the tendency of the syringe plungers to bind and of the quartz tube to leak at the junction points. However, where only one operator is using this apparatus it is necessary to use an automatic drive mechanism of this nature.

Mixing chamber and channels (see Fig. 2). The base plate is fused directly to the Plexiglas block containing the mixing chamber (19). The upper section of the mixing-chamber block has triangular sections cut out of each side directly below the syringes to provide room for four stopcocks (20). Each syringe empties into a stopcock constructed of nylon, with the axis of rotation at right angles to the diagonal of the mixing-chamber block. The tapered stopcock bore is approximately 0.4 mm. in diameter. A second channel (21) enters the stopcock horizontally along the diagonal of and through an extension (22) fused to the mixing-chamber block. The entrance to this channel is tapered (23) to receive intravenous tygon tubing (24, Fig. 1) (external diameter 0.4 cm.) by means of which the syringes are filled. Attempts to fill the syringes directly by inserting a Luer syringe into this joint were abandoned because of frequent breakage of the syringe tip.

A third channel (25) enters the stopcock vertically from below along the axis of the syringe. Approximately 6 mm. below the stopcock each channel makes a 45° turn toward the centre of the mixing-chamber block. All the channels leading to the stopcock are 1.5 mm. in diameter. At a distance of 6 mm. from the mixing chamber the bore of the channel is reduced to 0.8 mm. and the direction changed another 60°, such that the channel is now directed obliquely upward into the mixing chamber. The length of these oblique jets (26) is 1 cm. Each jet enters the mixing chamber at 90° with respect to the horizontal plane and 15° with respect to the vertical plane. The mixer is 3 mm. in diameter and 12.5 mm. long, and extends 6 mm. above and 6 mm. below the jets. The exit of the mixer is tapered (27) to receive the nylon bushing of the quartz tube. In order to construct this particular geometric arrangement of the jets it was necessary to cut away a portion of the mixing-chamber block until the four 45° channels were reached. Then the jets were drilled in the Plexiglas as shown in Fig. 2. Finally, a split ring (28) was sealed over the central portion of the mixing block. The split-ring portion of the mixing block (7 × 13.5 cm.) is inserted into a hole cut in a Plexiglas plate (29) and held in place by two set screws (30) (Fig. 1). This plate provides the means for attaching the mixing chamber and drive mechanism to the brass

block containing the quartz reaction vessel. Affixed to the brass block (31) by two brass screws (33) is a brass plate (32) with the same dimensions as the Plexiglas plate, which is fastened to the brass plate by four brass screws at each corner (34). The pitch of the screw thread is slightly less in the Plexiglas plate than in the brass plate (no. 10 American standard thread, 24 threads/in. opposed to $\frac{1}{4}$ in., 20 threads/in.). This affords a delicate control of the pressure of the mixing block exerted on the nylon bushing (35), which apart from the screws provides the only point of contact between the mixer and the quartz reaction vessel unit.

Quartz reaction vessel (see Fig. 2). Drilled vertically through the centre of the brass plate and block is a cylindrical column 1.5 cm. in diameter. This is partly filled at the top and bottom with two nylon bushings (35, 36), which serve to support the quartz reaction vessel (37) as well as to provide channels (38, 39) for the flow of material into and out of the reaction vessel. The upper bushing is tapered at the top, with a gradient slightly less than that of the mixing block, thereby providing a watertight seal when pressure is exerted on the bushing. Recessed half-way down the brass plate on the nylon bushing is a shoulder (40), which is actually clear of any contact with the brass plate itself. Pressure is exerted against this shoulder by means of a spanner draw-nut (41), the purpose of which is to exert pressure on a nylon bushing against the quartz reaction vessel.

The central portions of both the upper and lower bushings have channels 1.5 mm. in diameter, the exit and entrances of which are flared to provide a smooth flow of fluid from the channel into and from the quartz reaction vessel. The nylon bushings, connected with the reaction vessel by means of a draw fit, extend 3 mm. into the unpolished quartz tube (37), which has an internal diameter of 8 mm. and a wall thickness of 1 mm. In the original design no gaskets were interposed at the nylon-quartz junctions. However, unless the cutting of the quartz is precise (but not necessarily polished) it is difficult to obtain a tight seal without undue pressure exerted by the spanner draw-nut. Consequently, as a safeguard against frequent breakage of the quartz tube from excessive strain, a gasket of dental rubber dam has been used. The lower nylon bushing (36) is fitted flush against the brass block and the joint made watertight with a rubber gasket. A central portion of this bushing extends into the copper tubing exit to serve as a trap (42) for any gas bubbles which may accumulate in the exit tubing. The joints of the various members in the reaction vessel unit are rendered watertight by pressure exerted at the junction of the nylon-bushing taper and the mixing-chamber block by four screws connecting the Plexiglas plate to the brass plate and at the shoulder of the nylon bushing by the spanner draw-nut. This means that all the weight and force of the mixing block and driving mechanism are supported by the quartz reaction vessel. The vertical thrust exerted has not proved to be great enough to cause any fracture of the quartz, except when the transverse cut of the quartz is not at right angles to the axis of the vessel. The length of the reaction chamber is 2.54 cm. The effective path length, measured by determining the optical density of a solution of known optical density at 1 cm. path length, is 0.805 cm.

Sealed with de Khotinsky cement to the upper portion of the quartz tube are five copper-constantan thermocouples

(43) connected in series to form a thermopile, designed to record directly on a Brown strip-chart recorder. The thermopile leads pass to the rear of the brass block and plate through a channel (44). Direct vision of the quartz reaction vessel is obtained by means of a channel cut obliquely (45) from the front of the brass block to the vessel. When the spectrophotometer is being used, this channel is covered with black tape. A window (46) has been cut on either side of the reaction vessel along the direction of the light path of the monochromator. Two cooling channels (47) have been cut in the brass block and connected in series with the cooling coils supplied with the spectrophotometer. All the brass parts have been chromium-plated to reduce corrosion, which occurs especially at low temperatures and high humidity.

The entire unit is assembled and then placed beside the cuvette housing unit of the spectrophotometer (Fig. 1). The cover of the cuvette housing unit is removed, as this area is covered by the brass plate. Extra-long screws clamp together the mixer, cooling coils, cuvette housing unit and phototube housing unit. Additional support for the extra weight of the mixer was found to be unnecessary. The front of the cuvette housing unit has been cut away and replaced with a removable door (48). This permits the spectrophotometer to be used in the conventional manner when the mixer is not being used.

Light-leaks from the mixer are avoided by covering most of the areas, except the nylon stopcocks, with black tape. In addition, the joints between the various sections of cooling coils, cuvette housing unit, etc., are sealed with black tape to prevent leakage of water between them. As an added precaution a long no. 20 needle is placed near the base of the quartz reaction vessel through the front viewing channel. With continuous suction we avoid the danger of a sudden flooding of the monochromator or of the phototube housing unit as a result of a leak in the system.

Recording system. The Beckman spectrophotometer has been converted into a semi-automatic recording system to increase the accuracy of the results. The signal is taken from the slide-wire potentiometer (density scale), which serves to balance the voltage output of the phototube (null operated system). In the operation of the spectrophotometer the density-scale potentiometer is used to 'track' the changes in optical density of the reacting system. Simultaneously and continuously the recorder plots the corresponding percentage transmission, a linear function of the voltage output of the slide-wire potentiometer.

Fig. 3 shows a diagram of the circuit used for recording the percentage transmission on a strip-chart Brown recorder, Model 153 $\times$ 17V-X-30, with a chart speed of 2 in./min. and a pen excursion time of 2 sec. The maximum output of the slide-wire potentiometer (at 110% transmission) is dependent on the output of the voltage divider used to vary the photometric resolution (sensitivity) of the spectrophotometer. According to the circuit given by the manufacturer this varies from 0.2 (maximum sensitivity) to 1.7 v (minimum sensitivity), values in close agreement with those measured directly (0.3 and 1.68 v respectively). The maximum input allowed to the Brown recorder is 10 mv. This large drop in the signal makes impedance matching in a voltage divider between the spectrophotometer and the recorder a relatively simple matter. The input impedance to the recorder has been set at 500 Ω with a fixed resistor, R_1 ,

which is slightly lower than that recommended by the manufacturer but satisfactory for this arrangement. In order to reduce the spectrophotometer signal to 10 mv, the impedance to the spectrophotometer circuit should range from 10 000 to 85 000 Ω . Since in the normal operation the maximum output is in the vicinity of 1 v, the maximum impedance has been set at 50 000 Ω with a fixed resistor R_2 and a variable resistor R_3 in series, each of 25 000 Ω . For values less than 25 000 Ω , the fixed resistor is short-circuited. Should one wish to work in a range of minimum photometric resolution it would be a simple matter to change the ratios $R_1:R_2$ and $R_1:R_3$.

Fig. 3. Circuit diagram of potential divider used to match the impedance of the Beckman DU spectrophotometer with that of Brown strip-chart recorder.

The error introduced into the circuit of the spectrophotometer by the voltage divider as a result of current losses has been found to be undetectable. This can be seen very quickly on inspection of the circuit in Fig. 3. Since the percentage transmission, T , is linearly proportional to the voltage output of the slide-wire potentiometer, we may express the error in terms of an error of the measured percentage transmission. Thus we have:

$$100(T_0 - T)/T_0 = 100R_4/(R_1 + R_2 + R_3 + R_4),$$

where T_0 and T are the percentage transmissions read on the density scale in the absence and in the presence of the voltage divider circuit, and R_4 is the impedance of the spectrophotometer output. The maximum error obtained is found when R_4 is large, R_3 is zero and R_2 is small, i.e. when the output from the sensitivity voltage divider is small (0.2 v). Setting R_4 at 100 Ω and R_2 at 10 000 Ω , ($R_1 = 500 \Omega$), the error in measured percentage transmission is only 0.95%. With the spectrophotometer set at this high photometric resolution this small error is scarcely detectable. It vanishes as R_4 becomes smaller (high optical density) or as the sensitivity of the spectrophotometer is decreased.

In addition to recording percentage transmission the recorder is also used to plot temperatures while the experiments are being performed. The output of the thermopiles from either the reaction vessel or the bath is fed directly into the Brown recorder through a mixing switch. This enables the operator to record simultaneously with the kinetic studies the temperature of each experimental run.

Materials

Crystalline beef-liver catalase. This was supplied by the Worthington Biochemical Co.

Bacterial catalase. This was prepared from *Micrococcus lysodeikticus* by the procedure described by Herbert & Pinsent (1948).

Hydrogen peroxide. Superoxol (Merck) was used.

Buffer. Phosphate buffer (0.02M) was used.

Procedure

Standardization of recorder circuit. The power supplies to both the spectrophotometer and the recorder are turned on for at least 30 min. before use. The density scale is set at 50% transmission and the sensitivity is set at some intermediate value. For tracking purposes it is desirable to keep the sensitivity rather high to damp the fluctuations of the ammeter needle. The resistance R_2 is varied until the recorder pen falls on the 5 mv position. For finer adjustment the sensitivity-control knob may be used. Linearity of response of the recorder to the density scale is then checked by varying the percentage transmission from approximately 10 to 90%. Non-linearity usually indicates one of two possibilities: (a) the spectrophotometer or the recorder is not earthed properly; (b) the potential divider is drawing excessive current. An additional check may be obtained as follows: Set the selector switch at position 1, open the shutter and balance the spectrophotometer with the slit width, then disconnect the potential-divider circuit. Any deviation of the ammeter needle as a result of this manoeuvre indicates a current leak in the system. After these operations have been completed the spectrophotometer is used in the normal manner.

Operation of the stop-flow mixer. The syringes are filled via the horizontal channels (21) by the Tygon (polyvinyl plastic) tubing. In the majority of studies we have connected the channels directly with the reservoirs of material which are kept in vessels supported in the water bath. Thus a simple manipulation of the stopcock and the syringe plunger is all that is required to fill the syringe. In a typical experiment the four syringes can be filled with solutions A, B, C, D: A contains the enzyme and buffer; B contains the substrate and buffer; C contains the substrate, buffer and an inhibitor; D contains buffer. If the inhibitor absorbs in the ultraviolet this absorption must be corrected for in the determination of the optical-density values. The exit needle valve is closed. The plunger block is fixed in a position which will allow the syringe plungers to be lifted to the 20 ml. mark on the syringes. Two syringes are filled with solutions A and D and the corresponding stopcocks opened directly into the mixing chamber. Care must be taken that the two remaining stopcocks do not open into the mixer but into the horizontal channel, or are closed completely. The set screws of the plunger platform are released, and with pressure exerted centrally on the platform the needle valve is opened. Approximately 15 ml. of material is flushed through the reaction vessel. The Beckman dark current and slit width are adjusted in the normal manner with the wavelength set at 2090–2200 Å for hydrogen peroxide measurements. This operation is repeated to make certain that the optical control is the same.

Syringe D is then emptied, usually back into the reservoir, and the filling procedure is repeated with solutions A and B. The selector switch is set at no. 1 position and the chart

motor is turned on. Approximately 6 ml. of combined solutions A and B is emptied into the mixer. The tracking of the optical density is begun immediately, usually within a second after the needle valve is closed. Successive kinetic runs can be made with the same solutions, A and B, or alternate kinetic runs can be made with solutions A and B, and A and C, respectively. During the filling procedure it is important that the temperature of the material in the Tygon tubing and reservoirs be approximately the same as that of the mixer. Otherwise, up to 6 min. may be required to bring the temperature of the solutions in the syringes to a steady state. Because the reaction vessel is not in close thermal contact with the temperature-controlled brass block, it is necessary to bring this rapidly to a steady temperature by flushing solutions through it. This is normally accomplished while the Beckman instrument is optically balanced.

There have been no problems in cleaning this apparatus. No grease is used in the syringes and only occasionally is a detergent used to clean the system. The solutions used must be free of any insoluble material which might block the jets. Before the quartz tube is inserted in position it is cleaned thoroughly with a detergent and then with boiling nitric acid. Provided that no fatty material is used in the reagents it is not necessary to repeat this procedure. Oxygen-gas bubbles remain the major source of difficulty. However, if the surface of the quartz vessel is hydrophilic, these bubbles do not adhere to the walls, but rise freely in the solution to the top of the reaction vessel. The presence of such bubbles can be readily detected by the erratic fluctuations of the ammeter. For the most part these fluctuations can be damped out by the operator.

RESULTS

Accuracy and speed of mixing. The accuracy of mixing has been studied by comparing the optical densities of an absorbing solution (hydrogen peroxide at 2100 Å) in one syringe mixed with water in each of the three remaining syringes. The accuracy of mixing with any given pair of syringes is precise. The ratios of volumes ejected from each syringe are

$$1.000:0.992 \pm 0.002:0.986 \pm 0.001:0.990 \pm 0.002,$$

reflecting the small differences in bore diameter of each syringe. Uncorrected, the maximum error is less than 1%.

The speed of mixing has been tested in two ways. First, the time and amount of material required to flush the reaction vessel completely were determined and found to be approximately 6 ml. in 1 sec. or less. The time-limiting factor is not the mixer but the rate of flow in the reaction vessel. Secondly, two solutions with large differences in refractive index, namely, water and saturated $(\text{NH}_4)_2\text{SO}_4$ solution, were mixed. After the initial flushing of the reaction vessel, the optical density showed no fluctuations due to currents in the solution. We may conclude that, within the limits of error detectable by the optical system of this

instrument, the mixing is complete in the mixing chamber before the material reaches the reaction chamber.

Temperature control. Of more practical importance than temperature stability has been the time required for the solutions to reach a steady temperature. We have studied this by measuring the temperature-changes within one of the syringes and the reaction vessel. The rate of flow of cooling liquid was approximately 50 ml./sec. through the cylinder and the brass block and cooling coils respectively. Increasing the rate of flow by 80–100 ml./sec. does not alter the kinetics of temperature-change appreciably. Fig. 4 shows the rates of temperature-change in the syringe and reaction vessel respectively. The difference in temperature at time t , T_t , and at steady state, T_s , where $\Delta T = T_t - T_s$ and $T_s = 1.5^\circ$, is plotted. Because there is considerable inertia in the thermopile-recording system (large heat capacities and insulation properties) these results are only approximate. However, they do give the minimum time necessary for the syringes and reaction vessel to reach a steady temperature.

Fig. 4. Change of temperature of syringe and reaction vessel recorded from thermopile output as a function of time. The temperature of the circulating fluid, T_s , is 1.5° . The temperature of the syringe contents or of the reaction vessel at time t is T_t . $\Delta T = T_t - T_s$.

We observe that the rate of cooling of the reaction vessel is considerably slower than that of the syringe. The rate of temperature-fall in both instances approximates to a first-order equation, i.e. the rate of temperature-change is proportional to the temperature. Raising the initial temper-

ature to 45° increases the initial rate of temperature-fall in the syringe, but as ΔT approaches 10° the rate approaches that seen in Fig. 4. The average minimum time required for the temperature of the syringe to reach a steady state is 6 ± 1 min. This is, of course, diminished considerably if the solutions are at the approximate temperature of the water bath.

During steady state at 1.5° , the temperature of the reaction vessel is approximately 0.5° higher than that of the syringe. At higher temperatures this difference vanishes. The stability of the temperature is a function almost exclusively of the water bath. It can be held to within 0.1° or to the limit of accuracy of the temperature-recording system.

Kinetic studies. Two types of experiments are described to illustrate the reliability of this apparatus. One advantage that the apparatus possesses is the speed with which a series of rate curves can be obtained. This reduces the errors resulting from enzyme instability. Table 1 shows

Table 1. First-order velocity constants for the decomposition of hydrogen peroxide by bacterial catalase, pH 7.0, 0.02 M phosphate buffer, 2.4°

The rate curves were obtained in groups of approximately seven, i.e. the syringes were filled once every six or seven consecutive kinetic runs.

$10^2 k_0$ (sec. ⁻¹)	
Expt. 1	Expt. 2
2.67	1.60
2.67	1.63
2.60	1.67
2.70	1.58
2.75	1.63
2.63	1.60
2.63	1.63
2.62	1.68
2.59	1.70
2.60	1.60
2.68	1.63
2.60	1.56
2.68	1.62
Mean 2.65 ± 0.027 (S.D.)	1.60
	1.60
	1.62
	1.69
	1.60
	1.59
	1.64
	Mean 1.62 ± 0.024 (S.D.)

the accuracy with which one can determine the first-order velocity constant, k_0 , for the decomposition of hydrogen peroxide by catalase. The average half-times of the two sets of experiments are 26.5 ± 0.4 and 42.9 ± 0.9 sec. respectively. In general a shorter half-time, not less than 20 sec.,

results in a greater accuracy. Fig. 5 is a reproduction of a set of the recorder rate curves at $\lambda = 2090 \text{ \AA}$. In order to obtain the results in Table 1 it was necessary to transcribe the percentage transmission values in the curves in Fig. 5 to optical-density values and replot the results in the usual manner (Beers & Sizer, 1952). Under most conditions the standard error can be kept to less than $\pm 2\%$ and frequently lower than $\pm 1\%$. A sufficient number of points can be obtained in 20 sec. for one rate

curve. Thus one can obtain a series of rate curves, numbering up to 7, with one filling of the syringes in a little over 2 min.

The second type of experiment, demonstrated by the inhibitory action of NaCl on catalase, involves first derivatives of the rate of decomposition of hydrogen peroxide (see Beers, 1955*b*; Beers & Sizer, 1956). In this study the optical density, at $\lambda = 2090 \text{ \AA}$, of the reaction mixture was obtained every 10 sec. in a total of twelve kinetic runs. The

Fig. 5. Reproduction of chart recording of percentage transmission of Beckman DU spectrophotometer as a function of time. The breaks in the curves result from switching the input to the recorder from the spectrophotometer to the thermopiles A (bath) and B (reaction vessel) respectively. The reaction followed is the first-order decomposition of hydrogen peroxide by bacterial catalase (Expt. 2 of Table 1).

average of optical densities every 10 sec. was then determined, and from successive pairs of optical densities the observed first-order velocity constant of decomposition of the substrate was calculated. This is plotted in Fig. 6. The rate of decrease in the first-order velocity constant is seen to be very smooth and to follow very closely a first-order equation. These results are to be compared with those of Beers & Sizer (1956).

Fig. 6. Change of observed first-order velocity constant, k_0 , of the catalysis of hydrogen peroxide by beef-liver catalase in 0.654 M-NaCl, pH 6.0, at 1.5°. □, k_0 versus time; ○, $\log k_0$ versus time. Data were obtained from twelve consecutive kinetic runs by determining the average optical density ($\log 1/T$) every 10 sec. and calculating the value of k_0 from each pair of optical densities.

DISCUSSION

The instrument described in this paper has been designed to study first- and second-order reactions with half-times lying between the extremes of those studied with the rapid-flow (Hartridge & Roughton, 1923), the stop-flow or the accelerated-flow (Chance, 1947) methods and those requiring no automatic-mixing devices. This range lies between 1 and 60 sec. The accuracy of this instrument in the determination of velocity and equilibrium constants is within the range demanded by the theoretical considerations of the inhibited catalase-hydrogen peroxide systems (Beers, 1955*a*, *b*). The features of this mixer, which are of parti-

cular importance in achieving a high degree of accuracy, are as follows:

(a) *Precise determination of zero time.* This is particularly important in experiments illustrated in Fig. 6. In this instance it is possible to know accurately the concentration of the substrate at specific time intervals after the initiation of the reaction. Earlier tracing methods (Beers & Sizer, 1952), which did not permit this precision, gave results that required laborious calculations of inhibition-rate constants by the method of least squares (Beers & Sizer, 1956).

(b) *Continuous recording.* The random error of time versus substrate concentration, which is particularly troublesome in studying second derivatives of the substrate concentration (Beers & Sizer, 1956), is for practical purposes abolished in this recording system.

(c) *Speed and ease of repeating experimental runs.* The advantage of obtaining many kinetic runs over a short interval of time lies in the statistical value of such a series. Unfortunately, with few exceptions the majority of papers dealing with the kinetics of rapid reactions have devoted too little attention to the statistical significance of the results published.

(d) *Ease of obtaining controls.* This feature is evident in the results given in Fig. 6, which show the inhibition slope passing through the control point. The use of controls permits the operator to discard promptly results which are the result of changes in the controls rather than of the experimental conditions.

(e) *Temperature control.* This is a feature which has not been developed extensively in mixers (see, however, Dalziel, 1954).

(f) *Versatility.* The presence of four channels increases the number of possible variations in experimental conditions that may be studied. These variations can be further increased by the use of 'pre-mixer' mixing devices in which two or more reagents are mixed before being placed in a syringe. By this method one can therefore study the action of an inhibitor on an enzyme in the absence of the substrate. We have used this technique for studying the kinetics of inhibition of free catalase by sodium chloride.

SUMMARY

1. A temperature-controlled four-channel stop-flow mixing apparatus has been designed and constructed for use with the Beckman DU spectrophotometer.

2. Each of four syringes empties into a three-way stopcock. One stopcock limb is used for filling the syringe and the other limb empties into the mixer via a small jet. Mixing is completed within

the mixing chamber before the reagents are emptied into the reaction vessel. The accuracy of mixing exceeds the limit of resolution of the spectrophotometer.

3. The reaction vessel consists of a cylindrical quartz tube held in position with unstretched nylon bushings in a brass block adjacent to the cuvette housing unit. The flow of material is controlled by a needle valve at the exit of the reaction-vessel housing unit. Approximately 6 ml. of fluid is required to flush out the reaction vessel.

4. Temperature control is maintained with a water jacket round the four syringes and with cooling channels within the reaction-vessel housing unit. Steady-state temperature is reached in the syringes in a maximum of 6 min.

5. The signal from the density-scale slide-wire potentiometer of the spectrophotometer is fed through a potential divider into a Brown strip-chart recorder. As the operator tracks the changes in optical density of the reaction mixture the percentage transmission is recorded continuously. By means of a mixing switch the temperature of the reaction vessel measured with a thermopile is recorded at intervals during the reaction time.

6. The percentage error (standard deviation) of the velocity constant of decomposition of hydrogen peroxide by bacterial catalase measured with this apparatus is less than $\pm 2\%$.

This investigation was supported by research grant C-2550 from the National Cancer Institute of the National Institutes of Health, Public Health Service, U.S.A. The apparatus was constructed by Mr W. G. Strovink and Mr J. W. Dunnell. Drawings of the instrument were made by Mr P. H. Lund. The author wishes to thank Mrs Elizabeth Gaudy for her technical assistance in these studies.

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Studies on Sulphatases

12. THE ARYLSULPHATASES OF HUMAN TISSUES*

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(Received 11 July 1955)

The arylsulphatase activity of ox and rat livers is due to three distinct enzymes, which may be readily separated and which possess widely different substrate affinities (Dodgson, Spencer & Thomas, 1955b; Roy, 1953, 1954). Two enzymes, A and B, are present in the water-soluble material of acetone-dried liver and are referred to as the 'soluble' arylsulphatases. The 'insoluble' arylsulphatase is localized in the microsomes of the liver cell and has only been obtained in a soluble form by the use of haemolytic surface-active agents or by the action of crude trypsin preparations (Dodgson, Rose, Spencer & Thomas, 1955a). Both rabbit and mouse livers possessed enzymes similarly divided into 'soluble' and 'insoluble' arylsulphatases (Dodgson *et al.* 1955b), but no investigation was made into the homogeneity of the 'soluble' enzyme.

As the initial part of a study of human sulphatases the presence of two 'soluble' and one 'insoluble' arylsulphatases in human liver has been demonstrated and their characteristics were investigated. Other tissues have been examined for the presence of these three enzymes.

EXPERIMENTAL

Enzyme preparation and assay. Post-mortem samples of normal livers and other tissues, obtained within 24 hr. after death, were frozen at -15° . Little arylsulphatase activity was lost over several weeks at this temperature. Alternatively, acetone-dried powders were prepared.

The activity of fresh and acetone-dried tissue suspensions against potassium *p*-acetylphenyl sulphate (APS), potassium *p*-nitrophenyl sulphate (NPS) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) was measured by spectrophotometric estimation of the respective liberated phenols (Dodgson *et al.* 1955b). Incubation was for 1 hr. at 37.5° in the presence of 0.5 M

* Part 11: Dodgson, Spencer & Williams (1955c).