KEYNOTE ADDRESS

SOME PROBLEMS CONCERNING MIXERS AND DETECTORS FOR STOPPED FLOW KINETIC STUDIES

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This conference is taking place on the 55th anniversary of the introduction of rapid mixing methods to the field of biology by Hartridge and Roughton (1923*a*). Their specific aim was to study the reactions of hemoglobin with its ligands (Hartridge and Roughton, 1923*b*). The tenacity with which nature holds onto its secrets is nowhere better exemplified than with this remarkable molecule. The very nature of the reaction of hemoglobin with its ligands (O_2, CO, and NO) and its effectors (H^+, 2,3-diphosphoglycerate[phosphates], CO_2, and salt), together with its tetrameric cooperative intramolecular interaction, taxes our ingenuity and technology to explore, and we still do not understand these reactions in detail. However, methods and techniques discussed at this meeting offer the greatest promise so far for forcing this important model of molecular dynamics to yield its secrets.

During the past ten years a number of advances have been made that are only now bearing fruit, not just in hemoglobin research but also in many other areas of biology. The wide range of papers and posters presented in this Discussion bears witness to the breadth of application that has occurred as reliable commercial instruments have become available. These instruments have in general a time resolution of 1 ms, limited primarily by flow velocity and stopping time; they are useful for solutions whose viscosity is essentially that of water.

The state of the art in mixing and flow systems up to 1972 has been reviewed recently by Chance (1974). In this talk I would like to point out some of the technical problems that still exist, with the hope that this discussion will stimulate research efforts to solve them. Since the introduction of the rapid reaction apparatus and early experimental work of Hartridge and Roughton (1923*a*, 1924, 1926), Roughton and Millikan (1936), Millikan (1936), and Chance (1940*a*,*b*), very little quantitative work has been done on the theory of mixing and flow for the particular needs of stopped flow. Schlichting (1968), Pai (1954), Kay (1963), Hinze (1959), Davies (1972), and

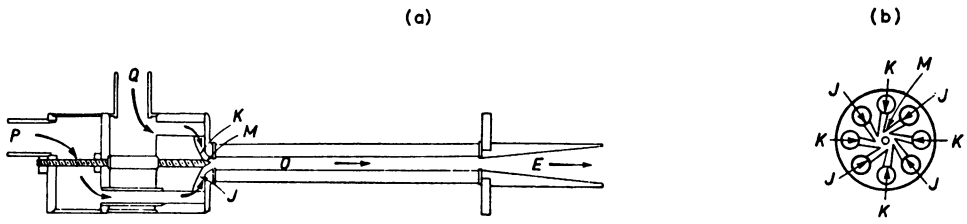


FIGURE 1 Original Hartridge-Roughton mixer.

Batchelor (1971) summarize much of the work on compressible and incompressible fluids. The fully developed turbulent flow of incompressible fluids in which eddy equilibrium has been reached and where Kolmogoroff radii can be measured has received some attention (Friedlander and Topper, 1961; Brodkey, 1975; Corrsin, 1961; and Toor, 1969). However, the mixer-flow-stop problem peculiar to the continuous, stopped, and accelerated flow systems used in the study of chemical and biochemical reactions needs to be considered in the light of a very great need to conserve solutions, increase velocity without cavitation, and stop the flow very rapidly. Smith (1973) has systemized the study of jet mixers and deals with scaled models. The problem of scaling turbulence and cavitation together is not dealt with. Work at very low temperatures (Chance, 1978) and at high viscosities (due to high protein concentration or high stabilizing medium concentration) demand efficient mixing. Moskowitz and Bowman (1966) have demonstrated the advantages of small multiple capillaries for knowing the precise time of the initiation of mixing, mixing of multiple components, and the use of a mesh to improve efficiency.

To define the problems more clearly, let me briefly remind all of you of the general

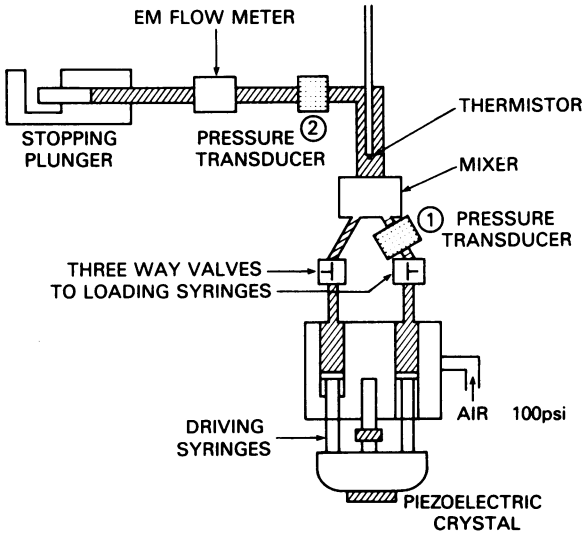


FIGURE 2 Stopped-flow method using the Gibson stopping syringe concept.

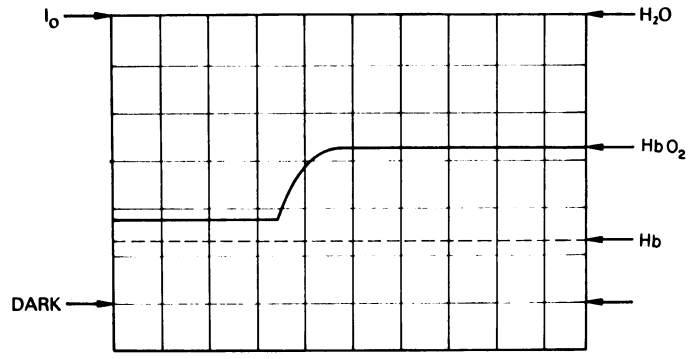
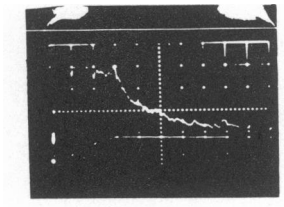
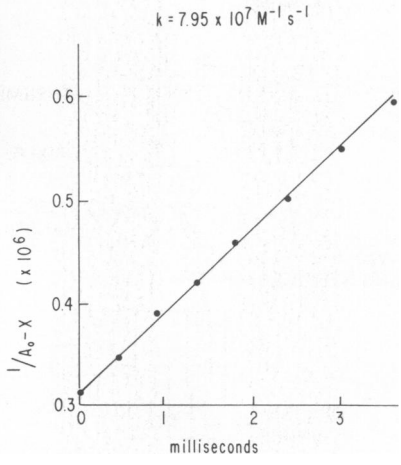


FIGURE 3 The reaction of 40 μM hemoglobin with 120 μM oxygen per iron.

scheme of a mixing system. Fig. 1 shows the first Hartridge-Roughton mixer made of brass from a Rolls-Royce carburetor. The volumes used in their first continuous flow experiment were 100 ml/point. In the mid-thirties a number of glass mixers were made and tested by Millikan (1936). These mixers together with stopped-flow brought about a reduction in volume to about 1 ml or less for the entire curve. Further improvement in optical detection, better mixers, and a greatly improved overall apparatus by Chance (1940*a*,*b*) brought the volume needed for a curve to 0.2 ml. Gibson's introduction of the stopping syringe in 1950 and a greatly improved apparatus with a double mixer (Chance, 1974) brought about the first commercial instrument. Fig. 2 shows the general stopped flow scheme, in which stopping is achieved by filling a syringe, which hits a stop. These were tested by visual observation of indicator reac-





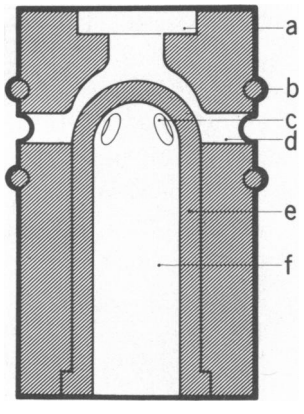


FIGURE 4 FIGURE 5

FIGURE 4 The reaction of 0.8 μM lactate dehydrogenase with 3.2 μM (AcPy) ADH (per monomer).

FIGURE 5 The ball mixer.

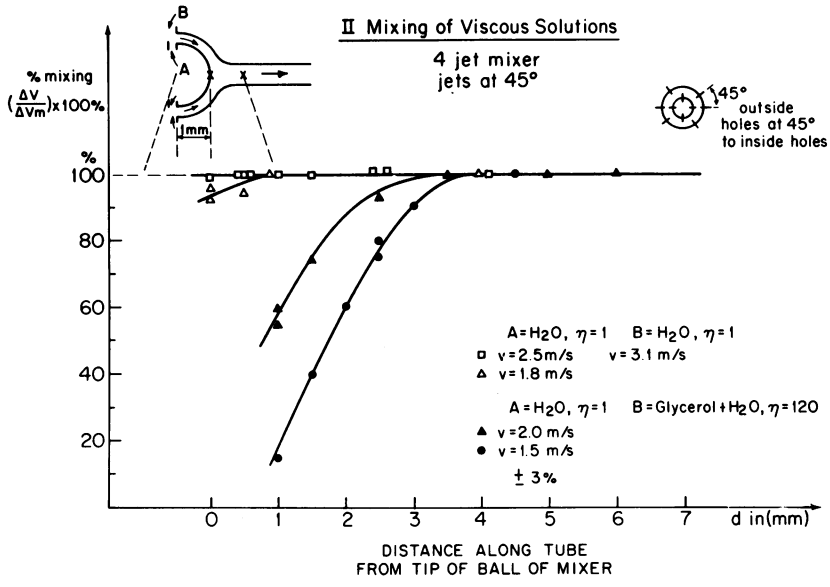


FIGURE 6 Mixing efficiency of the ball mixer. Reproduced by permission from Berger et al. (1968*a*). Copyright © 1968 American Institute of Physics.

tions and the use of characterized reactions. Roughton used equal titers of NaOH and HCl and a thermocouple moved along the observation tube as a test of mixing. This has proved useful, particularly when mixing glycerol and water, where the high heat of hydration can be used.

If we consider the hemoglobin reaction with oxygen at a concentration after mixing of 40 μM in heme, we see in Fig. 3 that at even 120 μM oxygen, part of the reaction is being missed. To simplify the analysis, we would like to use at least 250 μM heme, thus reducing dimerization as oxygen binds. This is particularly true for isoionic hemoglobin. Thus, if a reaction has a 1 ms half-life at 40 μM, it will move to roughly 150 μs half-life at 250 μM heme and become even faster as we go to 1 mM oxygen. If the "on" rate is of the order of 10^7 1/M-s, the half-life will be of the order of 75 μs. 10-μs resolution would be desirable. If 1 mm is the median point of closest approach to the point of mixing, a velocity of 100 m/s is required. We must clearly stop in this length of time as well, or characterize the system so that accelerated flow (Chance, 1940*b*) and simultaneous mixing and chemical reaction theory (Corrsin, 1961; Toor, 1969) can be used. Fig. 4 indicates the additional problem of light intensity insufficient to measure the reaction. This can likely be improved with a continuous wave laser ratioed to correct for variable intensity, if variable wavelength from 260 to 700 nM is available at a power level of 1 W. Lastly, when whole blood, high protein concentrations, etc, are mixed with plasma or buffer solutions, or low temperatures are used, severe viscous mixing problems arise. The ball mixer introduced by Berger and Bowman (1964) and extended to flow systems by Berger et al. (1968*a*) pushed the resolution time for mixing to less than 200 μs at flow velocities of 40 m/s. This mixer, shown in

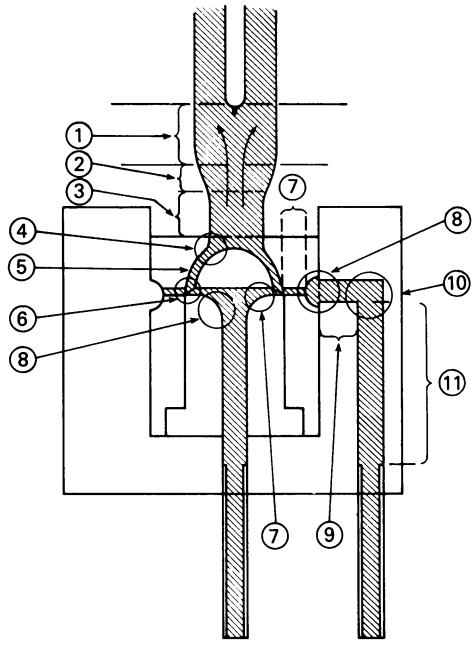


FIGURE 7 High-energy dissipation points in the micro ball mixer used in stopped flow calorimetry.

Fig. 5, is basically a simple turbulence-creating device, as mentioned by Goldstein (1938). The ball is in a flow stream and a turbulent wake is created on the downstream side of the ball, as if the stream were stationary and the ball moving. Most important, turbulence moves back against the ball as flow velocity increases. As shown by Berger et al. (1968*a*) in Fig. 6, optimal mixing occurs with the entry jets at a 45° angle to each other so that a close approach to a sheet of fluid flowing over the ball occurs. Balko et al.[1] have shown that energy dissipation in the form of heat in this mixer occurs as right-angle turns, rough points, etc. This is seen as the circled areas in Fig. 7. These points also act as nucleating centers for cavitation.

The development of the Parschall flume laid the foundation for the necessary shaping of the change in diameter of the passages to minimize cavitation. These principles have had to be modified in the design of the ball mixer, entrance, and exit tubes etc. shown in Fig. 7, due to the short distance available if time resolution, fluid conservation, heat losses, and cavitation minimization were to be achieved. These mixers have been made in a range of physical sizes from 1.5 mm diameter of the mixer on up (C. L. Gwen. 1977. 3860 Mt. Aladin, San Diego, Calif., and Update Instruments, Inc., Madison, Wis.). They are normally made of Kel-F (3M Company, St. Paul, Minn.) or any of a variety of stainless steels, Hastaloy C (Union Carbide Corp., New York), or Carpenter 20Cb being excellent for HCl and KCl solutions (1977, Carpen-

[1] Balko, B., R. L. Berger, and P. D. Bowen. 1978. High speed stopped-flow microcalorimeter. In preparation.

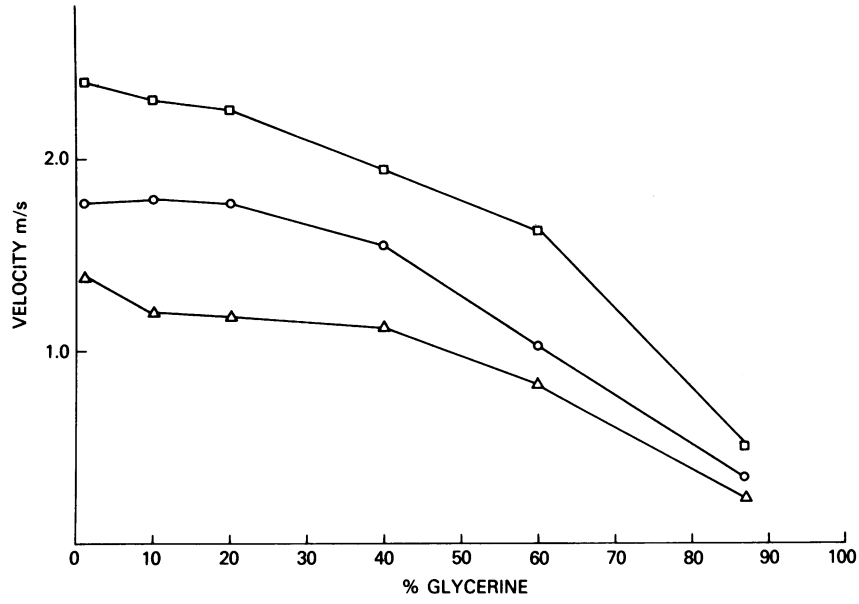


FIGURE 8 Velocity changes with driving pressures through a 3-mm diameter tube.

ter Technology Corp., Reading, Penn.). McClune and Fee (1976) report the use of this mixer as an augmenter to the Gibson-Durrum mixer (Durrum Instrument Corp., Sunnyvale, Calif.), but equal results are achieved with the ball mixer alone as shown by the mixing experiments in Fig. 6 with glycerol and water. An excellent discussion of reactions suitable for the testing of flow devices has recently been given by Tonomura et al. (1978) and should be referred to for details. Fig. 8 shows the change in velocity through a 3-mm diameter observation tube at three different driving pressures and various glycerine concentrations. Fig. 9 shows the nonlinear variation of viscosity with glycerine concentration. Fig. 10 is a plot of kinetic energy versus dissipation (temperature rise) through the observation tube.

For stopped flow at 85 m/s, a speed realized in the Berger-Science Products apparatus (Science Products Corp., Dover, N.J.) (Berger et al. 1968*b*), 11.8 μs are required to travel 1 mm. Minimum distance to center of the observation point is 2.5 mm; thus, a resolution time of 30 μs would be achieved if the solution can be stopped in less time and if cavitation can be avoided. The stopped valve used in the Berger-Science Products apparatus (see Chance, 1974), shown in Fig. 11, is a modification of an earlier valve (Berger et al., 1968*b*). It is normally closed. Flow is started by opening the valve and at the same time pressurizing an acoustic timer located in the body of the valve, the RC of which is set by a needle valve and the chamber volume. At a preset time, usually 5 ms, the valve closes in 25 μs, as measured with a phototube and light pipe. Due to cavitation, no better than 200-μs resolution at 40 m/s has been obtained (Berger et al., 1973; Berger et al. 1978).

It would appear that a 25–30 μs resolution time to the point of observation after stop

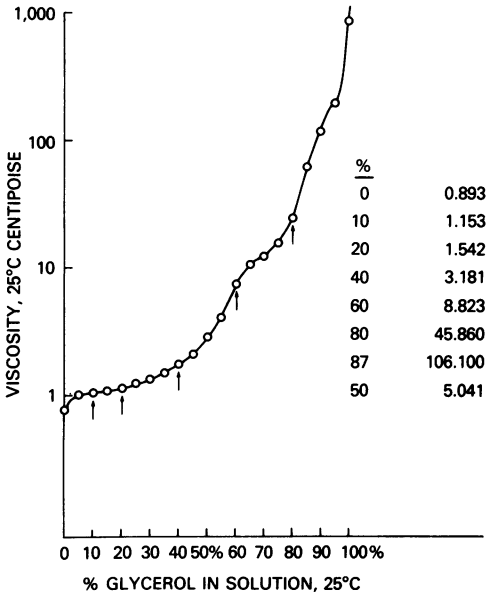


FIGURE 9

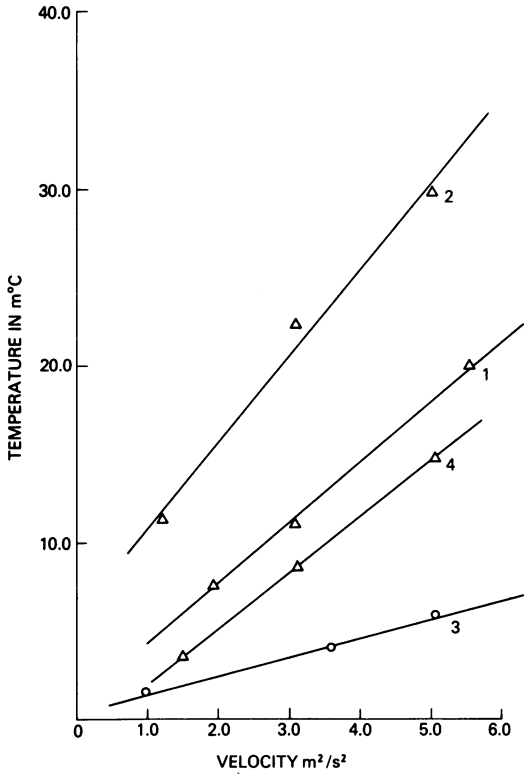


FIGURE 10

FIGURE 9 Nonlinear viscosity changes versus percentage of glycerine.
FIGURE 10 Energy dissipation versus fluid kinetic energy.

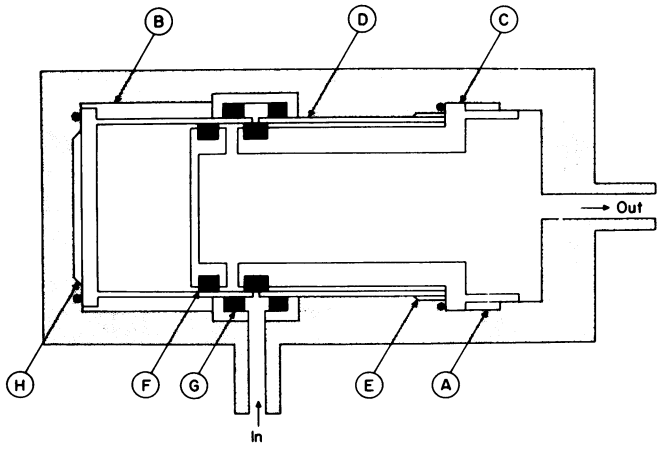


FIGURE 11 High-speed acoustic stop-valve. Reproduced by permission from page 40 of Chance (1974). Copyright © 1974 by John Wiley & Sons, Inc.

is obtainable, providing cavitation can be reduced to zero (Duffy and Staerk, 1969). One does have the problem of a pressure pulse at the front of the stop valve, which we have measured at about 1,000 psi. It appears to dissipate rapidly and, unless an air bubble or cells are involved, does not seem to affect the optical path, about 2–4 cm below the valve. There is no reason this valve cannot be closed in 10 μs but a larger pressure pulse will certainly occur. The theoretical and practical problem is the design of a highly turbulent system free of cavitation. While turbulence can be reasonably well scaled, turbulence and cavitation have offered severe problems. Nevertheless, work is proceeding on this problem with a time resolution of 10 μs the goal.

The major optical improvements made in stopped flow work since the last reviews (Schechter, 1970; Reich, 1971; Chance, 1974), are mainly in the various rapid scan spectrophotometers that have appeared. These have been discussed by Ridder and Margerum (1977). The apparatus of Kawania is described by Wightman et al. (1974); it has the ability to scan 250 nm in 1.2 ms with an optical density sensitivity of 0.005 absorbance units, and at 0.5 nm resolution 50 points can be taken. A new Tracor Northern unit offers a scan rate of 10 μs/diode with a 512-diode array (Tracor Northern, Middleton, Wis.). Optical densities to over 3 can be utilized with an intensified detector.

For single cell work, the system described by Benedetti et al. (1976) and Benedetti and Lenci (1977) is most interesting. The condenser stage is moved under computer control. Both spectra and location can be scanned as well as total shape; the latter is done independently with an infrared vidicon. Thus for stopped-flow work on single red cells, reactants could be flushed past the cell and the reactions followed inside the cells as, for example, oxygen is either added or taken away. Dr. Giardina will discuss its uses for red cell work later at this meeting (Antonini et al., 1978), with flash photolysis.

A variety of detection methods has been applied to stopped flow devices in the last several years. Of particular interest is the nuclear magnetic resonance flow system of Manuck et al. (1973); the circular dichorism system of Luchins (1977), used for the study of hemoglobin denaturation; and the combined stopped-flow laser flash photolysis system of Sawicki and Gibson (1978) and McCray and Smith[2] for the study of carbon monoxide and oxyhemoglobin. Less than 20-ms resolution times are obtained with the first two methods and about 0.2 ms with the flow-flash system. To study the hemoglobin reaction inside the red cell, a new dual-wavelength detection system has been developed (Berger et al., 1978) for use with the laser flash-flow system. It has a 1-μs response time and utilizes the near infrared region of the spectrum. It is a modification of the Optisat (Vurek, 1973) and, as shown in Fig. 12, utilizes two light-emitting diodes, one at 660 nm and one at 900 nm. Two phototransistors receive the light. Dichromatic filters separate the beams. The new fiber optic-single chip laser detector systems developed at the Bell Laboratories, (Murray Hill, N.J.) (Tien, 1977)

[2] McCray, J., and P. D. Smith. 1978. Kinetic binding to tetramers. Submitted for publication.

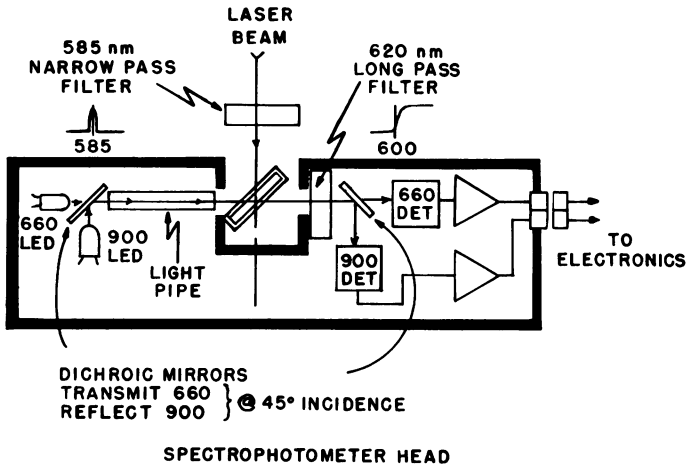


FIGURE 12 100 kHz dual-wavelength spectrometer.

for light communication lines offer fascinating opportunities for future single cell research.

Considerable effort has been expended over the years in looking at other detection methods. Some of these have been reviewed by Roughton (1963). Chance (1974) has reviewed quench flow by acid or freezing. Since these reviews, advances have been made in pH detectors (Crandall et al., 1971, 1978) and thermodetection (Balko and Berger, 1968; Balko et al. 1969).[3] Recently we have developed a thermistor probe, shown in Fig. 13 (Thermometrics, Inc., Edison, N.J.) and a modified differential bridge (Berger et al., 1974; Linear Research, San Diego, Calif.) shown in Fig. 14, which

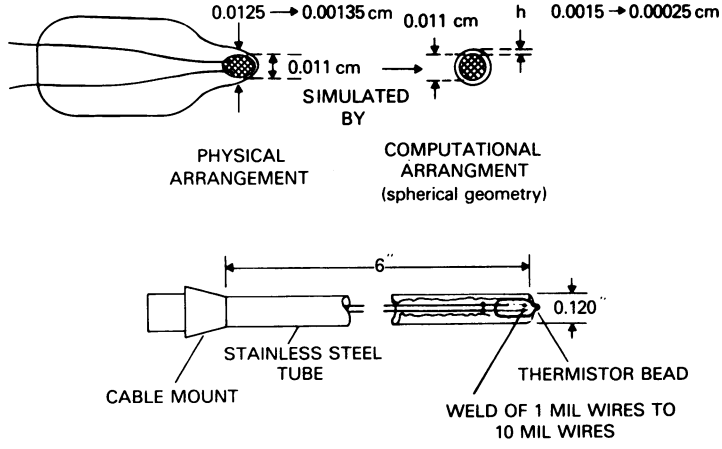


FIGURE 13 High-speed thermistor probe.

[3]See note 1.

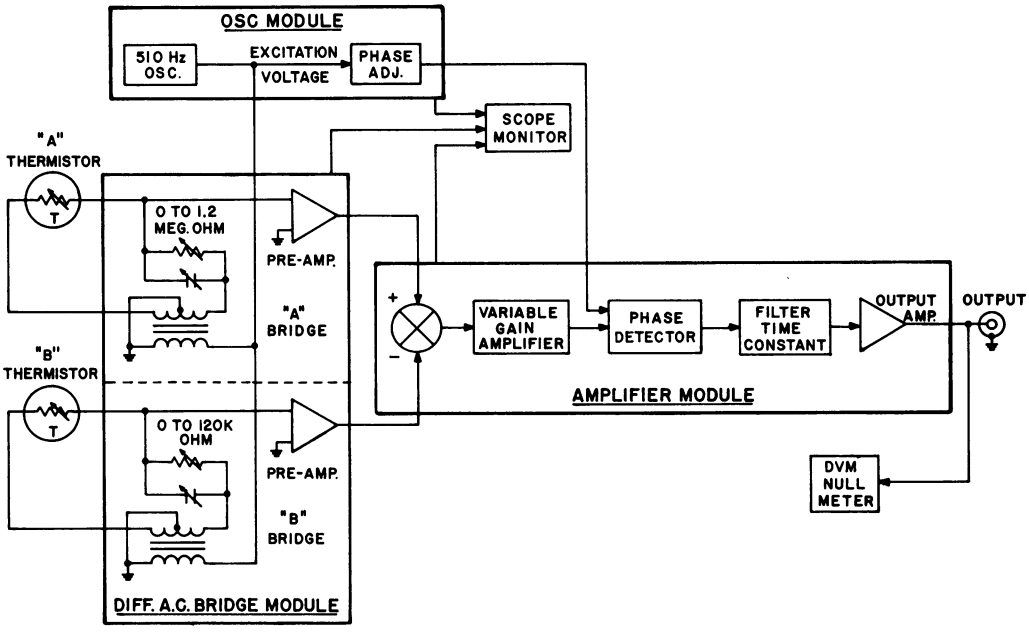


FIGURE 14 Differential 200 AC bridge.

has a response time to $1/e$ of 3 to 7 ms. The response is measured with the spring-loaded plunger shown in Fig. 15 (Berger and Balko, 1972). Fig. 16 shows a typical thermistor response to this plunge test. A detailed study of thermal losses in flow systems was carried out (Balko et al., 1978) with the result that the areas shown encircled in Fig. 7 have been smoothed, greatly decreasing heat losses. Fig. 17 shows sodium bicarbonate plus HCl (1.2 and 1.2 kcal/mol for each of the reactions given in Eqs. 1 and 2) at 28°C

$$H^+ + HCO_3^- \leftrightarrow H_2CO_3 \quad (1)$$

$$H_2CO_3 \rightarrow CO_2 + H_2O \quad (2)$$

Heats of flow, mixing, etc., can be kept to about 5 millidegrees or less. A number of problems have appeared. A pressure effect on the thermistor occurs when the glass coating is too thin, so that it begins to develop microcracks, or when bubbles exist in the thin glass coating of the thermistor. It is hoped this can be corrected by the evaporation of silicon dioxide onto the thermistor to hermetically seal it. The need for much faster and more sensitive thermal sensors has led to some work on thin film capacitance devices (Maserjian, 1972). Work is continuing on these devices in collaboration with the Thermometry Section of the National Bureau of Standards.

pH detectors also have proven troublesome for several reasons. In general, pH electrodes have resistances of 100–500 MΩ. Electrode and lead capacitance amount to

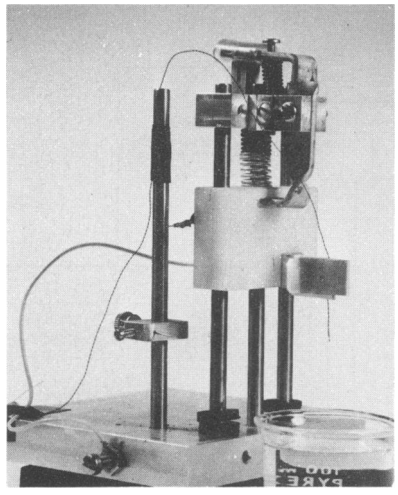


FIGURE 15 Spring-loaded guillotine for testing thermistor and pH electrodes. Reproduced by permission from Berger and Balko (1972). Copyright © 1972 Instrument Society of America.

about 300 pF. Thus, a time response of about 90 ms to $1/e$ is to be expected. A 3-mm-diameter combination electrode such an Ingold type (1977, W. Ingold AG, Urdorf-Zurich, Switzerland) made of LOT glass, a calomel reference, and a porous plug, together with a pH meter such as the one shown in Fig. 18, is presently being used. This pH meter uses a driven shield, thus reducing the lead capacitance to nearly zero. The electrode has about 150 pF capacitance and a resistance, at 25°C, of about 100–200 MΩ. We thus expect a response time of 15 ms. Fig. 19 shows the time of response to the plunge test (Berger and Balko, 1972), to be about 10 ms uncoated (*a*) and 14 ms coated (*b*). Faster times may be achievable, as suggested by Crandall (1978) at this discussion. $t_{1/2}$ of only several milliseconds using L & N electrodes (Leeds & Northrup

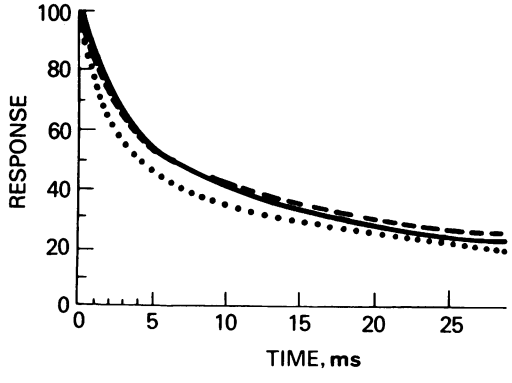


FIGURE 16

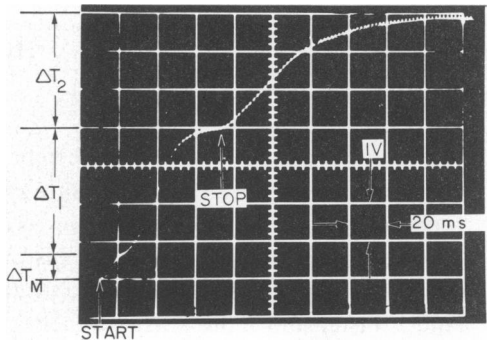


FIGURE 17

FIGURE 16 Thermistor response to plunge test.
FIGURE 17 Stopped-flow thermal reaction of 0.02 M $NaHCO_3$ with 0.01 M HCl.

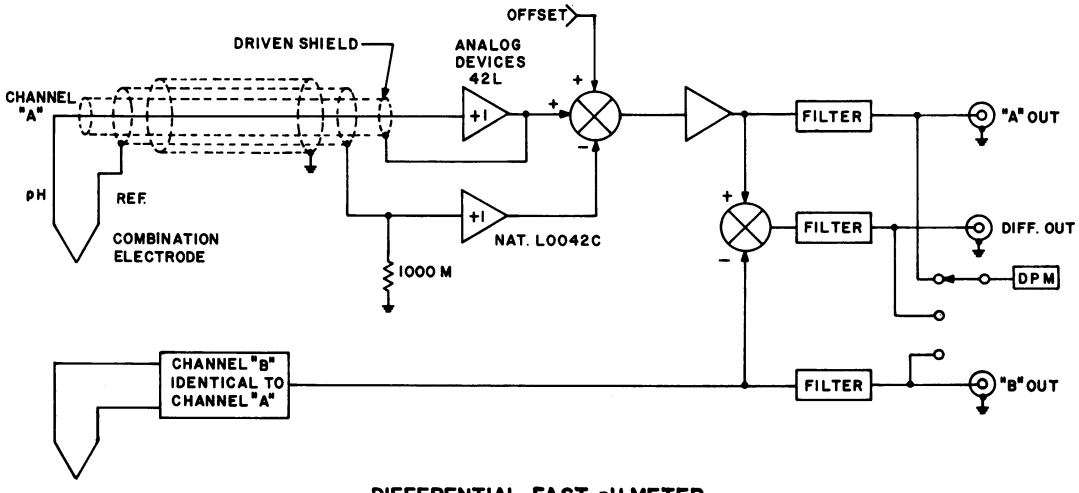


FIGURE 18 Differential fast pH meter.

Co., North Wales, Pa.) were estimated. The electrodes, in our hands, have a resistance of $5 \times 10^8\ \Omega$ and a capacitance of 140 pF. Since the driven shield eliminates the cable capacitance, probe capacitance is about 20–30 pF, giving a time constant of about 10–15 ms. Plunge tests give a response time of 9 ms, as shown in Fig. 20*a* and *b*. An additional problem is the loss of sensitivity and response time of the electrode caused by protein and cell contamination. This has been solved by the use of Lycra (1976; Ethicon, Inc., Somerville, N.J.) without serious loss of response time or sensitivity. Biocompatibility appears to be excellent (Kolobow et al., 1977). Studies of pH elec-

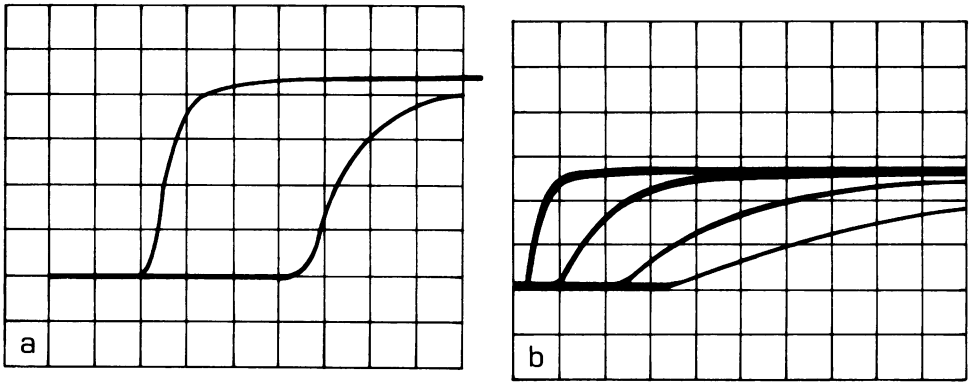


FIGURE 19 Response of Ingold-type lot 405 3472 3 mm combination pH electrode (Calomel ref. with porous plug) to the plunge test: *a*. uncoated $t_{1/2}$ = 10 ms; *b*. coated with Lycra (Ethicon) $t_{1/2}$ = 14 ms.

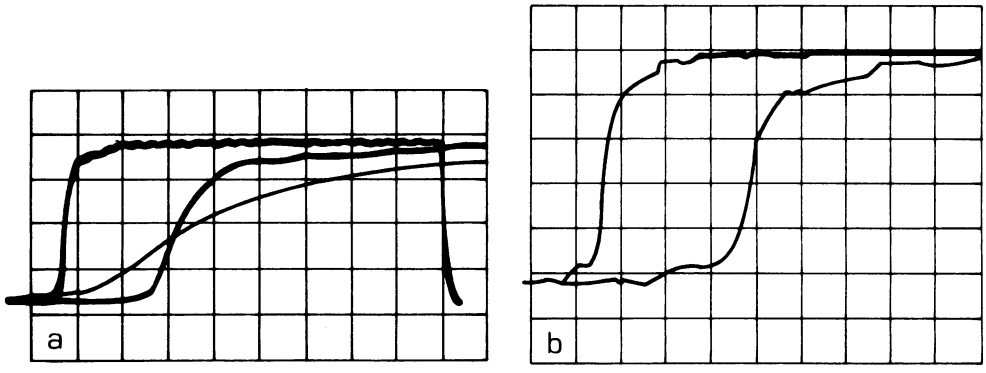


FIGURE 20 Plunge tests of Leeds and Northrup electrode type. Reference is silver-silver chloride with a crack-type leak. *a*. uncoated, $t_{1/2}$ = 9 ms; *b*. coated with Lycra, $t_{1/2}$ = 10 ms.

trode response times by Wikby (1972) made by electron flow measurements suggest a large number of time constants; one, accounting for about 10% of the electrode response, is as slow as 2–20 s. We do not see this. A careful investigation of this will use the plunge method developed for thermistors (Berger and Balko, 1972).

Froelich et al. (1976) has recently described a quench flow apparatus using a stepping motor and a ball-mixer system for multiple mixing experiments. Time resolutions of 1.5 ms are reported for sodium-potassium ATPase and a sacroplasmic reticulum-CaATPase. Multiple mixing can be carried out in up to three mixers in the present apparatus (Commonwealth Technology, Alexandria, Va.; Update Instrument, Inc.). An automatic sampler adds to the speed of operation. Four points on the rate curve can be obtained with each particular size tubing by changing the speed of the advance of the stepping motors. About 0.25 ml of each reagent are required per point. Application to several systems will be discussed by Froelich in a poster session at this discussion. Brahm (1977) has improved on the original Tosteson continuous flow filtration apparatus for use with red cells; he reports a time resolution of about 7 ms. Utilization of Nuclepore filters (Nuclepore Corp., Pleasanton, Calif.) pushed up into the flow path has proven very successful. It would appear that current attempts to make such an adapter for red cell volume regulation studies to the Froelich quench flow apparatus are meeting with success.[4]

Finally, I would like to say a word about the advances being made in data handling over the last several years. Stopped flow systems produce vast amounts of data and this is not a trivial problem. DeSa and Gibson (1969) worked out a minicomputer-based system for taking, correcting, calculating, and storing the data from a rapid flow apparatus. A commercial system to do this is available (OLS, Athens, Ga.), as are several systems that allow some data manipulation (Wightman et al., 1974; Mieling et

[4]Froelich, J. P., F. Kregenow, and R. L. Berger. In preparation.

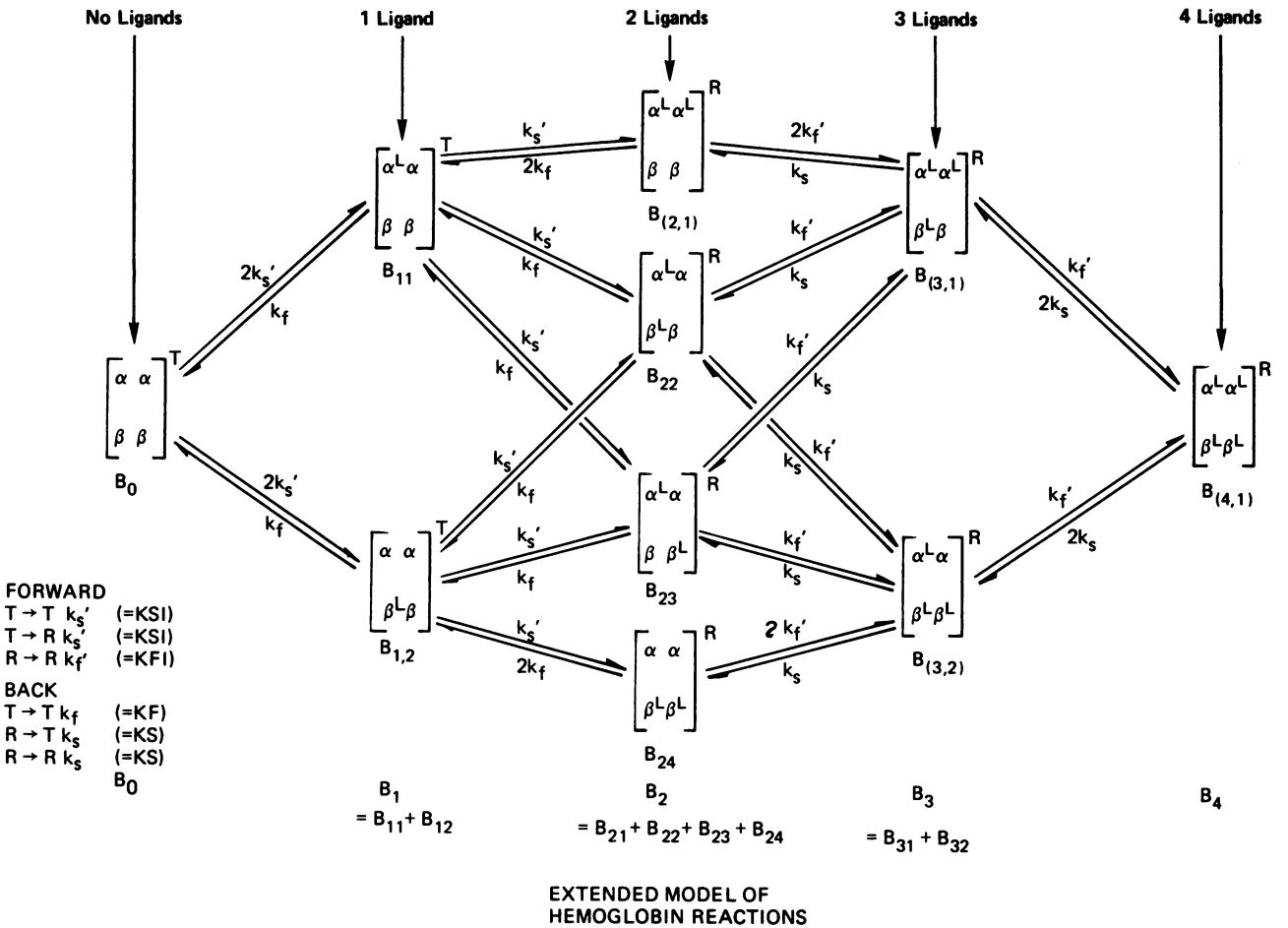


FIGURE 21 Expanded two-state finite element model of hemoglobin.

al., 1976). These tasks are rapidly being expanded to microprocessor based systems. The apparatus described by Winslow et al. (1978), while not a stopped flow instrument, is an example of automation where the entire experiment is carried out to the point of writing out the final graph. This machine is interesting from a different standpoint; it produces an oxygen dissociation curve of hemoglobin in whole blood or hemoglobin solution to an accuracy of about 0.1% over the entire range of saturation. This affords the possibility of testing kinetic models by forcing the kinetic data to fit the much more accurate equilibrium data. In the Adair-Perutz two-state model, a large number of pathways are possible, as shown in Fig. 21. McCray and Smith[2] 1978 and Berger et al. (1978) have analyzed this model by LaPlace transforms and the finite element simulation technique (FEST) of Davids and Berger (1960, 1969, 1977). This latter technique was developed to correct heat conduction calorimeters so they could be used for kinetics (Rehak et al., 1976; Berger et al., 1975). By coupling this to MLAB (Knott and Shrager, 1972), a curve-fitting technique, FEST can be used to determine automatically the correct rate constants to make the kinetic data fit the equilibrium curve. FEST is now being used on a microprocessor-based system to correct for the response

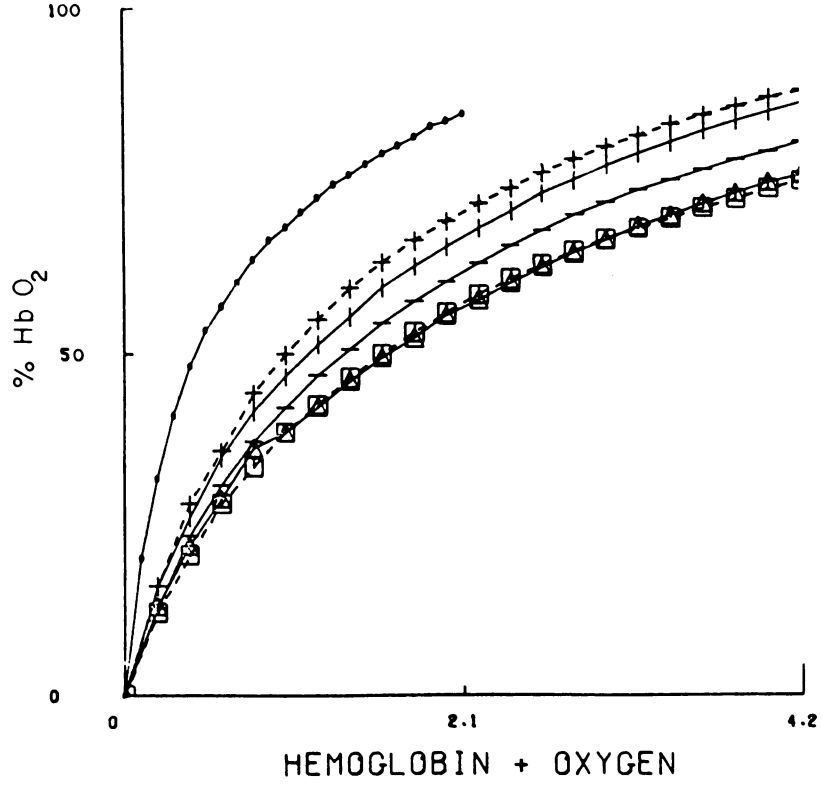


FIGURE 22 Reaction of 40 μM (in heme) hemoglobin with 120 μM oxygen as a. • Isoionic; *b.* +0.02 M bis-tris, pH 7.4; *c.* □ 1 M/M 2,3-DPG; *d.* Δ 2 M/M 2,3-DPG; *e.* | 0.1 M PO_4, pH 7.4; *f.* — 0.3 M KCl and 1 M/M 2, 3-DPG. Abscissa in milliseconds.

time of detectors[1] and to fit kinetics and thermodynamic models to obtain rate and equilibrium constants. While many schemes are presently available (see Garfinkel et al., 1977; Berman and Weiss, 1972), the finite element simulation technique offers the advantage of allowing the investigator to remain with the physics and chemistry of the problem.

The improvement in the data obtained by use of the microprocessor to "run" the experiment, by adaptations of modern signal processing methods, and by simulation methods makes it possible to perform biological experiments on a single small sample, thus greatly improving our ability to analyze complex biochemical reactions. The rising importance of this field is indicated by the number of discussions and poster papers at this meeting and by the large increase in descriptions of stopped-flow investigations appearing in the literature.

With our ability to produce isoionic proteins (Righetti and Drysdale, 1976), we must begin to work on the effect on reactions of the various constituents of the "real" world in which the reactions run. Fig. 22 shows such effects on the kinetics of the oxygen and hemoglobin reaction (Berger et al., 1973). The discussions of Moelwyn-Hughes (1967) and Guggenheim and Turgeion (1955) on pressure, salt, and dielectric constant effects, and Schurr (1970) on diffusion and, of course, the better-known temperature and entropy problems will add greatly to our understanding of the system with which we are dealing. That this system is, in a very real sense, a solid-state system is becoming clearer as our membrane friends demonstrate that one major metabolic system after another is clearly bound to the membrane. One would hope that solid-state physicists would team up with biochemists to explore, with what I am sure will be a new generation of solid-state experimental methods, the molecular interactions on the membrane surface.

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DISCUSSION

SUTTER: At 1000–2000 psi, what precautions are necessary to keep from fracturing the windows or otherwise blowing them out?

BERGER: Considerable work has gone into the construction of our observation tubes. They are presently made by casting the circular quartz windows in Stycast 2057 resin (Emerson & Cum-