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A Stopped-Flow Dual-Wavelength Spectrophotometer Suitable for the Study of Respiratory Chains

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The requirements for a dual-wavelength stopped-flow spectrophotometer to be suitable for studying limited quantities of respiratory-chain preparations are described. They can be met by a design using mainly commercially available components. The constructed apparatus has a dead-time of approx. 2.6ms, a mixing ratio of 17:1, and a minimal requirement for 0.5ml of mixed reactants per flow.

The considerable value of stopped-flow rapid mixing in association with dual-wavelength spectrophotometry for studying respiratory chains in suspensions of whole cells, subcellular organelles or membrane fragments is well-illustrated by the work of Chance and his associates over the last two decades [for a review, see Chance (1974)]. Surprisingly, the technique has not been widely adopted for these particular applications, and this may be in part due to the apparent complexity of the published designs for the mixing and observation chambers and associated driving and stopping syringes, as for instance in the regenerative-flow apparatus described by Chance (1974). Such complexity is certainly needed when it is desired to approach the limit of performance for the technique, with dead-times (i.e. the earliest age of mixed reactants that can be observed) of 0.2ms or thereabouts (Berger *et al.*, 1968; Chance *et al.*, 1967). However, there are many respiratory carriers whose oxidation kinetics are amenable to more leisurely measurement by using dead-times of 2–5ms, and for which a simpler apparatus is therefore acceptable. And, although the oxidation kinetics of more commonly studied respiratory chains such as those of rat liver mitochondria have been thoroughly described (Chance *et al.*, 1967; Chance, 1974), there remain for study many unusual and for the most part kinetically unexplored systems such as those found in bacteria, particularly of chemolithotrophic or combined oxidative and photosynthetic capacity.

The purpose of this paper is to describe the constructional and design features and performance of a stopped-flow dual-wavelength spectrophotometer using mainly commercially available components. The apparatus has a dead-time of 2.6ms, a mixing ratio of 17:1, a minimal requirement of 0.5ml per flow, and has proved to be suitable for studying limited amounts of respiratory systems.

Materials and Methods

Optical design for the dual-wavelength spectrophotometer

This follows the principles described by Chance (1951), and is centred around two newly introduced high-intensity, f/4, 200mm-focal-length grating monochromators that are well suited for this purpose. These monochromators, from Applied Photophysics, 20 Albemarle Street, London W1X 3HA, U.K., are compact, mount on optical benching, may be used in a variety of configurations relating the entrance to the exit beam, and have continuous mechanical controls for the height and width of the exit slits. The models used had 50mm × 50mm plane gratings of 1200 lines/mm, blazed at 300nm, with a reciprocal dispersion of 4nm/mm. The light-gathering power, defined as h/f^2m where h is the slit height, f the f number and m the focal length, is 0.33. The stray light level is <0.1% in the visible range. Time-sharing of the two wavelengths was arranged with a mirror oscillating at 150Hz (optical chopper type L42 with type 4A drive circuit from W. S. Kirkpatrick and Co., 22 Audrey Place, Fairfield, N.J. 07006, U.S.A.). Higher-frequency choppers (e.g. 400Hz) are available, and could be used with due attention to their smaller amplitude of mirror oscillation. Alignment of the lamp and optical chopper was greatly facilitated by mounting each on X–Y stage drives from microscopes. Details for mounting lenses in tubes and making light-tight boxes with inspection lids for the lamp and optical chopper are not given; these aspects of design are not critical and followed normal workshop practice. The box containing the optical chopper was, like the monochromators, mounted on optical benching firmly attached to a substantial base plate. The stopped-flow accessory (see below) was bolted directly to the side of the box containing the optical

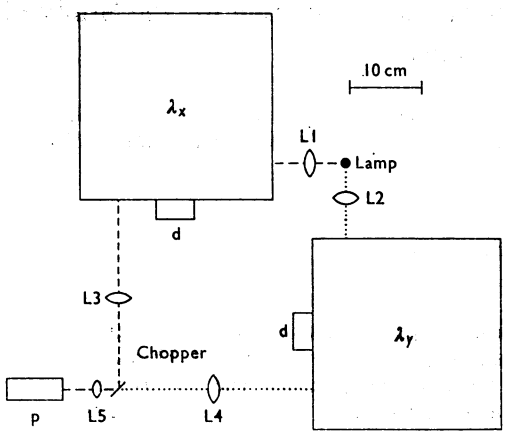

Fig. 1. *Optical plan of the spectrophotometer*

The two monochromators (Applied Photophysics) are labelled λ_x and λ_y to denote their wavelengths and relationships to the electronic circuits given below. The monochromator wavelength dial is labelled d. The lamp was a 12V, 55W quartz–iodide tungsten filament lamp (Philips no. 12258/59, from motor accessory shops). Lenses L1 and L2 were of 1.5in (3.81cm) diameter and 1in (2.54cm) focal length, L3 and L4 were 1.5in (3.81cm) diameter and 2in (5.08cm) focal length, and L5 was 1in (2.54cm) diameter and 1in (2.54cm) focal length. All lenses were made of Spectrosil and were from Optro Ltd., P.O. Box No. 2, Hornchurch, Essex, U.K. The optical chopper and the lamp were mounted on *X–Y* mechanical drives that allowed movement along the two optical axes, shown as interrupted and dotted lines for λ_x and λ_y respectively. The photomultiplier tube p was a 1in-diameter end-window type with a trialkali red-sensitive photocathode (type 125/B from E.M.I. Electronics, Electron Tube Division, Hayes, Middx. UB3 1HJ, U.K.). An approximate indication of scale is given by the 10cm horizontal bar. Important dimensions along optical axes, measured from the external face of the monochromator castings rather than from the jaws of slits, were: monochromator to chopper, 26.0cm; monochromator to lamp, 11.0cm; chopper to lens L5, 6.5cm. Lens L5 focused combined images of the two exit slits on to the front window of the flow cell (not shown). The end of the photomultiplier tube was positioned about 1cm from the rear window of the flow cell. The position of lenses L1 and L2 at about 5.0cm from the lamp was adjustable to give an image of the lamp filament at the entrance slits. Lenses L3 and L4 were also adjustable to give overlapping images of the exit slits at the chopper. Repositioning of the exit and entrance slits from the positions occupied on the commercially available monochromators was necessary to provide the continuously adjustable slits at the new exit positions. The monochromators are designed to allow this. The monochromators were calibrated for wavelength accuracy on completion of the apparatus by use of a medium-pressure arc lamp as the source.

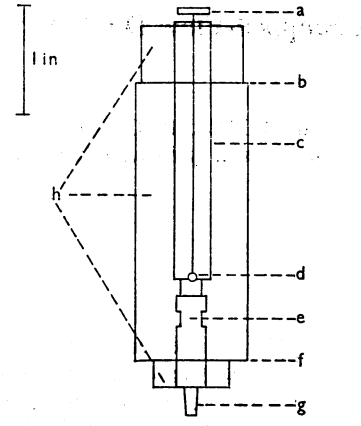

Fig. 2. *Construction of the minor syringe*

The plunger of a 250μl male Luer-tipped gas-tight Hamilton syringe (type 1725 LT, from V. A. Howe and Co., 88 Peterborough Road, London S.W.6, U.K.) was shortened to a length of 6.2cm, and the drive button (a) replaced. The top of the barrel of the syringe was shortened to about the 210μl mark, and its Luer fitting end pushed into a male–female plastic Luer adaptor (e) (Hamilton part no. 86507). The syringe and Luer adaptor were then embedded in clear polyester casting resin (available from hobby and art-craft shops). A plastic 50ml centrifuge tube made a convenient mould, and the male tip (g) of the Luer adaptor served to locate the syringe in a small central hole in the bottom of the tube during embedding. After casting, the resin (h) was machined to overall dimensions identical with the original drive syringes provided with the Aminco–Morrow stopped-flow accessory. The critical dimensions were diameter, 2.54cm (1in); height from the shoulder (f) to shoulder (b), 6.35cm (2.5in); height above shoulder (b), 1.27cm (0.5in); height of the hole (d) above the shoulder (f), 1.9cm (0.75in). It was essential that the syringe be central in the machined casting. The hole (d) was made through the resin casting with a 1mm diameter diamond-tipped drill, and went through the wall of the Hamilton syringe to join with the lumen at the zero μl mark. The hole (d) in the plastic casting was then enlarged to be able to receive a male Luer fitting. Finally the male tip (g) of the plastic Luer adaptor was removed and a $\frac{1}{4}$in × 28 UNC threaded hole made upwards into the body of the adaptor for 1cm. This threaded hole was used for connecting an adaptor and tube from the mixing chamber. Hole (d) connected the drive syringe to the reservoir syringe through a tap.

chopper. The layout and critical dimensions for lenses are shown in Fig. 1.

Design of the stopped-flow accessory

This is based on modifications to a commercially available apparatus (Aminco–Morrow stopped-flow apparatus, Aminco part no. 4-8409; American Instrument Co., Silver Spring, Md. 20910, U.S.A.),

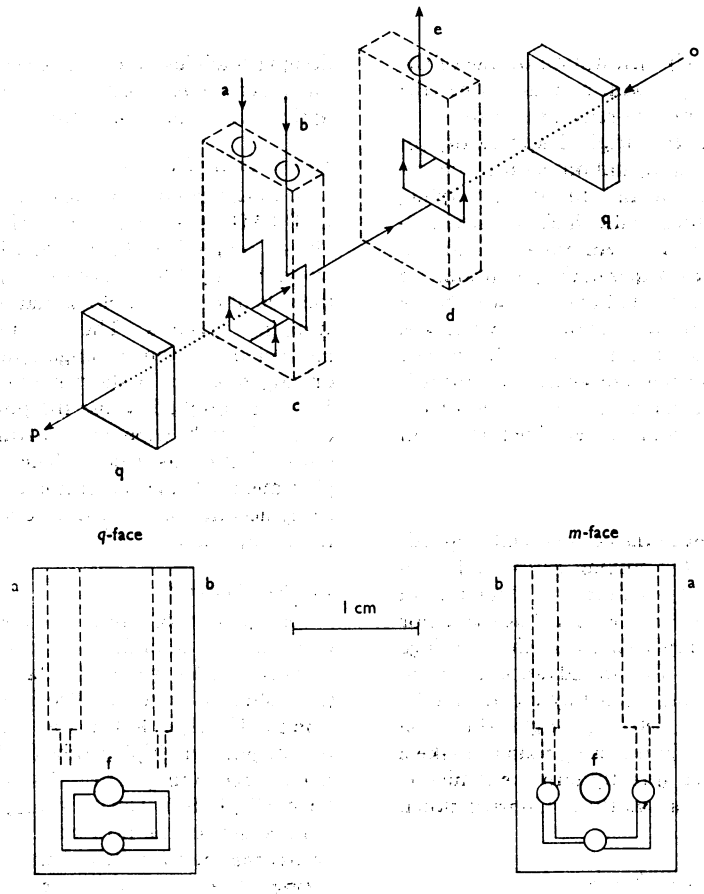

Fig. 3. *Construction of the mixing and observation chamber*

The design is based on that of Morrow (1970). The upper part of the Figure (not to scale) shows an exploded view of the mixing chamber (c), the exhaust chamber (d) and two quartz plates (q). These four components were clamped together to form the mixing and observation chamber. The act of clamping converted grooves cut in the faces of the mixing and observation chambers into closed channels. The optical path was from (o) to (p), the latter being the position of the photomultiplier tube. Each of the two chambers (c) and (d) can be regarded as having two faces: an *m*-face facing the middle of the assembly and abutting against the other chamber, and a *q*-face adjacent to a quartz plate. The grooves on the exhaust chamber (d) were on the *q*-face only, whereas they occur on both faces of the mixing chamber (c). The reactant path was via Teflon tubes from the major and minor syringes entering the mixing chamber as a push-fit at (a) and (b) respectively. The reactants flowed to separate channels on the *m*-face of the mixing chamber, mixed at the junction of the channels, and then traversed the mixing chamber from the *m*-face to the *q*-face. The once-mixed reactants were then divided along two separate channels on the *q*-face, and recombined again at the start of the observation chamber that ran from the *q*-face of the mixing chamber (c) to the *q*-face of the exhaust chamber (d). The reactants left the *q*-face of the exhaust chamber by two channels which recombined and communicated with the Teflon exhaust tube (e) which led to the stopping syringe. The optical path-length of the observation can be increased by inserting a quartz spacer with a central hole between the *m*-faces of the two chambers, and the same arrangements permit measurements of fluorescence (Morrow, 1970). Alternatively, the channels and holes of the mixing and exhaust chambers can all be machined in a single 6mm-thick Teflon block to give a 6mm path-length. The overall dimensions of the components were: quartz plates, 12.5mm×12.5cm×0.14mm; Teflon mixing chamber 24mm×12.5mm×6mm; Teflon exhaust chamber 24mm×12.5mm×4mm. The two lower Figures are to the scale shown and give the dimensions of holes and channels in the mixing chamber. The left-hand Figure is of the *q*-face; the right-hand Figure is the *m*-face. The important feature is the relative cross-sectional area of the two grooves carrying the unmixed reactants on the *m*-face. That from the major syringe was 1.0mm square, whereas that from the minor syringe was cut with a 90° end-mill to a triangular cross-section approx. 0.25mm deep. The ratio of the cross-sectional areas was therefore 17:1, corresponding to that of the major and minor syringes. This ensured identical linear velocities for the two reactants at mixing, and decreased unwanted mixing by diffusion between the channels before the mixing points. All other grooves were 1.0mm^2. The observation path (f) was along a 1.5mm-diameter hole in both chambers. The holes (a) and (b) receiving Teflon tubes are shown as interrupted lines and were drilled to the external diameter of the tube. All other holes were 1.0mm diameter. The construction of the exhaust chamber was on similar lines.

The modifications were (i) substitution of one of the two original drive syringes with a smaller one (the minor syringe) giving a mixing ratio of approx. 17:1 in place of the 1:1 on the original apparatus, (ii) construction of a new mixing and observation chamber suitable for 17:1 mixing and (iii) direct coupling of the syringe-driving block to the shaft of a mechanically and electrically linear potentiometer. Differentiation of the voltage at the wiper of the potentiometer indicated the flow velocity (Chance, 1940). Noise intrinsic in the use of wire-wound potentiometers was avoided by selection of a potentiometer with a plastic resistance track. Each of these three modifications is important to the performance and operation of the stopped-flow accessory, and is described in detail below.

The minor syringe

The two drive syringes in the commercial stopped-flow accessory gave a 1:1 mixing ratio, and each syringe of 3.5ml capacity delivered 0.71ml for an advance of 1cm. One of these remained as the major syringe, the other was replaced by a minor syringe that delivered 0.0416ml for an advance of 1cm. The minor syringe was constructed by shortening the barrel and plunger of a 250μl gas-tight Hamilton syringe and embedding it into casting resin to make a robust syringe that fitted directly into the Aminco–Morrow stopped-flow apparatus. Constructional details are given in Fig. 2.

The mixing and observation chamber

This is similar to that described by Morrow (1970), except that the cross-sectional areas of the channels fed by the minor syringe were decreased, which is necessary both to prevent back diffusion from the mixing chamber and to maintain the linear velocity at which the solution from the minor syringe is injected into the larger volume from the major syringe. The chamber was constructed from polytetrafluoroethylene ('Teflon') from G. H. Bloor Ltd., 14 Midland Street, Manchester 12, U.K. Dimensions of the mixing chamber are shown in Fig. 3. The mixing chamber (6mm thick) was clamped between two quartz plates directly on to the exhaust chamber (4mm thick) provided with the Aminco–Morrow 1:1 mixer, thereby forming an observation chamber of 10mm total path-length. The inputs from the major and minor syringes and exhaust to the stopping syringe were made with Teflon tubes that were a tight push-fit into their respective parts of the mixing and observation chamber. The other ends of these tubes were flared and attached to their respective syringes through $\frac{1}{4}$in×28UNC threaded inserts (Hamilton part no. 86419). The Teflon tubing to the major and exhaust syringes was 2.3mm external diam., 1.5mm internal diam., and that to the minor syringe 1.8mm external diam., 1.2mm internal diam. The same design principles were used to construct an alternative mixing chamber of 4mm thickness, giving an overall optical path-length of 8mm.

Flow-velocity indicator

A linear conductive plastic potentiometer (type LCP/FMI, 1kΩ±0.5% linearity, from Penny and Giles Conductive Plastics, Newbridge Road, Blackwood, Mon., U.K.) was mounted with a bracket on to the side of the metal block that holds the driving and stopping syringes. The potentiometer shaft was parallel to the syringe plungers, and was coupled to the driving block by a short metal rod. A potential of 5.0V was applied across the potentiometer, and the output from the wiper taken to the input of a differentiator circuit (see below). The maximum linear displacement of the potentiometer was 5.0cm, and easily accommodated the 3.5cm maximum stroke of the syringe driving block.

Electrical circuits

The power supplies for the lamp (12V, 5A), operational amplifiers (±15V) and gate-timing circuits (+5V) were standard commercial items (Kingshill Electronic Products, Torrens Street, London E.C.1, U.K.), as was the high-voltage supply for the photomultiplier tube (EHT supply type 472R from Bradenburg Ltd., Thornton Heath, Surrey CR4 6JE, U.K.). The +5V supply also provided the voltage across the flow-velocity potentiometer, and across the micro-switch at the stop for the stopping syringe. The drive unit for the optical chopper requires 28V d.c., and this was obtained from the ±15V supply by dropping approx. 2.0V across a series connexion with two silicon diodes.

The gate-timing circuit is shown in Fig. 4. It accepts an a.c. coupled reference wave from the optical chopper unit and generates ±12V pulses that drive the sample-and-hold amplifiers for each wavelength in the correct phase and duration.

The photomultiplier anode current was converted into a voltage, demodulated in phase with the time-shared wavelengths, provided with offset and actively filtered by the circuit of Fig. 5. Principles rather than details of design are given; the details may follow local practice and preference for components, none of which are particularly critical other than low offset currents for the input and, to a lesser extent, sample-and-hold amplifiers. Waveforms at important points in the circuits are shown in Fig. 6.

Differentiation of the flow-velocity potentiometer wiper voltage with respect to time was effected with an operational circuit by following normal practice to eliminate high-frequency noise (Graeme *et al.*, 1971). The response of the differentiator to a changing voltage at its input was calibrated by using a triangular waveform generator. From this calibration, the voltage across the flow-velocity potentiometer (5.0V

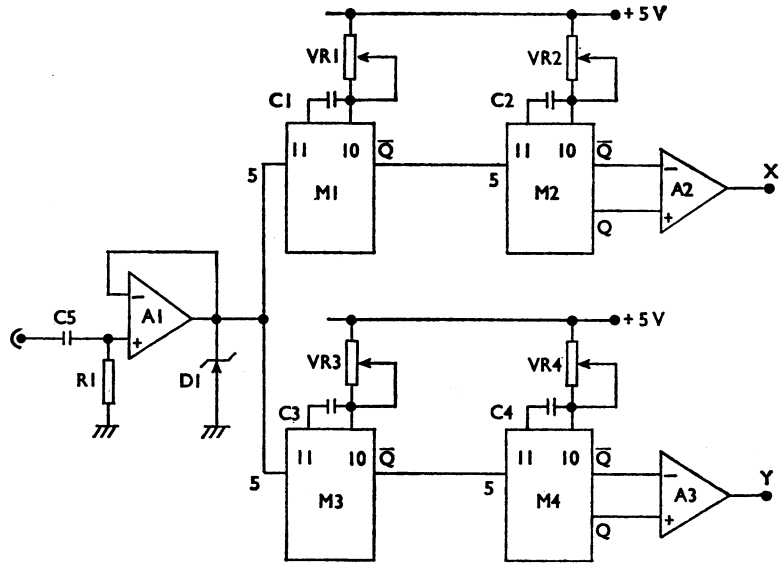

Fig. 4. *Gating and timing circuit*

The reference waveform from the optical chopper was a.c. coupled by C5 (0.1 μF) and R1 (100 KΩ) to the voltage follower A1. The output of A1 was kept within limits from −0.5 V to +4.3 V by the zener diode D1. M1, M2, M3 and M4 were monostable devices (Texas Instruments SN 74121N), triggered by a positive edge at pin 5. The other two inputs at pins 3 and 4 were grounded. The values for the timing capacitors across pins 10 and 11 were C1, 0.1 μF; C2, 0.22 μF; C3, 0.47 μF; C4, 0.22 μF. Adjustment of the duration of the monostable pulses was made with VR1–VR4 (50 kΩ, 24-turn printed circuit mounting trim-potentiometers). A1, A2 and A3 were operational amplifiers (Texas Instruments SN 72741N). A2 and A3 acted as open-loop comparators and provided ±12 V outputs at X and Y driven by the outputs of M2 and M4 respectively. The ±12 V pulses at X and Y switched the sample-and-hold amplifiers of the demodulating circuit (Fig. 5). The width of the gating pulses at X and Y was adjusted by VR2 and VR4 respectively. The timing of the gating pulses with respect to the reference waveform from the optical chopper, and therefore the photomultiplier signal, was adjusted by VR1 and VR3. Q and $\overline{Q}$ are the positive and negative logic outputs respectively.

across 5.0 cm), and the dimensions of the drive syringes (a total of 0.752 ml delivered per cm travel) it was possible to calculate the volume flow velocity of the mixed reactants, as ml/s, for a given voltage output from the differentiator.

Recording of data

A four-channel storage oscilloscope was used in the earlier part of this work, one channel being used for the flow-velocity trace and another for the spectrophotometric trace. This method suffers from two major drawbacks. One is the need to photograph the oscilloscope screen if a permanent record is needed, the other is the relative inaccuracy with which the photographs can be measured. These drawbacks are readily overcome by substituting a digital transient recorder, display oscilloscope and pen recorder for the storage oscilloscope. A moderately priced single-channel 8-bit 1000-word transient recorder with a pre-trigger memory facility (model DL 901 from Data Laboratories, 28 Wates Way, Mitcham, Surrey CR4 4HR, U.K.) has proved entirely satisfactory for our purposes. Each experiment was displayed on an oscilloscope as soon as stored, and permanently recorded when desired in a 50 s plot on a pen recorder. A separate transient recorder and display and pen-recorder channel for the flow-velocity trace was not required, for it was shown that the flow velocity and its duration and relationship to the moment of triggering by the stopping syringe micro-switch were highly repeatable for any given combination of flow volume and pneumatic drive pressure. A number of flow-velocity recordings were therefore made separately for the flow conditions usually used, and related to the spectrophotometric recordings by reference to the common trigger point. Examples of measurements stored with a transient recorder are shown in Figs. 2 and 3 of the following paper (Haddock *et al.*, 1976).

Results and Discussion

Flow velocity, mixing efficiency and dead-time

A differentiator output of 1.0 V corresponded to a volume flow velocity of 11.0 ml/s. In practice a

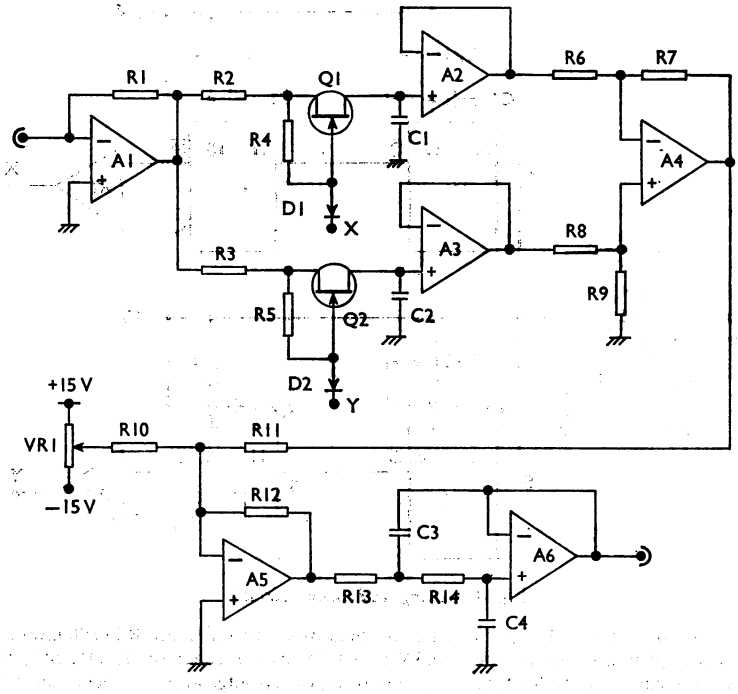

Fig. 5. *Amplifying, demodulating and filtering circuit for photomultiplier signal*

The inverting input of amplifier A1 led directly to the photomultiplier anode. A1 was a current-to-voltage converter, Q1 and Q2 were field-effect transistors acting as switches driven by the gating pulses X and Y in phase with one or the other of the two wavelengths. The gating pulses were generated by the circuit of Fig. 4. A2 and A3 together with Q1 and Q2 and capacitors C1 and C2 constituted the sample-and-hold amplifiers. A4 substracted the output of A2 from A3. A5 provided a summing point for zero offset of ±20% transmission provided through potentiometer VR1. A6 and associated network was an active low-pass filter. For operation of the spectrophotometer, the photomultiplier voltage and monochromator slit width was adjusted to give a 1.0 V signal monitored at the output of A2. The width of the other exit slit was then adjusted to give 1.0 V at the output of A3. Final balancing of the output signal from A6, which went to the transient recorder input, was made with VR1. The overall sensitivity was 1.0 V for 100% T. Components were: A1, operational amplifier type P501A from Analog Devices, 59 Eden Street, Kingston-on-Thames, Surrey, U.K.; A2–A6, operational amplifier type N5556 V (Signetics) Quarndon Electronics, Slack Lane, Derby, U.K.; Q1 and Q2, N-channel field effect transistors type 2N4391 from Siliconix, Station Approach, Buckhurst Hill, Essex, U.K.; D1 and D2, silicon diodes 1S 920 from Texas Instruments; R1, 1 MΩ; R2 and R3, 1 kΩ; R4 and R5, 220 kΩ; R6, 7, 8 and 9 all 3.3 kΩ and 1% tolerance; R10, 1.64 MΩ; R11 and R12 both 22 K and 1% tolerance; VR1, 5 kΩ, 10 turn; C1 and C2, 0.47 μF polyester; C3, 0.1 μF polyester; C4, 0.047 μF polyester. R13 and R14 were in fact on a switch that could set each at one of six values (10, 20, 50, 100, 200 and 500 KΩ) corresponding in that order to time-constants of approx. 1, 2, 5, 10, 20 and 50 ms for the active filter. The time-constant of the sample-and-hold amplifiers was 0.5 ms. Capacitors of a few pF in feedback loops of amplifiers are not shown. Voltage offset trim for the amplifiers was not necessary. If logarithmic operation with a sensitivity of 1.0 V per extinction unit was required, a switch made the two following substitutions. (i) An NPN transistor type 2N1613 (Texas Instruments) in a transdiode connexion (Patterson, 1963) in place of R1. (ii) A 1.3 kΩ, 1% tolerance resistor in place of 22 kΩ for R11. The zero offset control VR1 still provided ±0.2 V of offset at the output, but this became equivalent to ±0.2 extinction instead of ±20% T. Except where stated otherwise, resistors were 5% tolerance.

volume flow capacity of 10 ml/s was obtained at a driving pressure of 480 kPa (70 lb/in²). The observation chamber was 1.5 mm in diameter, and the corresponding linear velocity along the tube at 10 ml/s is calculated to be 5.7 m/s. This is well below the value at which cavitation commences, stated by Chance (1964*a*) as 8–25 m/s according to the mixer design. The Reynolds number (Reynolds, 1883) for flow in the observation tube was greater than 1000, in keeping with the requirement for turbulent flow and avoidance of timing errors that could arise from laminar flow. The mixing efficiency was not tested in any detail. It was sufficient to demonstrate that mixing was complete by the time that the mixed

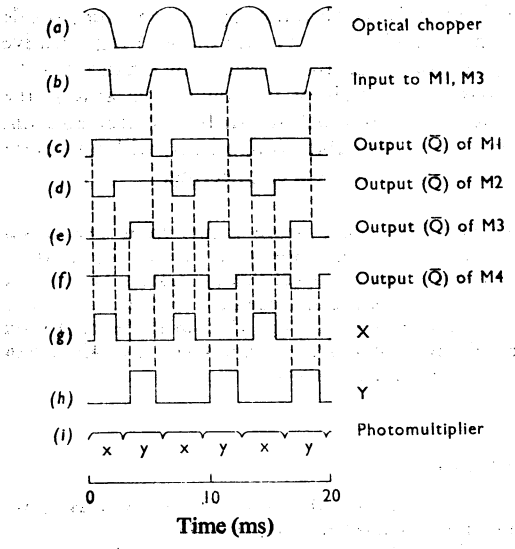

Fig. 6. *Oscilloscope display of waveforms in the circuits*

In all traces a positive voltage is upwards. Sensitivities are not given, but the peak-to-peak voltages were (*a*) reference waveform from the optical chopper, from −7 to about 0V; (*b*) −0.5V to 4.3V; (*c*–*f*), 0.2V to 3.3V; (*g*), (*h*), ±12V. The photomultiplier output was monitored at the output of the current-to-voltage converter A1 of Fig. 5. The photomultiplier signals corresponding to the two wavelengths from the monochromators are labelled 'x' and 'y', and they are sampled by the gating voltages X and Y without interference from the unwanted signal occurring when the optical chopper is switching wavelengths. Waveforms that are triggered simultaneously by the same transient are shown connected at the transient by vertical interrupted lines. The flow of information is from M1 to M2 to X, and from M3 to M4 to Y.

solutions entered the observation tube, as shown by the usual procedure of mixing acid and alkali in the presence of a pH-indicator.

At a linear flow velocity of 5.8m/s, the mixed reactants would take 0.85ms to traverse 5mm along the observation chamber. The beginning of the observation chamber was approx. 10mm from the earliest point at which the reactants first mixed, and the total cross-sectional area of the channels was similar to that of the observation tube. It follows that the dead-time would be about 2.6ms half-way along the 10mm-length observation chamber. This value was confirmed experimentally by observing the extent of completion, before cessation of flow, for two second-order reactions as the concentration of one of the reactants was increased. The reactions were the reduction of cytochrome *c* by dithionite and of 2,6-dichlorophenol-indophenol by ascorbate, and the dead-times so measured were between 2.3 and 2.8ms.

Spectrophotometric performance

Discussion of the signal-to-noise ratio must be related to the reponse time of the measuring circuits. Also, the relative concentration of a respiratory component in the light-scattering preparation under study will influence the signal-to-noise ratio, which in general is limited by photomultiplier shot noise. Thus it is difficult to quote meaningful absolute values for the noise level. However, the oscilloscope recording shown in Fig. 7 demonstrates noise of about 0.002 extinction unit at a measuring time-constant of 2.5ms in a measurement of the oxidation of cytochrome *c* occurring when an anaerobic suspension of *Saccharomyces cerevisiae* cells was rapidly mixed with oxygen. The half-time for this oxidation can be calculated from Fig. 3(*a*) of Chance (1964*b*) to be approx. 4ms. This is entirely consistent with our own determination, where 25% of the cytochrome *c* was oxidized during the flow, the remainder becoming fully oxidized within a few ms of the flow stopping (Fig. 7). With longer time-constants of 20–50ms the noise level falls to about 0.0004 extinction unit, or 0.1% transmission. The experiment of Fig. 7 was carried out by flowing a total of 0.75ml of mixed

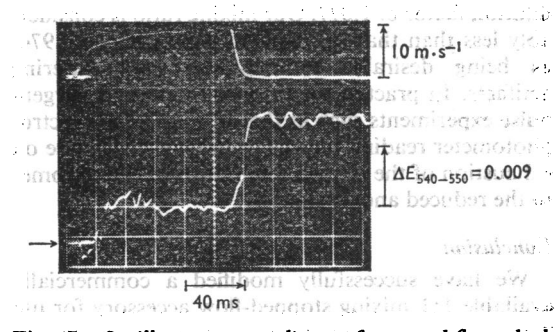

Fig. 7. *Oscilloscope recordings of stopped-flow dual-wavelength measurements of the oxidation of cytochrome c in cells of S. cerevisiae*

The major syringe contained an anaerobic suspension of baker's yeast, about 30mg dry wt./ml in water at 24°C. The minor syringe contained air-saturated water. The driving pressure was 483kPa (70lb/in²), and the measuring time-constant 2.5ms. The path-length of the observation chamber was 8mm and a total of 0.75ml of mixed reactants was flowed. The wavelength pair was 550 and 540nm; an upward deflexion of the spectrophotometer recording [lower trace] indicates cytochrome *c* oxidation (i.e. a decrease in extinction at 550nm with respect to 540nm). The horizontal arrow shows the spectrophotometer reading both just before mixing and a few seconds after when the oxygen pulse was exhausted. The upper trace is the output of the flow-velocity differentiator at an oscilloscope sensitivity of 0.5V/cm. Commencement of flow is shown by an upward deflexion of the flow-velocity trace, and cessation by a rapid fall about 100ms later.

reactant, and shows that a smaller flow of 0.5ml would have been sufficient. In practice we find that 0.5ml is a reasonable minimal volume to use unless a relatively long measuring time-constant such as 50ms is used, in which case the brief steady state set up during the period of flow lasting about 100ms will not be properly measured unless the flow volume is increased.

Further examples of the performance of this apparatus are given in the accompanying paper (Haddock *et al.*, 1976) in an investigation of the kinetics of oxidation of the cytochromes of *Escherichia coli*.

Economy

The volume of the preparation under study that was required for each flow is 0.5ml. The contents of the major syringe are diluted 5.9% for each 17:1 mixing with the minor syringe contents. Thus ignoring dead volumes, six stopped-flow measurements could be made with 3.0ml of preparation while incurring a dilution factor of 1.059. This compares well with the 80:1 regenerative-flow apparatus described by Chance (1974), where each stopped-flow measurement requires 3.0ml (again ignoring dead volumes), and six successive measurements made by recycling 3ml through the 80:1 mixer would incur a dilution factor of 1.077. Our mixing ratio is considerably less than that recommended by Chance (1974) as being desirable to minimize light-scattering artifacts. In practice we have found that in oxygen-pulse experiments such as that of Fig. 7, the spectrophotometer reading returns to the pre-flow value on exhaustion of the oxygen and return of cytochromes to the reduced anoxic state.

Conclusion

We have successfully modified a commercially available 1:1 mixing stopped-flow accessory for use with a dual-wavelength spectrophotometer and to give 17:1 mixing. Except for analysis of reactions with half-times of 2ms or less the apparatus is comparable in economy and sensitivity with the 80:1 regenerative stopped-flow apparatus described by Chance (1974). The particular merit of our own apparatus is the relative ease with which it can be constructed from commercially available components, without severe demands for mechanical engineering.

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