A Double-Beam Rapid-Scanning Stopped-Flow Spectrophotometer

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A double-beam rapid-wavelength-scanning stopped-flow spectrophotometer system based on the Norcon model 501 spectrometer was constructed, which enables u.v.- or visible absorbance spectra to be recorded at the rate of 800/s after the rapid mixing (within 3ms) of two reactant solutions. Each spectrum spans about 200nm in 1ms. It is possible to record difference spectra during reactions with half-lives less than 10ms involving absorbance changes of less than 0.1 absorbance unit. Analogue circuitry is used to produce spectra of absorbance against wavelength. Up to 32 such spectra can be recorded at pre-selected times during a reaction and stored in an 8K×8-bit-word hard-wired data-capture system to be subsequently displayed individually or simultaneously. Time-courses at different wavelengths can also be displayed. By averaging up to 2¹⁶ spectra it is possible to record spectra under conditions of low signal-to-noise ratios. Examples are given of the application of the system in recording: (a) the spectrum of NADH (2mol/mol of enzyme) produced in the pre-steady-state phase of the horse liver alcohol dehydrogenase-catalysed oxidation of ethanol; (b) the spectrum of NADH (4mol/mol of enzyme) produced in a single turnover in the glyceraldehyde 3-phosphate dehydrogenase reaction; (c) spectral changes during the reaction of aquocobalamin and dithionite; (d) spectral changes during the reaction of cyanmethaemoglobin and dithionite; (e) the release of thionitrobenzoate and production of NADH when thiol-modified glyceraldehyde 3-phosphate dehydrogenase is mixed with dithiothreitol and substrates.

Porter (1967) pointed out that many of the fast techniques used in biochemistry give only relaxation times without necessarily providing information about the nature of the process giving these relaxations. A first step in gaining such information is to record electronic absorption spectra of transient intermediates in fast reactions. To this end we describe here a double-beam wavelength-scanning stopped-flow u.v.-visible light spectrophotometer system that permits 800 absorption spectra/s to be recorded from 2 to 3ms after the mixing of two reactant solutions. This apparatus can be used to gain information about transient intermediates in chemical reactions which would be unobtainable by using conventional fixed-wavelength single-beam stopped-flow spectrophotometers except at great cost in time and materials. Each wavelength scan covers approx. 200nm in 1ms and the next scan follows after a 0.25ms delay giving the scanning rate of 800 spectra/s. The signal outputs from the reference and sample beam photomultiplier tubes are processed electronically to give a voltage that is proportional to the difference in absorbance between the contents of the stopped-flow sample cuvette and the reference cuvette. After analogue-into-digital conversion this signal is fed into an 8K-word data-capture system, which can be used to store 32 spectra (each of 256 8-bit words), taken at pre-selected times throughout the reaction. These spectra can be displayed on an

oscilloscope screen either individually or superimposed. In addition, the *i*th point from the *j*th spectrum can be displayed to give time-courses at any wavelength. An added feature of this system, made possible by the high frequency with which spectra are generated, is signal enhancement by averaging large numbers of spectra in a way normally applied in recording high resolution n.m.r. (nuclear-magnetic-resonance) spectra (Computation of Averaged Transients = CATting). Thus low-noise spectra can be generated under conditions of very low signal-to-noise ratios.

Although several different rapid-scanning systems have been developed (e.g. Strojek *et al.*, 1969; Delhay 1968; Pimental, 1968; Niesel *et al.*, 1964) only the Norcon (132 Water Street, South Norwalk, Conn., U.S.A.) (formerly Warner and Swasey) model 501 has seen much application in stopped-flow studies (Dolin *et al.*, 1967; Warner and Swasey Company Bulletins PB-1 and PB-5).

In our system the Norcon 501 serves as a scanning monochromator. Scanning is effected in the 501 by sweeping right-angled mirror pairs through an intermediate focal plane in a double-pass Czerny–Turner grating monochromator. There are 24 mirror pairs attached to the circumference of a dynamically balanced rapidly rotating wheel, which is the only moving part in the device. Each mirror pair generates a spectrum by an effect equivalent to

moving a slit across a stationary spectrum (Dolin *et al.*, 1967). The wavelength region to be scanned and the dispersion required are determined by the grating selected.

The Norcon 501 has been coupled to stopped-flow devices by others (Warner and Swasey Company Bulletins PB-1 and PB-5), but always in single-beam mode. We have used a double-beam system for two reasons. First, so that the displayed output is immediately available as the absorbance difference between sample and reference cells. Single-beam systems have the disadvantage that they superimpose the changes (often relatively small) in the absorption spectrum of the sample on to the large composite spectrum made up from the sample absorption, detector wavelength response and energy spectrum of the light-source. Absorption spectra must then be generated by laborious manual subtractions or by the use of a computer. Even when a computer is available, much of its information-storage capacity is wasted on parts of the spectrum to be discarded after subtraction. A double-beam system not only avoids this but also permits the use of a reference solution to compensate for absorption by other components in the sample solution. 'Difference spectra' can be produced directly by using the unmixed components as a reference in a split-compartment cell. Second, a double-beam system corrects for fluctuations in light-source intensity (especially important when xenon arc lamps are used) as well as correcting for differences in reflectivity of the corner mirror pairs. It is noteworthy that the system described here does not use abnormally high light intensities which would degrade photolabile substances. For example, a 100W tungsten-halogen lamp serves to cover the visible and near-u.v. regions of the spectrum.

Materials and Methods

NAD⁺ (Boehringer, Mannheim, Germany; grade I) was repurified by chromatography on DEAE-cellulose in an ammonium hydrogen carbonate gradient as described by Coulson *et al.* (1973). DL-Glyceraldehyde 3-phosphate (diethylacetal) was purchased from Boehringer (London) Corp., London W.5, U.K. Ethanol was 'Aristar' grade from BDH Chemicals, Poole, Dorset, U.K. Aquacobalamin was a gift from Dr. K. Joblin, University of Sussex.

Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) was purchased from R. N. Emmanuel, Wembley, Middx., U.K., and recrystallized from water-ethanol (1:1, v/v). Dithiothreitol was purchased from Cyclochemicals, Los Angeles, Calif., U.S.A.

Rabbit skeletal muscle glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) was prepared and enzyme concentration determined as described by Osborne & Hollaway (1974).

Horse liver alcohol dehydrogenase (EC 1.1.1.1) was the kind gift of Professor H. Gutfreund, University of Bristol. The molar concentrations of solutions of this enzyme were calculated by using a molar extinction coefficient of $183000\text{M}^{-1}\text{cm}^{-1}$ at 280nm (Theorell & McKinley-McKee, 1961).

Detailed description

Schematic diagrams of the optical system external to the Norcon 501 and the signal-processing system are shown in Fig. 1.

Optical system. The arrangement is shown in Fig. 1. The optical components are mounted on a $0.9\text{m} \times 1.5\text{m}$ (3ft $\times$ 5ft) cast-iron optical table (Scientifica and Cook, Acton, London W.3, U.K.). The Norcon 501 stands directly on this table, but the other optical components are mounted on a 76cm square $\times$ 5cm thick (2.5ft square $\times$ 2in thick) steel sub-table which is placed on pillars to bring the optical path of the stopped-flow apparatus in line with the output beam of the Norcon 501.

Light, either from a 450W Osram Wotan xenon-arc lamp [XBO450; AEG-Telefunken (U.K.), Slough, Bucks., U.K.] powered by a Thorn Automation, (New Basford, Nottingham, Notts., U.K.) model 703 xenon-arc power supply, or from a 100W Atlas A1/209 (Atlas Lighting, London W.C.2, U.K.) tungsten-halogen lamp [with a Sorenson (Norwalk, Conn., U.S.A.) QSB power supply] is focused with the quartz compound lens L_1 (Fig. 1) on to either of the two entrance slits, S_1 . (Note that when the Norcon 501 is used as a monochromator the light-path is the reverse of that intended by the makers. Thus our entrance slits are their exit slits etc.) The lens L_1 was custom-designed and constructed by Scientific Optics, Hastings, Sussex, U.K. The lamp housing (based on the design of Dr. J. J. Holbrook, Department of Biochemistry, University of Bristol) is mounted on a Zeiss triangle optical bench. The two entrance slits allow different regions of the diffraction-grating's dispersion to be scanned and the entire lamp housing shifts to illuminate either one or the other of the entrance slits marked S_1 in Fig. 1. For work in the visible region (where the strong xenon lines above 400nm interfere) a tungsten-halogen lamp replaces the xenon-arc lamp. Heat is removed before the entrance slits either by a water filter (for the u.v.) or by an i.r.-absorbing filter (Rofin, Barnet, Herts., U.K.) placed at F_1 (see Fig. 1).

Virtually any standard 65mm $\times$ 76mm ruled area plane diffraction grating can be used in the Norcon 501. We use a 258 line/mm grating (blazed at 320nm) which gives nominal scan regions of 250–460nm and 430–650nm as well as a 300 line/mm grating (blazed at 500nm) giving scans of 400–610nm and

Fig. 1. Optical system and signal processing system

Schematic diagram of the optical system external to the Norcon 501 Rapid-Scanning Monochromator and block diagram of the signal-processing system used in the rapid-scanning stopped-flow spectrophotometer. The diverging light-beam from the Norcon Model 501 is turned through 90° and made to converge by the system of spherical mirrors (M_1 and M_3) and reflecting prism (M_2). The variable beam-splitter (see the text) reflects from 50 to 100% of the light through the converging lens L_2 and thence to the quartz window of the stopped-flow observation cell. The remainder of the light passes through the reference cell to the reference photomultiplier. The AC-coupled signals from the reference and sample photomultipliers are processed by analogue circuits to give an output linear in absorbance difference between the sample and reference cells. The absorbance difference output voltage is 'corrected' for baseline perturbation caused by geometric differences between the 24 corner mirrors by the 'baseline corrector', amplified and fed to the ESL 'data-capture system'. The 'scan selector' enables the selection of every spectrum or every 5th, 10th, 25th, 50th or 99th spectrum for entry to the 32-spectrum memory of the 'data-capture system'.

570–760 nm. Entrance and exit slit-widths are available from 2 mm to 0.025 mm in seven steps. The approximate spectral band-width at 0.5 mm slits is 4 nm with the 258 line/mm grating (Warner and Swasey Company, Technical Bulletin PB-5).

The start of each scan is signalled by an electrical pulse, which can be used to synchronize the sweep of an oscilloscope or other storage-display device. Wavelength calibration is simple since the time elapsed from the start of each scan is proportional to the wavelength (Dolin *et al.*, 1967). We use a low-pressure mercury-vapour lamp as a primary standard to determine the dispersion of each grating in nm/100 μ s. The 258 line/mm grating gives 30 nm/100 μ s and the 300 line/mm grating 25 nm/100 μ s. For each experiment, known wavelength points are provided for calibration by the sharp absorption

lines of either a holmium oxide or a 'didymium' glass filter (Corning 5120, from Optiglass, Walthamstow, London E17, U.K.) placed in the sample beam.

Behind the exit slit, S_2 , is a filter, F_2 , which is selected to remove light of higher orders of diffraction from the emergent beam. The diverging beam of monochromatic light is collected by a 127 mm (5 in) diameter spherical front-surface mirror [focal length 304 mm (12 in)], M_1 , which focuses it on to a 5 mm square plane mirror, M_2 , and thence to another 127 mm (5 in)-diameter spherical mirror, M_3 . (This optical transfer system was designed by Mr. J. Bristow, lately of Techmation, 58 Edgware Way, Middx. HA8 8JP, U.K.)

To avoid problems arising from non-homogeneity of light intensity across the beam, the light beam is split uniformly over its whole area (the beam is

4.5 cm in diameter at this point) by a variable beam-splitter comprising two 10 cm square quartz plates, aluminized with 3 mm-wide parallel bands at 3 mm spacing (manufactured by Scientific Optics). These plates are mounted so that the stripes are at right angles to the direction of the slits. One plate is fixed and the other is moved by a micrometer drive such that the ratio reflected/transmitted light can be changed evenly from 50:50 to 100:0.

The reference beam passes through a standard 1 cm-path-length spectrophotometer cell and the reflected sample beam is focused by a 5 cm-focal-length silica lens, L_2 , on to the 2 mm-diameter entrance window of the 1 cm-path-length flow cell of the stopped-flow apparatus [based on the design of Gutfreund (1967) and built by Mr. P. Austen, National Institute for Research in Dairying, Shinfield, Berks., U.K.]. Both sample and reference beams then impinge on matched EMI (Hayes, Middx., U.K.) 9635QA, 50 mm-diameter photomultiplier tubes. These are normally operated at 1000–1500 V from a Brandenburg (Thornton Heath, Surrey, U.K.) 472R power supply.

Signal-processing system. The system for signal processing is also shown in Fig. 1.

The outputs from the photomultiplier tubes are AC-coupled to high slew-rate, FET operational amplifiers (Philbrick Nexus 101101) and show a response only to light-levels changing intensity with a frequency component greater than 5 Hz and less than 1 MHz. These signals first undergo DC restoration and then are amplified further in standard circuits with high slew-rate operational amplifiers ($>50 \text{ V}/\mu\text{s}$). The signals (in the range of 0.2–12 V at most wavelengths) are input to a Philbrick Nexus (Cranford, Middx., U.K.) 4358 logarithmic module used in conjunction with Philbrick Nexus model 101101 operational amplifiers which gives an output proportional to $\log(E_{\text{ref.}}/E_{\text{sample}})$ and is adjusted so that 1 V corresponds to 1 absorbance unit. The effective band-width of the overall system is from 5 Hz to 1 MHz.

The Norcon 501 generates a trigger pulse at the start of each spectrum. These pulses are first turned into square pulses by a 'Schmitt trigger' circuit and then input to the 'scan selector' (Fig. 1). This logic device can be set to pass either every pulse or every 5th, 10th, 25th, 50th or 99th pulse, thus enabling the selection of spectra for storage or display according to the rate of reaction of interest. The apparatus can also be set so that there is a logarithmic time-base by recording the 1st, 2nd, 4th, 8th, 16th, etc., spectra. (We are indebted to Dr. J. J. Holbrook for suggesting the latter recording mode.) The selected pulses pass to the logic 'gate', which blocks the passage of pulses until it is 'enabled' by the closing of a switch actuated by the stopping syringe of the stopped-flow device. Thus the system can be set such that it does not

record spectra until activated by the signal from the stopped-flow apparatus indicating that the flow has stopped.

Small geometric variations in the mounting of the 24 pairs of corner mirrors cause an up or down displacement of each observed spectrum, so that the spectrum given by each mirror pair has a slightly different baseline. The baseline variation is not wholly abolished by the log-ratio circuits as slight changes in angle from mirror pair to mirror pair cause a small but significant change in the amount of light impinging on the entry window of the flow cuvette. This problem is much more serious when the xenon-arc lamp with its intense 'hot-spot' is used. To correct the variation, a device was built which switches in an adjustable DC correcting voltage to the signal for each pair of mirrors. This 'scan baseline corrector' receives a pulse from the 'Schmitt trigger' for every spectrum and a synchronizing pulse for every scan-wheel revolution [this is generated by a light-emitting diode-photodiode reflective transducer (STRT-850A from Brookes and Green, Guildford, Surrey, U.K.), which detects a single reflective strip mounted on the black cover of the scan wheel]. A high-speed switching circuit switches in and out 24 adjustable resistors at the appropriate times to provide the appropriate correcting voltage for each pair of mirrors.

The baseline-corrected signal then passes to another 'amplifier' which serves to amplify the signal to the 0 to +10 V scale required by the data-capture system. A DC backoff is incorporated at this stage so that small changes riding on large absorption peaks can be observed at high amplification.

The data-capture system (designed and constructed by Enquiry Systems, High Wycombe, Bucks., U.K.) is triggered by the pulses from the scan-selector unit when the gate is open. It will store 32 spectra in digital form with each spectrum corresponding to 256×8 -bit words. These spectra can be displayed repetitively on an oscilloscope screen after suitable digital-into-analogue conversion. The spectra can be displayed in the following modes: (a) all 32 spectra can be displayed together in overlay mode (Fig. 2); (b) each spectrum can be displayed separately; (c) the i th point from each of the 32 spectra may be displayed, thus giving the time-course at the wavelength represented by the selected point. Fig. 2 shows the time-course at two wavelengths as well as an overlay of 32 spectra. The wavelength of the point selected can be determined by displaying the i th point pulse superimposed on an entire spectrum.

The stored spectra can be read out on punched tape or magnetic tape if required for further analysis.

The data-capture system also has an average or 'CATting' (Computation of Averaged Transients) mode. In this mode either 64 or 2048 (2^{11}) or 65536 (2^{16}) spectra are averaged. The noise, which is

Fig. 2. Reaction of aquocobalamin with dithionite

Aquocobalamin ($150\ \mu\text{M}$) was mixed with 10 mM -sodium dithionite in 0.01 M -potassium phosphate buffer, pH 7.4, at 23°C . (a) Overlay of 32 spectra (scan from 390 to 566 nm). Each spectrum took 1 ms and they are at 62 ms intervals, commencing when the flow stopped; 0.1 absorption unit/scale division. (b) Time-course at 412 nm taken from the experiment in (a). The vertical scale is 0.1 absorbance unit/division. Points are 62 ms apart. (c) Time-course at 540 nm .

Fig. 3. Effect of signal averaging

The spectra shown are of a 5 mm -thick 'didymium', glass filter and cover the range from 370 to 580 nm . The large peaks at the left of the spectra are artifacts resulting from too low signals from the photomultipliers. A single spectrum is shown in (a) and the result of averaging 64 such spectra in (b). Spectrum (c) is a single scan of the same filter but with a solution of diluted milk (2 absorbance units at 540 nm) placed in the reference and sample cuvettes to attenuate the light. The signal-to-noise ratio improvement by averaging 64 scans and 2048 (2^{11}) scans is shown in (d) and (e). Spectrum (f) is a baseline with water in the reference and sample cuvettes.

random, cancels, and the signal summates. As long as the signal changes negligibly in the time taken for collection of the spectra, low-noise absorption spectra of extremely dilute species may be recorded, such as the spectra of enzyme intermediates in the steady state.

At present the stored spectra are displayed on an

oscilloscope screen and photographed. The photographs are enlarged and sketched on graph paper. The errors at this stage are greater than at any other and it is intended to use computer processing to obviate this difficulty. (We consider that it is important to 'see' the data during experiments so that computation has been left to the final stage.)

Performance

Signal-to-noise ratio and signal averaging (CATting). Fig. 3 shows spectra of a 'didymium' glass filter recorded in different modes of operation of the apparatus with wavelength resolution and signal-to-noise ratio of a single 1 ms scan. This signal-to-noise ratio enables the recording of useful spectra in reactions with half-lives as low as 5 ms involving maximum absorbance changes of less than 0.1 absorbance unit. (Spectra recorded which give significant absorbance changes in 1 ms can be 'corrected' by suitable extrapolation procedures.) If the signal-averaging mode is used, an enhanced signal-to-noise ratio can be obtained (Fig. 3b). Although this spectrum clearly has a higher signal-to-noise ratio than the single spectrum of Fig. 3(a) the power of this application is better illustrated by the spectra in Figs. 3(c), 3(d) and 3(e). These spectra were recorded with diluted milk placed in each light beam to simulate high background absorbance (averaging about 2 absorbance units). It can be seen that the signal-to-noise ratio is hopelessly low except when the 2^{11} accumulated scan mode is used. Comparison of Fig. 3(b) and 3(e) reveals that the signal-to-noise ratio of the averaged spectrum with the high-background absorbance approaches

Fig. 4. Authenticity of a recorded spectrum of a 'didymium' glass filter

The continuous line represents a spectrum of a 5 mm thick 'didymium' filter obtained by using the rapid-scanning spectrophotometer (band-pass: 4 nm) and the points shown (Δ) are values recorded by a Cary 14 spectrophotometer. The Cary 14 spectrum of the filter is also shown (----) raised 0.04 absorbance unit above the spectrum recorded by the rapid-scanning spectrophotometer system (—).

that of the averaged spectrum with no signal attenuation.

Accuracy. Fig. 4 shows the spectrum of a 'didymium' glass filter recorded by the rapid-scanning spectrophotometer, compared with a spectrum recorded by a Cary 14 (Varian Associates, Walton-on-Thames, Surrey, U.K.) spectrophotometer and Fig. 5 shows a similar comparison of the spectrum for Chlorophenol Red (acid form). The agreement between the rapid-scan spectrophotometer and the Cary is within 3% at all wavelengths in each case and much of this error results from the drawing and processing of the photographs of the oscilloscope screen.

The response of the rapid-scanning spectrophotometer is linear in absorbance up to 1.5 absorbance units after which significant deviations from linearity become apparent (Fig. 6).

It is possible to achieve satisfactory results when the overall absorbance change is as low as 0.02 unit without the use of the average mode, since the noise in a single spectrum is normally less than 0.02 absorbance unit (0.05 absorbance unit/scale division). In practice useful spectra have been obtained in reactions with half-lives of 5–10 ms giving total absorbance changes of less than 0.1 unit.

Applications

The following examples have been selected to give some indication of the scope and performance of the apparatus. More detailed presentations of these experiments, and conclusions arising from the results will be given elsewhere.

Fig. 5. Spectrum of Chlorophenol Red (acid form)

Chlorophenol Red in 0.2 M-sodium acetate buffer, pH 4.4. —, Spectrum recorded on the rapid-scanning spectrophotometer; Δ , values recorded with a Cary 14 spectrophotometer.

Fig. 6. Absorbance linearity

Absorbance values for solutions of Chlorophenol Red in 0.2M-sodium acetate buffer, pH4.4, determined on the rapid-scanning spectrophotometer are plotted against the values determined on the Cary 14 spectrophotometer. Δ , 400 nm; $\bullet$, 433 nm (peak); $\blacksquare$, 465 nm.

Reaction of aquocobalamin with dithionite. This example illustrates the use of the 'overlay' and 'point i ' time-course modes. Fig. 2(a) shows an 'overlay' of 32 spectra recorded during the reaction of 75 μ M-aquocobalamin with 5 mM-dithionite in a phosphate buffer, pH7.4, at 23°C. The spectra, each of 1 ms duration and spanning the range of 390–566 nm, were recorded at 62 ms intervals. Also shown (Figs. 2b and 2c) are two time-courses at 412 and 540 nm which were generated from the same stored data as the overlay by using the i th point from the j th spectrum mode of the ESL data-capture system. The presence of the isosbestic point at 487 nm and the similarity (on the time axis) of the selected time-courses indicate that there is only a single observable process with a half-life of about 0.25 s ($k_2^{\text{obs}} = 560 \text{ M}^{-1} \cdot \text{s}^{-1}$). The identity of the product formed in the reaction is not clear from comparison with standard spectra. However, it seems unlikely that the cobalt(III) species has changed redox state. A likely possibility, arising from work by Pratt (1972), would be that the cobalt(III) ion is liganded to

Fig. 7. Pre-steady-state NADH formation in the alcohol dehydrogenase reaction

Horse liver alcohol dehydrogenase (11.5 μ M) was mixed with 3 mM-NAD⁺ and 0.1 M-ethanol (all in 0.067 M-potassium phosphate buffer, pH7.0, at 23°C). The time-course at 340 nm (a) shows the pre-steady-state 'burst' and the slower steady-state phase. (b) Spectra at different stages of the reaction are shown at 8 ms ($\bullet$), 20 ms ($\square$), 64 ms ($\circ$) and 4 s (values $\times 2$; $\blacktriangle$) after the flow stopped. The concentrations given are those in the reaction mixture. The intercept on the ordinate obtained by a backwards-extrapolation of the steady-state phase of the reaction (a) corresponds to about 2 mol of NADH produced/mol of enzyme.

Fig. 8. Pre-steady-state NADH formation in a single-turnover glyceraldehyde 3-phosphate dehydrogenase reaction

Enzyme (17 μM) in one syringe of the stopped-flow apparatus was mixed with NAD^+ (4.5 mM) and D-glyceraldehyde 3-phosphate (5 mM). The concentrations in parentheses are those in the final reaction mixture. The reaction medium comprised a 0.05 M-imidazole buffer, pH 7.0, containing 0.1 M-KCl, 1 mM-EDTA and 5 mM-dithiothreitol. (a) Time-course of the reaction at 340 nm. (b) The spectra shown are each of 1 ms duration and were recorded at 1.4 ms (●), 12.6 ms (□) and at the completion of the reaction (○). The absorbance at the end of the reaction and the spectrum at the end of the reaction are designated ∞ in (a) and (b) respectively. Assuming a molar extinction coefficient of $6.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for NADH it can be calculated that about 4.7 mol of NADH are produced/mol of enzyme.

a sulphonylate ion (SO_3^{2-}) and a water molecule in the axial positions.

Pre-steady-state NADH formation in the reactions catalysed by horse liver alcohol dehydrogenase and rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. This example illustrates the application of the apparatus to the recording of the spectra of enzyme-bound intermediates.

It is known that when horse liver alcohol dehydrogenase is mixed with NAD^+ and ethanol there is a pre-steady-state 'burst' of NADH production which is proportional to enzyme concentration (Shore & Gutfreund, 1970). We have used the rapid-scanning apparatus to record the spectrum of this 'burst-phase' NADH. Fig. 7 shows spectra recorded at different times after mixing enzyme with NAD^+ and ethanol, and the time-course of the absorption change at 340 nm showing the 'burst' and steady-state phases. The magnitude of the 'burst' phase is proportional to the enzyme concentration and corresponds to about 1 equiv. of NADH per subunit of enzyme protein (assuming that the molar extinction coefficient of enzyme-bound NADH is $6.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 340 nm). It can be seen that the initially produced

NADH has λ_{max} at 320 nm but that the absorption maximum shifts towards 340 nm as the reaction enters the steady-state phase where non-liganded NADH accumulates. The results demonstrate that the NADH produced during the 'burst' phase is bound to the enzyme and so excludes any mechanism that would involve a rapid NADH release before the rate-limiting step in catalysis.

Fig. 8 shows results from a single turnover experiment with glyceraldehyde 3-phosphate dehydrogenase from rabbit skeletal muscle. The enzyme was mixed with NAD^+ and glyceraldehyde 3-phosphate in the absence of phosphate (so that the reaction could not go beyond one turnover of the enzyme). Fig. 8 shows the time-course of NADH production and spectra taken at different times during the reaction and shows that there is little difference between the spectrum of NADH formed in the rapid phase of the reaction and that at the end of the turnover, in contrast with the results with alcohol dehydrogenase. We do not attach any significance to this result at present other than that the spectra of the NADH molecules produced in the different phases are closely similar.

Fig. 9. Reaction of cysteine-substituted glyceraldehyde 3-phosphate dehydrogenase with a mixture of thiol and substrates

The appearance of NADH at 340nm lags behind the release of thionitrobenzoate (peak at 412nm) after mixing thionitrophenyl enzyme (7 μ M) with NAD⁺ (100 μ M), D-glyceraldehyde 3-phosphate (50 μ M), arsenate (10mM) and dithiothreitol (5mM) all in 0.1M-imidazole buffer, pH7.0, at 23°C. Concentrations in parentheses are those in the reaction mixture. These are selected frames of a 16mm cine film taken of the oscilloscope during the stopped-flow experiment with a high-speed cine-camera. Each frame has a 3ms exposure but persistence of the image on the oscilloscope screen causes many traces to be visible. The scan is from 300 to 530nm. The times in ms after the flow stopped are shown below each frame. The first frame shows the spectrum before the 'push' was started, i.e. the spectrum at the end of the reaction and the second frame (-3ms) shows the flushing out of these spent reactants before the flow stopped.

Reaction of cysteine-substituted glyceraldehyde 3-phosphate dehydrogenase with a mixture of substrates and dithiothreitol. This example serves to illustrate the application of the method to a reaction involving two separate events. The process under investigation is given in eqn. 1 (M. R. Hollaway, H. H. Osborne & M. A. Rosemeyer, unpublished work).

Where E-S-S-X represents a catalytically inactive form of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase obtained by treating the enzyme with four molar equivalents of Ellman's (1959) reagent [5,5'-dithiobis-(2-nitrobenzoic acid)]. ESH represents the native enzyme, RSH, dithiothreitol, and X⁻, the thionitrobenzoate anion, which absorbs maximally at 412nm. The object of the experiment was to determine in a single experiment whether the process of X⁻ release was accompanied by the synchronous appearance of catalytic activity as measured by NADH formation. This single experiment (see Fig. 9) gave sufficient information to estab-

lish that the process of X⁻ release was complete before enzyme activity started to appear. The significance of this lag in the appearance of activity will be discussed elsewhere.

Reaction of cyanomethaemoglobin with dithionite. This example demonstrates the recording of difference spectra during a reaction.

The reaction involves the reduction of the haem-linked Fe³⁺ to Fe²⁺ and release of the CN⁻ from the haem group (E. Antonini, personal communication). The time-dependence of the spectral changes in the reaction are shown in Fig. 10 together with a time-course of the change in difference spectrum recorded with reactants placed in the reference cuvette. The difference spectra generated in this mode have the same advantages for interpretation as difference spectra recorded in conventional spectrophotometry.

Discussion

The examples of use of the rapid-scanning stopped-flow apparatus demonstrate the ease with which a large amount of both spectral and kinetic information

Fig. 10. *Difference spectra; reaction of cyanmethaemoglobin with dithionite*

Difference spectra observed after the mixing of cyanomet-haemoglobin (1 mg/ml) with 0.02M-sodium dithionite, both in cyanide-phosphate buffer (0.05M- Na_2HPO_4 -0.15M- NaH_2PO_4 -0.05M-KCN, pH 6.8). The unmixed components were used in a split-compartment cuvette in the reference beam. The spectra were recorded with a high-speed cine camera as in Fig. 9. The spectra were recorded at 7.4 ms ($t = 0$ in the Figure), 148, 303, 518 and 770 ms and 1.25, 2.0, 2.7, 3.9 and 6.0 s respectively.

can be obtained rapidly and with a small amount of material. It could be argued that this information could be obtained with a conventional stopped-flow apparatus and simply carrying out a large number of experiments at different wavelengths. However, 100 stopped-flow experiments would be required to approach the order of the wavelength resolution of the apparatus described here and this would not only be a time-consuming exercise (say 1.5 h at least), but would also involve the use of large amounts of material, which in the case of most enzyme-catalysed reactions would render the experiment impractical. In addition, on the time-scale of such an experiment reactants could undergo changes leading to errors in the final spectrum.

The potential applications of the apparatus can be listed as follows. (1) Direct observation of the electronic absorption spectra of short-lived intermediates with simultaneous determination of time-courses for their formation and breakdown (e.g. Figs. 7 and 8). The recording of the spectra of very-short-lived intermediates is possible by using the apparatus in the continuous-flow mode (it is possible to record spectra in solutions that have been mixed only 1 ms before).

(2) Direct observation of difference spectra during a reaction (e.g. Fig. 10). The presence of discrete numbers of isosbestic points should be useful for delineating the minimum number of observable intermediates.

(3) Simultaneous recording of the spectra of two or more processes that take place with different spectral changes. An example has been given (see Fig. 9) but other applications include the recording of H^+ release and product formation in the same reaction by inclusion of a pH indicator in the reaction mixture. Again it could be argued that use of a conventional stopped-flow apparatus could provide the same information by carrying out the reaction at two different wavelengths. However, it is not always easy to see which two wavelengths should be selected since changes in one chromophore could overlap changes in another. Also, the scanning experiment has the advantage that absorbance changes at different wavelengths are recorded in the same reaction, so that there cannot be uncertainties arising from comparisons of two separate time-courses, i.e. in terms of the time-bases being exactly the same or the reactants changing in some way during the resetting of the wavelength.

(4) Signal averaging. The ability to record spectra under conditions of low signal-to-noise ratio by addition of large numbers of spectra (see Fig. 3) has wide potential application in biochemical systems such as mitochondria and membrane-bound enzymes.

(5) Flash photolysis. The geometry of the apparatus is such that it is possible to irradiate the contents of the observation cuvette with light from a flash lamp or laser. Also the stopped-flow assembly can be removed and any photolabile system investigated by flash irradiation followed by rapid scanning.

The rapid-scanning stopped-flow apparatus has proved particularly useful in a number of systems as a means of making a preliminary investigation of the nature of spectral changes. This has enabled subsequent detailed kinetic studies in a conventional stopped-flow apparatus to be carried out at wavelengths where the signal change can be identified with known events.

In conclusion it is hoped that the application of the apparatus described here to chemical and biochemical problems will assist further progress along the pathway towards explaining kinetic data in terms of X-ray crystallographic structure and directly observed chemical events.

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