

- Burton, K. (1956). *Biochem. J.* **62**, 315.
- Burton, K. (1959). *Biochem. J.* **71**, 388.
- Butler, J. A. V., Cohn, P. & Crathorn, A. R. (1957). In *Advances in Radiobiology*, p. 33. Edinburgh: Oliver and Boyd.
- Creasey, W. A. (1960). *Biochim. biophys. Acta*, **38**, 181.
- Creasey, W. A. & Stocken, L. A. (1959). *Biochem. J.* **72**, 519.
- Davie, E. W., Koningsberger, V. V. & Lipmann, F. (1956). *Arch. Biochem. Biophys.* **65**, 21.
- Deakin, H., Ord, M. G. & Stocken, L. A. (1963). *Biochem. J.* **89**, 296.
- Dubois, K. P. & Petersen, D. F. (1954). *Amer. J. Physiol.* **176**, 282.
- Edelmann, A. (1951). *Amer. J. Physiol.* **167**, 345.
- Ellman, G. L. (1959). *Arch. Biochem. Biophys.* **82**, 70.
- Faurès, E. & Errera, M. (1962). *Int. J. Radiat. Biol.* **4**, 477.
- Fitzgerald, P. J., Simmel, E., Weinstein, J. & Martin, C. (1953). *Lab. Invest.* **2**, 181.
- Hempelmann, L. H., Carr, S., Frantz, I. D., Masters, R. & Lamdin, E. (1950). *Fed. Proc.* **9**, 183.
- Herranen, A. & Brunkhorst, W. (1962). *Biochim. biophys. Acta*, **65**, 523.
- Hevesy, G. (1949). *Nature, Lond.*, **163**, 869.
- Hoagland, M. B. (1955). *Biochim. biophys. Acta*, **16**, 288.
- Holley, R. W. & Goldstein, J. (1959). *J. biol. Chem.* **234**, 1765.
- Holmes, B. E. & Mee, L. K. (1952). *Brit. J. Radiol. N.S.* **25**, 273.
- Holton, P. (1959). *J. Physiol.* **145**, 494.
- Hülsmann, W. C. & Lipmann, F. (1960). *Biochim. biophys. Acta*, **43**, 123.
- Kay, R. E. & Entenman, C. (1956). *Arch. Biochem. Biophys.* **62**, 419.
- Killander, D., Ribbing, C., Ringertz, N. R. & Richards, B. M. (1962). *Exp. Cell Res.* **27**, 63.
- Klouwen, H. M. & Betel, I. (1963). *Int. J. Radiat. Biol.* **6**, 441.
- Logan, R., Errera, M. & Ficq, A. (1959). *Biochim. biophys. Acta*, **32**, 147.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Mazia, D. (1954). *Proc. nat. Acad. Sci., Wash.*, **40**, 521.
- Ord, M. G. & Stocken, L. A. (1963). *Nature, Lond.*, **200**, 136.
- Osawa, S., Allfrey, V. G. & Mirsky, A. E. (1957). *J. gen. Physiol.* **40**, 491.
- Rees, K. R. & Rowland, G. F. (1961). *Biochem. J.* **78**, 89.
- Rendi, R. & Hultin, T. (1960). *Exp. Cell Res.* **19**, 253.
- Richmond, J. E., Altman, K. I. & Salomon, K. (1951). *J. biol. Chem.* **190**, 817.
- Richmond, J. E., Ord, M. G. & Stocken, L. A. (1957). *Biochem. J.* **66**, 123.
- Salganik, R. I. (1958). *Biokhimiya*, **23**, 377.
- Schram, A. & Brachet, J. (1962). *Biochim. biophys. Acta*, **57**, 596.
- Selye, H. (1957). *The Stress of Life*, p. 227. London: Longmans, Green and Co.
- Smit, J. A. & Stocken, L. A. (1963a). *Biochem. J.* **89**, 10p.
- Smit, J. A. & Stocken, L. A. (1963b). *Biochem. J.* **89**, 37.
- Stricks, W., Kolthoff, I. M. & Tanaka, N. (1954). *Analyt. Chem.* **26**, 299.

*Biochem. J.* (1964) **91**, 161

## Apparatus for Rapid and Sensitive Spectrophotometry

BY Q. H. GIBSON\* AND L. MILNES

*Department of Biochemistry, University of Sheffield, Sheffield 10*

(Received 29 May 1963)

Although the first application of flow methods for following a rapid chemical reaction in solution was made by Hartridge & Roughton (1923) during work on a biochemical problem, and although many of the technical advances in the meantime have been dictated by special biochemical requirements (for reviews see Roughton, 1953; Chance, 1953), the methods have been restricted to a very few Laboratories. The apparatus described in the present paper is the result of several years' experience and is intended for work with clear solutions with extinction changes of 0.02–0.5 in observation tubes of 2–20 mm. optical path. The aim has been to build a robust and reliable apparatus for use in general

biochemical work. The need for reliability is particularly evident with enzymes where a week's work may be needed to yield enough material for a single set of kinetic determinations. The apparatus described does not exist only as a single example; ten complete sets are already in use in different Laboratories and it is hoped that the description is full enough to be followed by any competent mechanic.

### CONSTRUCTION

*Layout and principle of operation.* This may be followed by reference to Figs. 1 and 2. The reagents are placed in the 25 ml. reservoir syringes (*A* in Figs. 1 and 2) from which they are transferred by proper setting of the valves to the 2 ml. driving syringes (*B* in Figs. 1 and 2). To make a determination the driving syringes are forced in together

\* Present address: Johnson Research Foundation, University of Pennsylvania, Philadelphia 4, Pa., U.S.A.

by the syringe-pushing block (*N* in Figs. 1 and 2) by using an hydraulic unit (*O* and *U* in Fig. 2). The reactants pass through the mixers (*C* in Fig. 1) and then flow past the observation window (*E* in Fig. 1) and on into the 2 ml. stop syringe (*F* in Figs. 1 and 2), driving its plunger upwards until the handle strikes the stop (*G* in Figs. 1 and 2). When this happens flow is stopped suddenly without having been slowed down previously, and the progress of the reaction is observed by a photomultiplier (*H* in Figs. 1 and 2). To repeat the observation the port valve (*I* in Fig. 1) is opened by using the key (*P* in Figs. 1 and 2), which allows the spent reaction mixture in the stop syringe to drain away through the drain port (*J* in Fig. 1) to the beaker (*R* in Fig. 2). The port valve (*I* in Fig. 1) is then closed and the cycle repeated by operating the hydraulic unit (*O* and *U* in Fig. 2).

*Details of construction of the flow unit.* The driving syringes are 2 ml. metal-and-glass syringes with ceramic plungers (S. and R. J. Everett and Co. Ltd., 939 London Road, Thornton Heath, Surrey). They are modified by removing the taper tip and replacing it by a threaded stainless-steel insert. The details are shown in Fig. 3. The insert (*E* in Fig. 3) is screwed into position from inside the syringe (*B* in Fig. 3) with a flat polythene seal (*H* in Fig. 3) under the head (*F* in Fig. 3), and is secured by pinning (*G* in Fig. 3). To help in doing the work on the metal end of the syringe, a jig was made as shown in Fig. 3 which allows the metal end (*C* in Fig. 3) to be held firmly by two faces (*D* in Fig. 3) without strain on the glass barrel. The view in Fig. 3 is a cross-section; the body of the jig is a hollow cylinder divided longitudinally into two halves which can be pulled together by four clamping screws (shown at *A* in Fig. 3). When the syringe has been grasped between the two halves of the jig it is further clamped longitudinally by screwing in

a collar (*I* in Fig. 3) which carries one of three hardened-steel drill bushes (*K* in Fig. 3) for carrying out the operations on the end of the syringe. The first bush guides a no. 7 (5.1 mm.) drill for tapping OBA (6 mm. diam.  $\times$  1 mm. pitch), the second passes an OBA tap, and the third guides a hollow mill used to cut a seating for an O-ring which seals the junction between the driving syringe and stainless-steel block; the ring slips over the threaded nozzle of the syringe and is compressed against the back of the valve block when the syringe is screwed home. The core diameter of the hollow mill is 6 mm. and the wall thickness 0.8 mm. Its use is important, not only to seat the sealing ring, but also to cut away the projecting part of the locking pin (*G* in Fig. 3) which holds the insert (*E* in Fig. 3) in the end of the driving syringe (*B* in Fig. 3).

*Valve block.* The driving syringes (*B* in Figs. 1, 2 and 4) screw into a stainless-steel valve block (*K* in Figs. 1, 2 and 4). This block, besides anchoring the driving syringes, also allows transfer of liquids to them from the reservoir syringes (*A* in Figs. 1, 2 and 4). Details are shown in end and side elevation in Fig. 5. The liquids from the reservoir syringes are admitted by the valves (*L* in Figs. 1, 2 and 4) at positions *B* (Fig. 5) which connect with the 1-in-10 tapers (*A'* in Fig. 4) at *A* (Fig. 5). The liquids from the driving syringes enter at *C* (Fig. 5) and leave at *D* (Fig. 5) to travel to the mixers. The layout of drillings used allows the block to be made by drilling alone without plugging, and there are only two intersections between 2 mm. diam. holes. The drilling from the driving syringes towards the delivery block (i.e. from *C* to *D* in Fig. 5) is a single hole passing straight through the block. The detailed fabrication procedure is: a piece of stainless steel is dressed to a finished size of 76.2 mm.  $\times$  38.1 mm. with sides accurately square and

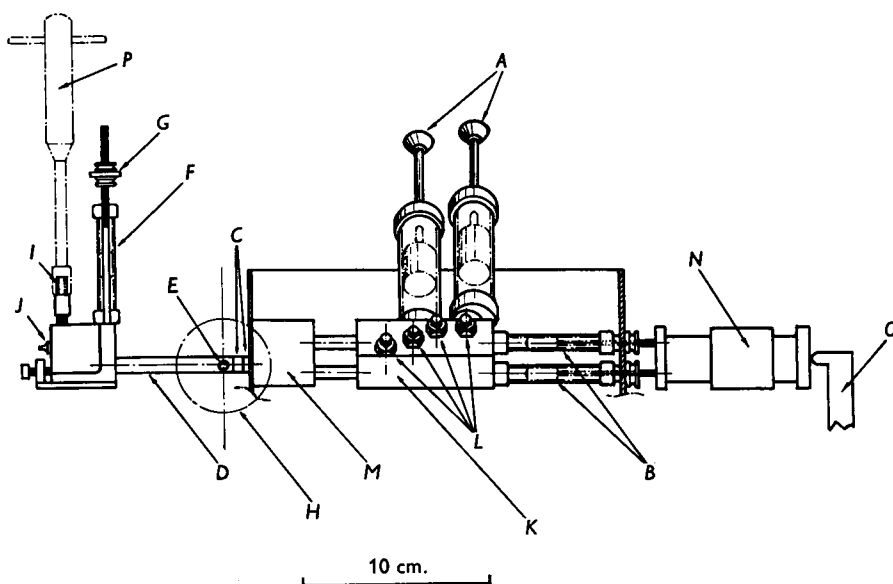


Fig. 1. Side elevation of flow apparatus. *A*, Reservoir syringes (25 ml.); *B*, driving syringes (2 ml.); *C*, two mixers; *D*, observation chamber; *E*, observation window; *F*, stop syringe (2 ml.); *G*, stop; *H*, position of photomultiplier; *I*, port valve; *J*, drain port; *K*, valve block; *L*, valves; *M*, delivery block; *N*, syringe-pushing block; *O*, lever to Armstrong R.C. 8 hydraulic actuator; *P*, position of key for operating stop valve.

parallel. Two parallel 2 mm. diam. holes are then drilled right through the block 17.7 mm. apart on a diagonal of the square face and are thus 76.2 mm. long (*C* to *D* in Fig. 5). At one end of the block (*D* in Fig. 5) the holes are opened out to a 60° cone 6 mm. deep. At the other end (*C* in Fig. 5) they are opened out with a no. 7 (5.1 mm.) drill and tapped OBA to a depth of 6 mm. Two more holes are then drilled at 90° to the first pair and to intersect with them. These are the holes starting from *A* (Fig. 5). Their centres are separated by 23 mm. in the longer direction of the block and by 12.5 mm. in the shorter direction. The hole nearer the end of the block tapped OBA is 13 mm. from that end. The ends of these holes at *A* (Fig. 5) are opened out with a 1-in-10 taper reamer to fit the type of reservoir syringe in use. Four holes (7.15 mm. diam.) are then drilled in at the valve positions *B* (Fig. 5) so that each meets one of the previous drillings and passes 3 mm. beyond it. These valve holes are opened out to 7.9 mm. diam. to a depth of 27 mm. for the two long valves (*l*) and to a depth of 14 mm. for the two short valves (*s*). These holes are counterbored to

11.4 mm. diam. to depths of 24 mm. and 11 mm. respectively, and then tapped 12.7 mm. diam.  $\times$  1 mm. pitch. The conical valve seatings are cut with a 60° centre reamer until the 2 mm. diam. drillings (the first drillings made in the block) are seen to pass through the centre of the face of the cone.

The block stands in a cradle (*K'* in Fig. 2; see Fig. 5) screwed to the bottom of the water bath and which holds it at an angle of 45° to the vertical. This makes it possible to plug in the reservoir syringes and to operate the valves

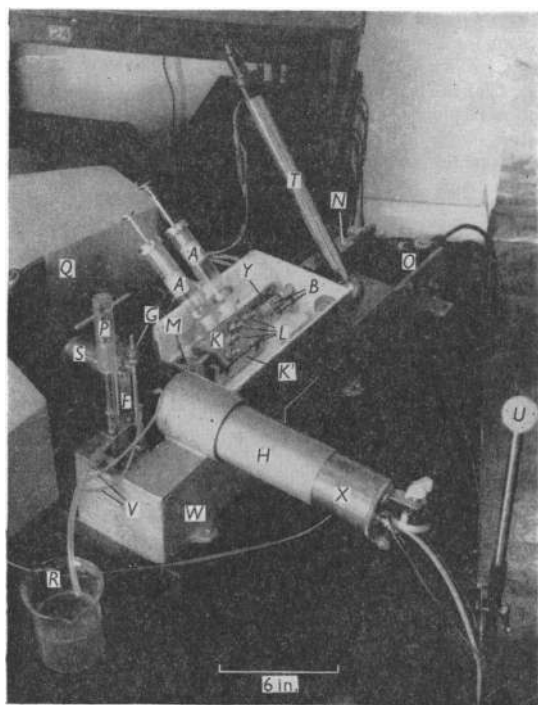


Fig. 2. Oblique view of flow apparatus. *A*, Reservoir syringes (25 ml.); *B*, driving syringes (2 ml.); *F*, stop syringe; *G*, stop; *H*, photomultiplier housing; *K*, valve block; *K'*, cradle for valve block; *L*, valves; *M*, delivery block; *N*, syringe-pushing block; *O*, Armstrong R.C. 8 hydraulic actuator; *P*, key for operating valves; *Q*, monochromator; *R*, beaker for overflow; *S*, lens holder; *T*, contact thermometer; *U*, operating lever for hydraulic drive; *V*, water leads to observation tube; *W*, main casting supporting the housing; *X*, housing for cathode follower; *Y*, heaters.

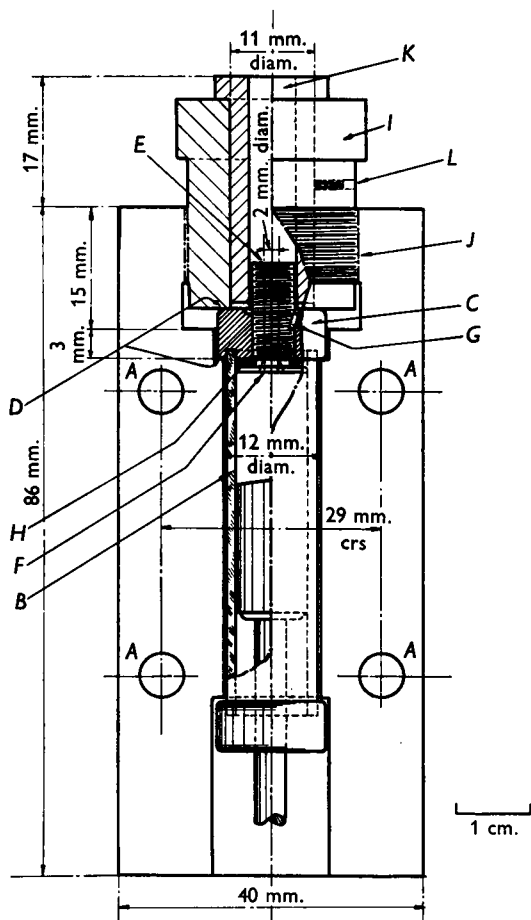


Fig. 3. Cross-sectional view of jig for holding syringes, with detail of the modification. *A*, Holes for clamping jig together; *B*, Everett interchangeable 2 ml. syringe held at the metal end (*C*) by two faces (*D*) to avoid strain on the glass barrel; *E*, threaded stainless-steel insert (OBA; 6 mm. diam.  $\times$  1 mm. pitch) with Phillips recessed head (*F*), secured by a 1.5 mm. diam. stainless-steel locking pin (*G*) at 10°; *H*, polythene seal; *I*, collar with 22 mm. diam.  $\times$  0.5 mm. pitch thread (*J*); *K*, removable hardened-steel drill bush (set of three: one for a no. 7 drill, one for tapping OBA clearance and one for a hollow facing mill), locked in position with a grub screw (*L*).

without fouling the sides of the water bath. The cradle is indicated and details given in Fig. 5. The block is located in the cradle by two 4.75 mm. round dowel pins fixed in the end with the 60° conical holes. These pins pass through holes (*E* in Fig. 5) in the cradle. The block is pressed against the leads to the mixer by two 2BA hexagon-headed stainless-steel screws (*F* in Fig. 5) which press against the end of the valve block.

**Valves and seatings.** The details of the valves (*L* in Figs. 1, 2 and 4) are shown in Fig. 6. The central valve spindle (*A* in Fig. 6) is made of three pieces (*D*, *E* and *F* in Fig. 6): the 60° tip (*D* in Fig. 6) is made from polytetrafluoroethylene (PTFE); the tip is pinned into the stainless-steel shank (*E* in Fig. 6), which in turn is held inside the stainless-steel collar (*B* in Fig. 6) by the nut (*F* in Fig. 6). This allows the tip to move relative to the collar (*B* in Fig. 6). The threaded collar (*B* in Fig. 6) is made to move up and down relative to the block by working in the double-threaded collar (*C* in Fig. 6). The internal thread of this double-threaded collar (*C* in Fig. 6) is the same as the external thread on the first collar (*B* in Fig. 6), and the external thread of the double-threaded collar (*C* in Fig. 6) allows it to be screwed into the stainless-steel block. Two dimensions are given for the two collars (*B* and *C* in Fig. 6) because two lengths of valve are needed to reach the channels in the block. To prevent reagent being forced into the thread holding the two collars (*B* and *C* in Fig. 6), the lower part of the central valve spindle (*D* and *E* in Fig. 6) is surrounded by a close-fitting polythene washer (*G* in Fig. 6) with a 90° inclusive bevel at its outer edge. This is forced into tight contact with the

valve spindle by the dished stainless-steel washer (*H* in Fig. 6) which is pressed firmly on to the polythene washer (*G* in Fig. 6) when the double-threaded collar (*C* in Fig. 6) is screwed into its place in the valve block. The valves are opened and closed, by using a 3 BA hexagonal end milled on the spindle, with the key *P* in Fig. 2.

**Delivery block.** This is shown at *M* in Figs. 1, 2 and 4, and a vertical section is given in Fig. 7. The block is made from a piece of stainless steel 4.5 cm. × 4.5 cm. × 3.75 cm. (*A* in Fig. 7). The two drillings which receive the reactants (*G* in Fig. 7) are 18 mm. and 36 mm. respectively from the bottom of the block and each is 43 mm. deep. A third hole to intersect the first two is drilled with centre 3 mm. from the front edge of the block to a depth of 28 mm. The part of this hole from the top of the block to the upper long hole is plugged (*E* in Fig. 7). All these holes are 2 mm. in diameter. After the drilling, the front face, marked *X* in Fig. 7, is milled down until only a thin partition of 0.5–1 mm. thickness separates the drilling (*G* in Fig. 7) from the surface. A coating 0.3 cm. thick (*B* in Fig. 7) of epoxy resin (Araldite; Aero Research, Duxford, Cambs.) is then put on the end surface to decrease condensation when the apparatus is being used at temperatures lower than room temperature. A recess (8 mm. diam.) is milled into the resin to provide a mount for the polythene seal (*D* in Fig. 7) and mixers (*C*<sub>1</sub> and *C*<sub>2</sub> in Fig. 7). The baffle (*F* in Fig. 7) in the drilling (*G* in Fig. 7) at the point where this leads to the mixers is a stainless-steel plate set at 90° to the direction of flow in both halves of the drilling, and it tapers from 1 mm. at the root to about 0.1 mm. thickness at the tip. It is inserted by

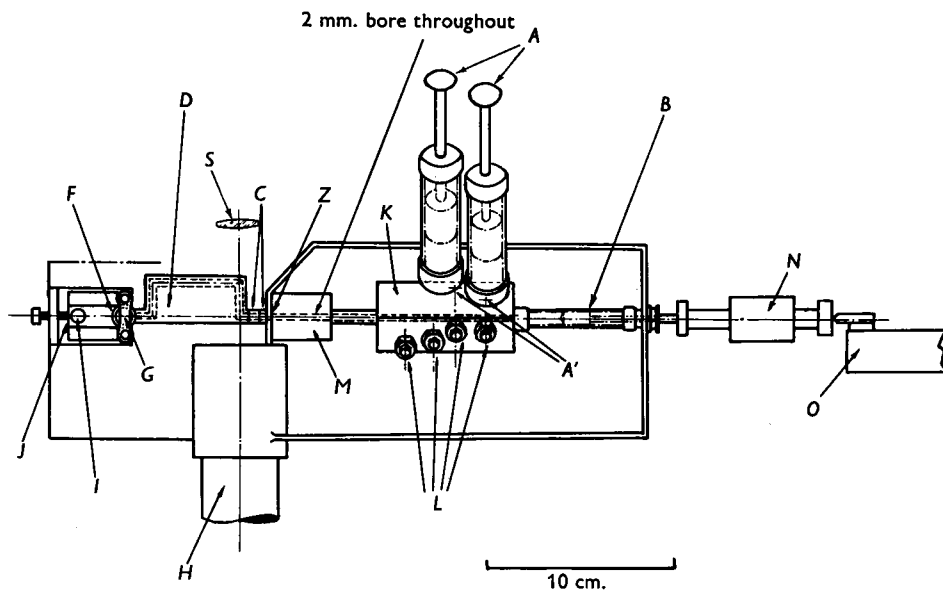


Fig. 4. Plan view of flow apparatus. *A*, Reservoir syringes (25 ml.); *A'*, tapered ports for reservoir syringes (1-in-10 record taper); *B*, driving syringe (2 ml.); *C*, two mixers; *D*, observation chamber; *F*, stop syringe (2 ml.); *G*, stop; *H*, photomultiplier housing; *I*, port valve; *J*, drain port; *K*, valve block (stainless steel); *L*, valves; *M*, delivery block (stainless steel); *N*, syringe-pushing block; *O*, Armstrong R.C. 8 hydraulic actuator; *S*, biconvex quartz lens (25 mm. diam. × 50 mm. focal length); *Z*, baffle. The crosses on the valve block (*K*) show the positions of the valves (*L*) and of the tapered ports (*A'*) for the reservoir syringes (*A*).

drilling a 2 mm. diam. hole into the block to a depth of 15 mm. at the centre of the recess in the resin. The baffle (*F* in Fig. 7), fabricated by shaping the end of a 2 mm. diam. stainless-steel rod, is pushed into the hole where it is held with a little epoxy resin.

**Mixers.** These are shown at *C* in Figs. 1 and 4. Details are shown in Fig. 8, which includes cross-sections parallel to (Fig. 8*a*) and at right angles to (Fig. 8*b*) the direction of flow, as well as a view of the surface by which fluid enters the mixer (Fig. 8*c*). Fluid from the delivery block reaches the mixer over the area shown by the inner broken circle in Fig. 8(*c*) and travels through the shallow depressions to four 1 mm. diam. holes (*C* in Fig. 8*c*) which lead to the mixing jets. Eight jets open tangentially into a central 2 mm. diam. hole (Fig. 8*a*). The jet diameter may be 0.5 mm. or 0.75 mm. (*B* in Figs. 8*a* and 8*b*) and two mixers are usually used in series. The mixers are made of polymethyl methacrylate resin (Perspex) by using a jig whose details are given in Figs. 9. In use the jig is laid on the table of a drilling machine between two gauge blocks set at 90° and a Perspex blank (8 mm. diam. × 5 mm. thick) (*A* in Fig. 9) put into it. The centre hole (2 mm. diam. × 4 mm. deep) is drilled first. A 2 mm. slip gauge is then put in to move the jig laterally for drilling the four 1 mm. diam. holes each of which is 4 mm. deep. The jig is rotated through 90° between the drilling of each hole. The slots on the rear surface of the mixer are then cut 0.5 mm. deep, by a 1 mm. engraving cutter, to connect with the four 1 mm.

diam. holes and overlapping the line of the central 2 mm. diam. hole without breaking into it. To drill the tangential jets the jig is set on its edge and the adjustable locating pin (*B* in Fig. 9) is used to set the drill path to pass through the centre of the 1 mm. diam. hole and into the 2 mm. diam. centre hole. A set of four holes is drilled and the locating pin reset by using the drill in the second drill bush. The second set of four tangential holes is then drilled. The mixer is finished by plugging the ends of the jet holes with Perspex rod by using the shank of a 1 mm. drill pushed in from the rear of the mixer as a stop. The ends of the mixers are lapped accurately flat (*A* in Fig. 8) and are pressed directly against the end of the observation tube without an intermediate washer.

**Observation chamber.** Details of an observation chamber (*D* in Figs. 1 and 4) with a 20 mm. optical-path observation tube are given in Fig. 10. Temperature-controlled water enters the inlet chamber *G* (Fig. 10) by the inlet port *H* (Fig. 10), passes to the main chamber *F* (Fig. 10) by the drillings *J* (Fig. 10) and escapes by the outlet port *I* (Fig. 10). The mixture of reagents enters at *A* (Fig. 10), and traverses *D* and *E* (Fig. 10) to leave at *B* (Fig. 10). The observation light beam passes through the polished Perspex plugs *C* from *X* to *X'* (Fig. 10), travelling through a 20 mm. depth of reaction mixture. The stainless-steel plugs *L* (Fig. 10) are pressed into place after the drillings *D* and *E* (Fig. 10) have been made. The tube is held in place with the end *A* (Fig. 10) which is lapped flat against the mixers and

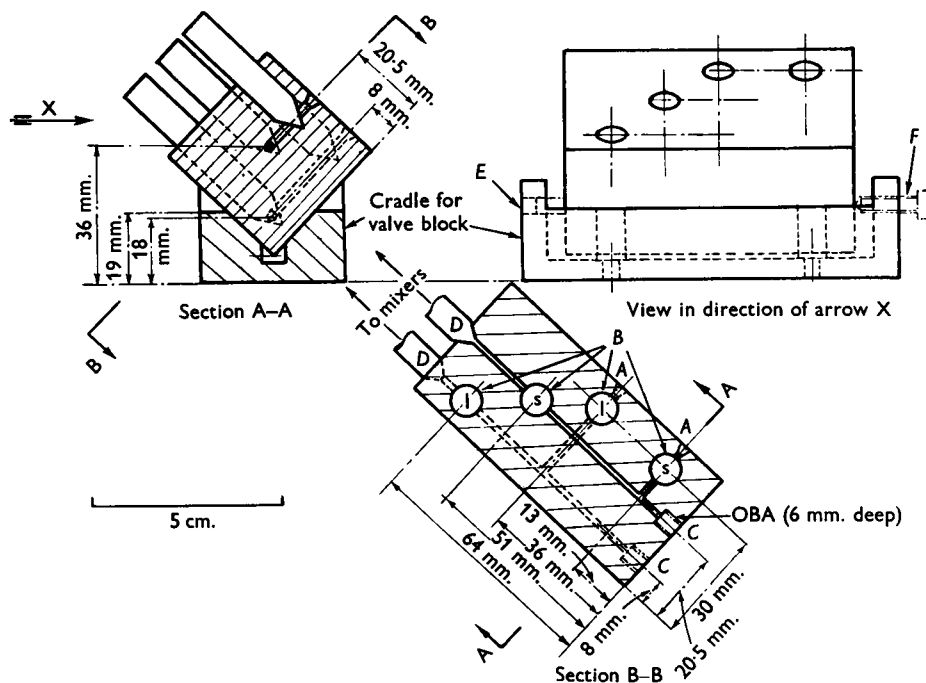


Fig. 5. Side and end elevations of valve block standing in its cradle, and diagrammatic section. *A*, Positions of tapered (1-in-10) ports for reservoir syringes; *B*, positions of valves (l, long valves; s, short valves); *C*, positions where liquids enter valve block from driving syringes; *D*, positions where liquids leave valve block; *E*, holes in cradle for valve block to take dowel pins attached to the valve block; *F*, two 2BA hexagon-headed stainless-steel screws pressing against the valve block.

*B* (Fig. 10) against the stop block. The design is similar in principle to that described by Gibson & Antonini (1960).

**Stop block.** A vertical section together with a plan is shown in Fig. 11. The block (*A* in Fig. 11) is made of stainless steel and has a simple port valve (*B* in Fig. 11) with a PTFE tip (*C* in Fig. 11) which allows liquid to flow either to the stop syringe (*E* in Fig. 11) or out through the drain port (*D* in Fig. 11). The block is 35 mm. × 35 mm. × 38 mm., but the exact dimensions are not critical. The stop syringe (*E* in Fig. 11) is the same size (2 ml.) as one of the driving syringes and is prepared in the same way. The drilling and tapping procedure required to receive the stop syringe is the same as that described for the valve block. The stop block carries an adjustable stop (*G* in Figs. 1, 2 and 4). This is made from two 3.2 mm. diam. stainless-steel rods which screw into the outside pair of the three holes shown at *I* in Fig. 11. The upper parts of these rods are threaded and each carries a pair of knurled nuts with a metal strip sandwiched between them spanning the gap between the two rods. The volume delivered at each operation of the apparatus is adjusted by screwing these nuts up and down. The whole stop block moves on a base-plate which has a large screw (*J* in Fig. 11) to press the block firmly against the end of the observation tube (*G* in Fig. 11) as shown. It is locked in position by set-screws passing through the slots *K* in Fig. 11, the movable part extending from *X* to *X'*.

**Other parts of the apparatus.** The remaining parts of the equipment may be laid out as required by the size of the photomultiplier and the optical parts. In Fig. 2 much of the ancillary equipment is shown including the water bath in which the valve block, driving syringes and delivery blocks are immersed. The delivery block is built into the wall of the water bath so that the Araldite-coated surface (*X* in Fig. 7), is flush with the front wall. The whole assembly is mounted on a heavy casting (*W* in Fig. 2) screwed to a wooden base. The monochromator (*Q* in Fig. 2) is mounted separately on a base with levelling screws, and an additional lens of 50 mm. focal length is mounted at *S* in Fig. 2 to concentrate the light from the monochromator on the opening of the observation tube. The light source is a tungsten-ribbon-filament lamp of 6v (108w) fed from a large lead storage battery without stabilization. The photomultiplier is an end-window type and a cathode follower is mounted at the end of its housing at *X* in Fig. 2. There is a wide latitude in the electronic accessories. A cathode follower is not imperative, and, provided that the anode load of the multiplier does not exceed about 0.1 MΩ, an oscilloscope may be operated directly. Rather high multiplier voltages may be needed, however, and a follower is perhaps desirable. With the equipment described, and with an 11-stage photomultiplier working into a 1 MΩ load, sufficient light is available to work over the visible spectrum with a nominal

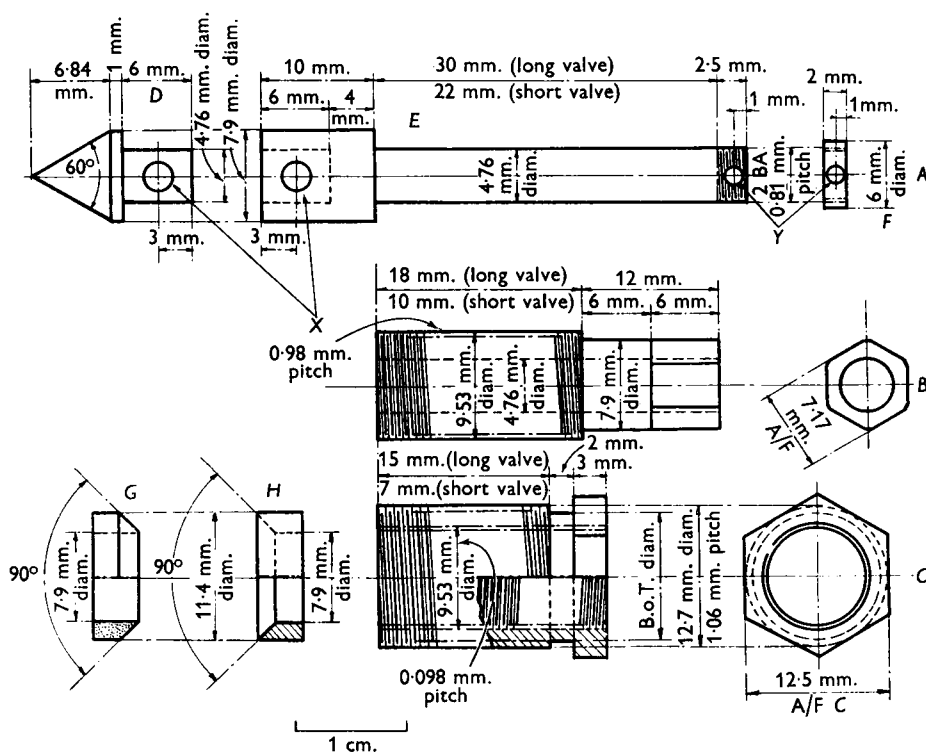


Fig. 6. Details of valves for controlling liquid flow. *A*, Central valve spindle (composed of three pieces: *D*, PTFE 60° tip; *E*, stainless-steel shank; *F*, stainless-steel nut); *B*, stainless-steel collar; *C*, double-threaded stainless-steel collar; *G*, polythene washer; *H*, dished stainless-steel washer. The holes *X* are drilled to take a 2.38 mm. diam. stainless-steel pin, and the holes *Y* to take a 1.58 mm. diam. stainless-steel pin.

band-width of 1–3  $m\mu$ , with a multiplier voltage of 400–800v and an output from the cathode follower of 1v (appearing in one particular case across 33 k $\Omega$ ). The response-time of the system is very short: less than 0.1 msec. So short a response-time gives a needlessly high noise level, and the output of the cathode follower may be shunted with small capacitances to bring the response time to about 5% of the half-time of the reaction being studied (the response time in msec. is equal to  $33 \times$  capacitance in  $\mu F$ ). Longer response-times lead to appreciable distortion of the traces, and, although correction is possible, the process is laborious. As the limiting factor in the use of the apparatus is the speed with which the records can be evaluated, anything which complicates this process is best avoided.

### PERFORMANCE

Chance (1955) has shown how the fastest irreversible second-order reaction that can be followed in a flow apparatus is related to the response-time of the recording system, the optical aperture of the monochromator, the sensitivity of the photomultiplier and the extinction coefficient of the reactants. Although his analysis was

primarily directed towards continuous-flow systems, much of his reasoning is equally applicable to the stopped-flow method. In general, it is advisable to use a light source of high surface brightness, a monochromator of large  $f$  number, set to the greatest nominal band-width the absorption spectrum under study will allow, and a detector of high quantum efficiency. Where small changes in extinction must be followed, filters are sometimes useful, but detailed calibration curves should be prepared in every case as serious errors in rate constants may occur when the pass bands of the filter are broad compared with the absorption bands of the substances being studied. Because there are so many factors that influence the performance of the apparatus, the values that follow must be regarded as giving the indication only. The greatest rate constant so far measured is  $7 \times 10^7 M^{-1} \text{sec.}^{-1}$  (Gibson & Roughton, 1962), and it seems likely that  $5 \times 10^8 M^{-1} \text{sec.}^{-1}$  could be reached in a favourable case.

The limit in studying first-order reactions is set by the time taken for the mixture of the reactants

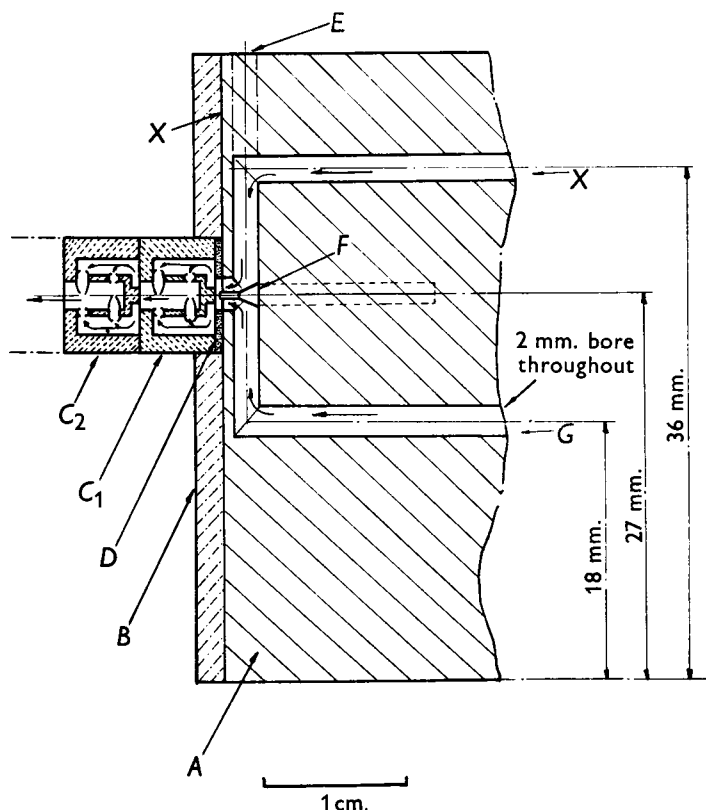


Fig. 7. Vertical section of delivery block with mixers in position. *A*, Body of stainless-steel delivery block; *B*, Araldite insulant on the front face (*X*) of the block; *C*<sub>1</sub> and *C*<sub>2</sub>, first and second mixers respectively; *D*, polythene seal; *E*, stainless-steel plug; *F*, baffle; *G*, drillings.

to flow from the mixing chamber into the observation tube. This is about 3.5 msec. for the 20 mm. observation tube fitted with two mixers as recommended, by pulling vigorously on the lever of the hydraulic unit to produce a pressure within the driving syringes estimated at 150 lb./in.<sup>2</sup> Measurements of the distribution of pressure decrease in the apparatus during flow show that there are only limited possibilities of improving on this value. Thus, in one set of measurements, the valve block was found to contribute 14 % of the total resistance, the delivery block 12 %, the mixers 22 %, the 20 mm. observation tube 40 %, and the stop block and stop syringe 13 %. It is possible, however, where a first-order reaction associated with sufficient extinction changes is studied, to obtain a worth-while improvement by substituting a 2 mm. observation tube for the 20 mm. one. The 2 mm. observation tube has a straight-through hole and decreases the total resistance to the flow to a little over two-thirds of that given by the system with a 20 mm. tube. Further, the total volume between the point of mixing and the point of observation is less with the 2 mm. tube, and the dead time is decreased to about 1.5 msec. The fastest first-order reaction that could usefully be studied would then have a rate constant of about 500 sec.<sup>-1</sup>.

The scatter of replicate determination depends on the amplitude of the extinction change being measured; if this is about 0.05 the standard error of a point on a reaction record is about  $5 \times 10^{-4}$  in extinction. The absolute error remains constant as

the total extinction change decreases below 0.05, whereas the percentage error remains roughly constant when the extinction change is increased. This is, in part, because of the difficulty of determining the elapsed time with sufficient precision, and because the photographs of the oscilloscope trace cannot be measured accurately enough.

The volume of fluid used to obtain each record should be about 0.6 ml. (i.e. 0.3 ml. from each syringe). In each operation it is necessary to use enough liquid to wash out the volume between the mixers and the point of observation, and the flow period must also last long enough to allow acceleration of the moving parts to their maximum speed. The volume recommended has been arrived at empirically and is satisfactory under most circumstances. It may have to be increased where diffusion across the T-mixer in the delivery block can give rise to trouble. For example, with 0.5 M-glucose and 10  $\mu$ M-glucose oxidase it was found that, if more than 2-3 min. was allowed to elapse between operations of the apparatus, decreased excursions in extinction were observed. This was attributed to diffusion of the very concentrated substrate solution across to the enzyme side of the apparatus.

To give concreteness to the discussion of the performance of the apparatus, an example of its application to an enzyme reaction is given. In this experiment the flavoprotein glucose oxidase was mixed with a solution containing glucose and oxygen and the reaction followed at 450 m $\mu$ . The

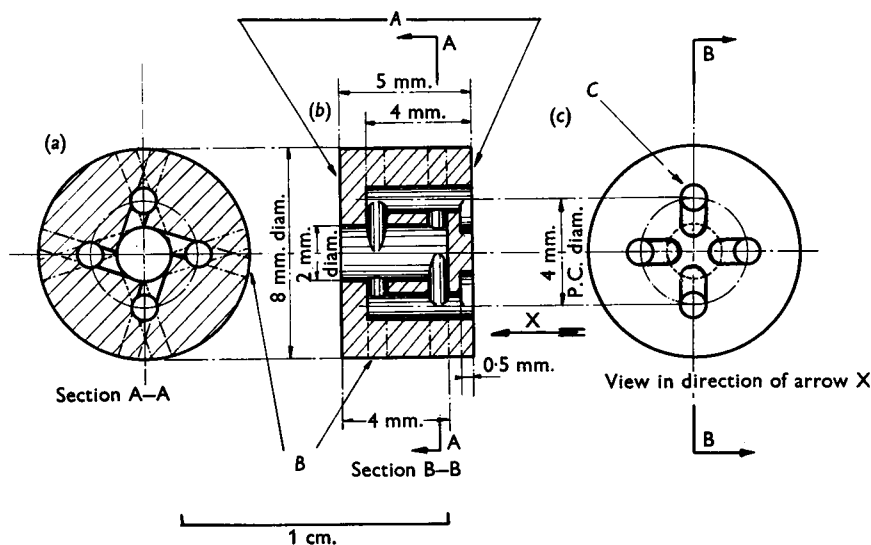


Fig. 8. End view (a), longitudinal section (b) and cross-section (c) of Perspex mixers. A, Both faces lapped flat and parallel; B, holes drilled 0.5 mm. or 0.75 mm. diam., plugged with Perspex where indicated by the broken lines; C, four holes drilled 1 mm. diam.



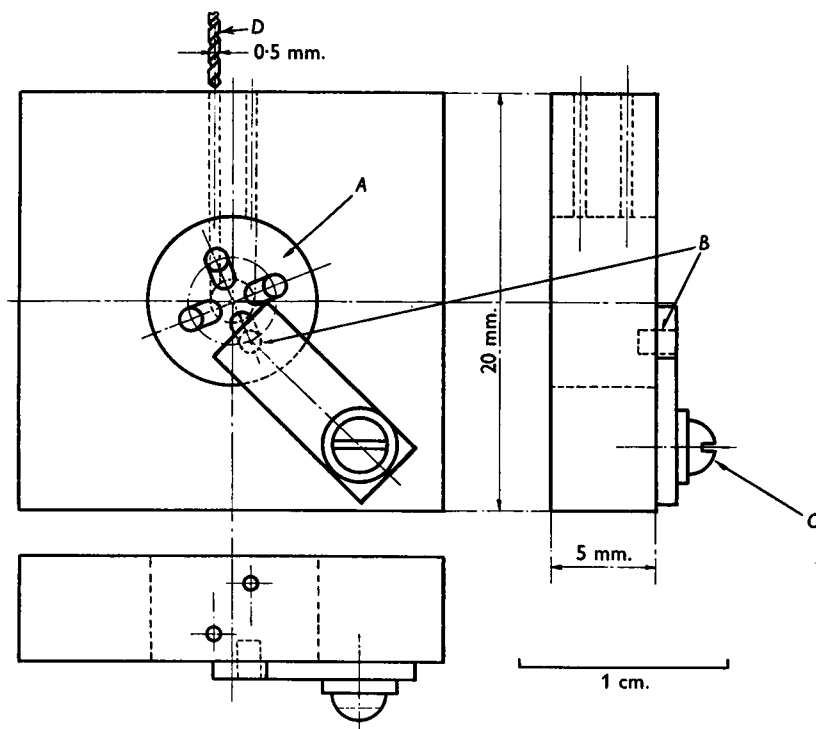


Fig. 9. Jig used for making Perspex mixers. *A*, Mixer in position; *B*, 1 mm. diam. locating pin; *C*, screw for positioning the locating pin; *D*, drill.

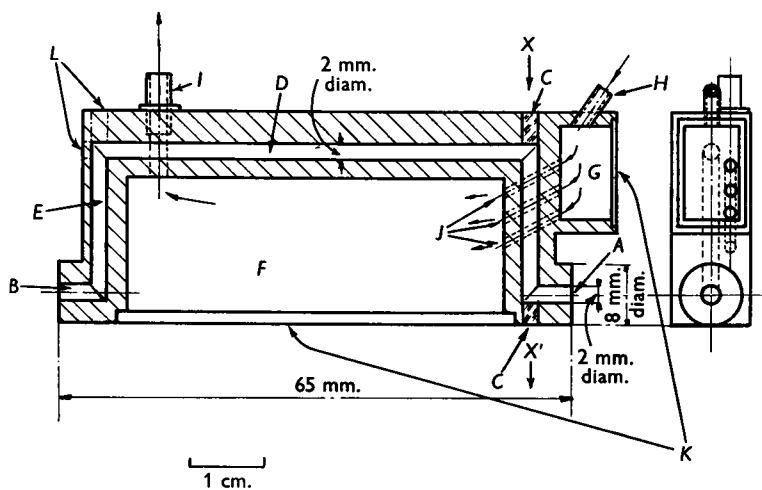


Fig. 10. Plan of observation chamber with a 20 mm. optical-path observation tube. *A* and *B*, Inlet and outlet respectively for reagent mixture; *C*, Perspex plugs polished at both ends to form windows; *D* and *E*, 2 mm. diam. drillings; *F*, main chamber; *G*, inlet chamber for temperature-controlled water; *H* and *I*, inlet and outlet ports respectively for temperature-controlled water; *J*, drillings between chambers *F* and *G*; *K*, chamber lids pressed into recesses after milling; *L*, stainless-steel plugs pressed in after drilling; *X-X'*, light-path.

enzyme goes to a steady state in which most of the flavin is in the oxidized form; then, as the oxygen is consumed, the amount of reduced flavin is progressively increased, until when it is all gone the enzyme is fully reduced. Details of the conditions of the experiment are given in Fig. 12.

#### Miscellaneous operating and maintenance points

The plungers of the driving syringes should be removed from the barrels about once a month, cleaned with detergent, and dried. They should be lubricated with an oil unlikely to release interfering substances into the solutions being studied. Liquid paraffin (B.P.) is suitable in many cases.

All gas bubbles must be removed from the system before starting work; small residual bubbles difficult to purge can be dissolved by filling the apparatus with gas-free water and then pushing the

liquid briskly from one driving syringe to another or from the driving syringes to a reservoir syringe. At the end of each day's work, after residual reagents have been washed out with water, the apparatus should be left full of gas-free water. This avoids the risk of gas bubbles appearing because of temperature changes in the room; with bubbles prevention is better than cure. For the same reason solutions should always be transferred under positive pressure from the reservoir syringes to the driving syringes. If rigorous exclusion of oxygen is required, reservoir syringes should not be employed but the vessel in which the reagent is prepared should be connected directly to the inlet of the flow apparatus. Over long periods of time (several months), the polythene washers around the valve spindles shrink and creep under the high pressure, and to maintain an adequate seal it is necessary to tighten the nuts periodically. If this precaution is neglected, the movement of the valve spindles can sometimes pump small quantities of air into the system and unsatisfactory operation follows.

About once a year, the valve stems should be removed from the valve block and the PTFE tips examined, as the prolonged heavy loads to

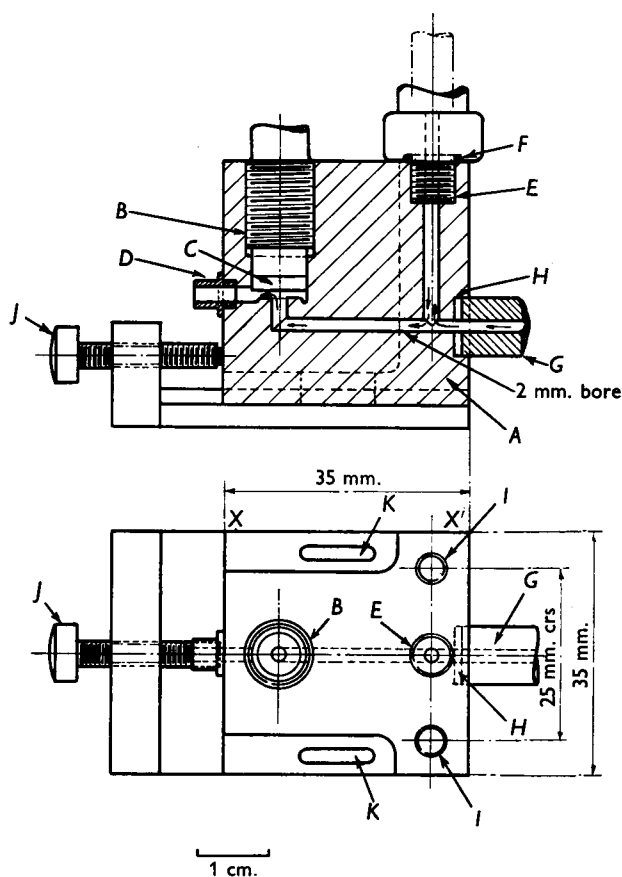


Fig. 11. Elevation and plan of stop block. A, Stainless-steel block; B, port valve with PTFE tip (C); D, drain port; E, stop syringe; F, O-ring seal; G, observation chamber; H, polythene seal; I, holes tapped for 2 BA rod; J, large screw; K, slots; X-X', extent of movable part.

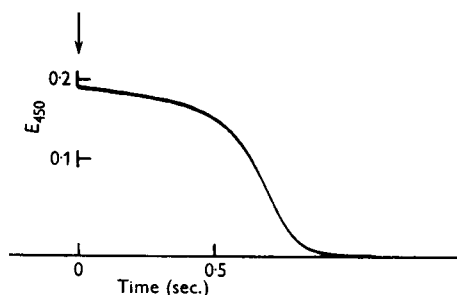


Fig. 12. Changes in extinction at 450 m $\mu$  during the oxidation of glucose by molecular oxygen catalysed by glucose oxidase from *Aspergillus niger*. At the point marked by the arrow a new portion of reactants was introduced into the observation tube and the extinction increased to a value 0.193 above that characteristic of the spent reaction mixture. The subsequent changes in extinction as oxygen disappears are recorded in the photograph. The conditions of the experiment were: enzyme concn., 11.7  $\mu$ M (expressed as flavin); glucose concn., 0.05M; oxygen concn., 0.68 mM; phosphate buffer, pH 5.9 (sodium:potassium ratio, 1:5), 0.1M; temperature, 0°. The optical path was 20 mm. and the nominal band-width 3.3 m $\mu$ , with the 108w tungsten lamp described in the text and a Bausch and Lomb Optical Inc. (Rochester, N.Y., U.S.A.), 'small grating' monochromator of aperture  $f.4$ . The trace was displayed on a Tektronix Inc. (Portland, Ore., U.S.A.) oscilloscope (type 503), and photographed on Ilford Ltd. (Ilford, London) recording film (type 5B52) by using a Cossor Ltd. (Highbury Grove, London) oscillograph camera. The scales for extinction and time were added subsequently.

which they are subjected can result in cutting into and fragmentation of the surface of the valve tips.

Finally, the 20 mm. observation tube described is not the only device that can be employed with the apparatus; the separation of the mixers from the observation tube makes it possible to use any recording device that can be introduced into the space between the mixer and the stop block. Alternative arrangements for following bioluminescent reactions (Gibson & Hastings, 1962) and fluorescence changes (Hastings & Gibson, 1963) have already been described and there is no reason why conductivity, pH or oxygen concentration should not also be followed with suitable equipment.

#### SUMMARY

1. The construction of a stopped-flow apparatus for following rapid reactions with half-times from 5 msec. upwards is described.

2. Some details of performance and suggestions for operation and maintenance are given.

#### REFERENCES

- Chance, B. (1953). In *Technique of Organic Chemistry*, vol. 8, p. 627. Ed. by Friess, S. L. & Weissberger, A. New York: Interscience Publishers Inc.
- Chance, B. (1955). *Disc. Faraday Soc.* **17**, 120.
- Gibson, Q. H. & Antonini, E. (1960). *Biochem. J.* **77**, 328.
- Gibson, Q. H. & Hastings, J. W. (1962). *Biochem. J.* **83**, 368.
- Gibson, Q. H. & Roughton, F. J. W. (1962). *J. Physiol.* **163**, 60.
- Hartridge, H. & Roughton, F. J. W. (1923). *Proc. Roy. Soc. A*, **104**, 376.
- Hastings, J. W. & Gibson, Q. H. (1963). *J. biol. Chem.* (in the Press).
- Roughton, F. J. W. (1953). In *Technique of Organic Chemistry*, vol. 8, p. 669. Ed. by Friess, S. L. & Weissberger, A. New York: Interscience Publishers Inc.

*Biochem. J.* (1964) **91**, 171

## Further Properties of the Diamine Oxidase of Pea Seedlings

BY J. M. HILL AND P. J. G. MANN

*Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts.*

(Received 14 August 1963)

Mann (1961) described a method yielding highly purified preparations of pea-seedling diamine oxidase (diamine-oxygen oxidoreductase, EC 1.4.3.6). The final preparations were pink solutions and the absorption spectra showed a band in the visible region with a maximum at about 500 m $\mu$ . Spectrophotometric investigations of the reactions of the preparations with 1,4-diaminobutane, sodium dithionite and hydrazine sulphate supported the suggestion that the pink colour is a property of the enzyme. The preparations contained 0.08–0.09% of copper which could be removed with sodium diethyldithiocarbamate; the copper-free preparations so obtained were catalytically inactive but most of the original activity was restored by adding Cu<sup>2+</sup> ions. Mann (1961) suggested that the copper is present in the enzyme as a complex with a carbonyl compound and that this complex forms the prosthetic group of the enzyme. A study of the inhibition of the diamine oxidase by chelating agents provided further evidence that the enzyme contains copper (Hill & Mann, 1962). Werle, Trautschold & Aures (1961)

also obtained highly purified preparations of the diamine oxidase that were rose-red in concentrated solution and suggested that the colour is a property of the enzyme. These preparations contained 0.12% of copper.

The results of Mann (1961) and Hill & Mann (1962) were obtained with 1,4-diaminobutane as the substrate. Extracts of pea- and clover-seedlings, and partially purified diamine-oxidase preparations made therefrom, catalyse the oxidation not only of aliphatic diamines but also of histamine, agmatine, aliphatic monoamines, phenylalkylamines and of the dibasic amino acids lysine and ornithine (Werle & Pechmann, 1949; Kenten & Mann, 1952; Mann, 1955; Werle & Hartung, 1956; Clarke & Mann, 1959). Mann (1955) concluded that the oxidation of all these compounds was catalysed by one enzyme of wide substrate specificity. Werle *et al.* (1961) showed that many of these compounds are also oxidized by highly purified preparations of the diamine oxidase.

The present work was started to investigate further the specificity of the enzyme and the mechanism of its action.