lock-in amplification techniques are utilized in conjunction with a special stopped-flow observation chamber to produce and detect the small, rapidly varying signals. We present initial results of the application of the instrument to a complicated protein subunit folding and assembly reaction system involving reorganization on the secondary, tertiary, and quaternary structural levels. These results demonstrate the instrument's utility for separating out the kinetics and, thus, for elucidating the interplay of the structural changes at those various levels.

To illustrate instrumental capabilities, the acid denaturation of ferrihemoglobin at three pH values and a pH-jump renaturation of denatured heme-free α globin are presented, as monitored by CD at 222-nm. The fastest of these reactions, exhibiting a 42-ms reaction half-time for a total CD change of 88 millidegrees (17.2×10^3-deg-cm^2dmol^{-1} mean residue ellipticity), was detected with a signal-to-noise ratio of 5 to 1.

At 222 nm, with an effective OD between 0.5 and 1.5 and a smoothing time constant of 4 ms, the noise level does not exceed approximately 17 millidegrees with current components and design. The noise is sharply reduced by increasing wavelength by even a few nanometers. Modifications to reduce the noise at 222 nm by an order of magnitude are outlined.

With a total accessible wavelength range from ~200 nm to ~800 nm, the instrument should prove a useful tool in kinetic investigation of a wide array of reactions involving altered optical activity and modification of chiral centers.

A SIMPLE SYSTEM FOR MIXING MISCIBLE ORGANIC SOLVENTS WITH WATER IN 10–20 ms FOR THE STUDY OF SUPEROXIDE CHEMISTRY BY STOPPED-FLOW METHODS

GREGORY J. MCCLUNE AND JAMES A. FEE, *Biophysics Research Division and Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109 U. S. A.*

We describe a simple device capable of mixing dimethyl sulfoxide (DMSO) and aqueous solutions for spectrophotometric observation of superoxide (O_2^-) chemistry with common stopped-flow methodology (1). Studies on superoxide and particularly on its dismutation catalyzed by so-called superoxide dismutases have been forced to rely on the expensive pulse radiolysis technique or poorly defined chemical or biochemical techniques to produce superoxide (2). Pulse radiolysis can generate superoxide concentrations up to 300 μM, while chemical methods produce steady-state concentrations 1,000 times lower. A method for dissolving high concentrations (>100 mM) of commercially available potassium superoxide in DMSO has been

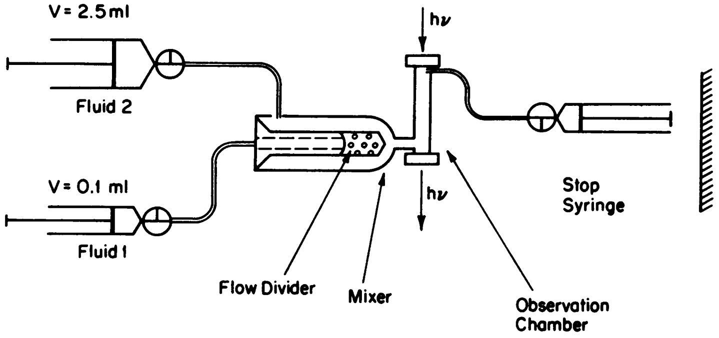
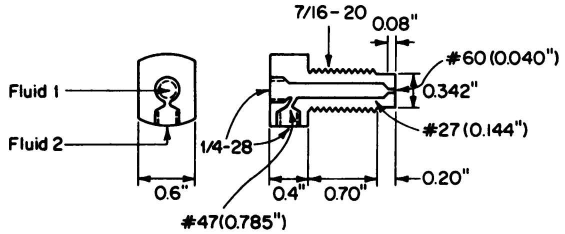
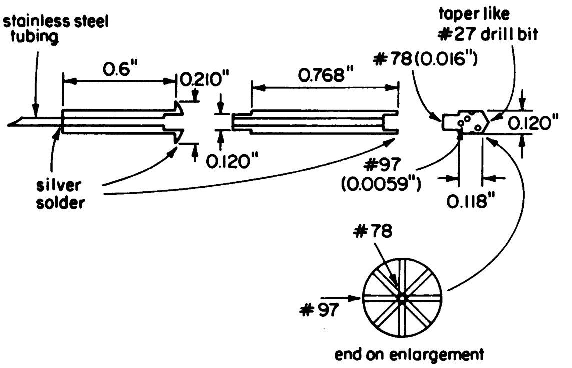


FIGURE 1 Schematic representation of stopped-flow apparatus with organic-aquo solution mixer (not drawn to scale).



MIXER SCREW(DELRIN®)



MIXER NOZZLE (STAINLESS STEEL)

FIGURE 2 Details of mixer screw and flow divider. The flow divider is inserted into hole 1 of the mixer screw and secured as described in Ref. 8. Fluid 2 enters hole 2 by a stainless steel connector similar to that described by Ballou (8). Holes ~0.003" can be placed in the stainless tip with a laser (Indiana Wire and Die Co., Fort Wayne, Ind.). The tip of the flow divider can also be constructed from Delrin and screwed into the central part of the flow divider. Holes ~0.006" can be drilled through the Delrin tip.

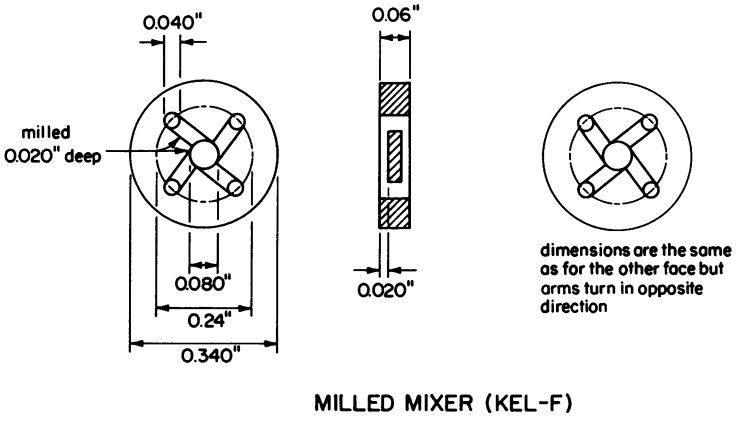


FIGURE 3 Detail of four-jet tangential mixer.

described (3, cf.4), making stopped-flow experiments with high superoxide concentrations feasible, provided mixing can be achieved in the time range of 10 ms.

Although DMSO and water are miscible, they have different refractive indices, a small volume contraction occurs on mixing, and there is a considerable heat of mixing (5). It is not easy to mix them rapidly. Our first attempts to mix equal volumes of DMSO and water using a variety of mixing chambers and different flow velocities revealed a propensity for these two liquids to layer on one another, forming a structure like a rolled blanket, which caused severe optical absorption artifacts and dissipated only over a period of minutes. Since useful kinetic data can usually be obtained only if the half-time for mixing, considered as a first-order process, is less than one-tenth of the half-time of the chemical reaction to be studied, it was clear that an alternative approach had to be taken.

The method we have developed to accomplish rapid, homogeneous mixing of DMSO and aqueous solutions involves (*a*) a ratio of aqueous to DMSO solution volume of at least 25:1, (*b*) a flow divider to separate the DMSO stream into eight or more parts and to force them into the aqueous solution stream normal to the net flow direction of the resultant mixture, (*c*) turbulence in the wake of the flow divider tip (6), and (*d*) a tangential mixer that further mixes the mixture produced by the flow divider. The mixing process is generally considered to involve the physical breakup of the solutions into small packets by turbulence. These packets then mix to homogeneity by diffusion (7). The flow divider, which aids in the physical mixing of the solutions, will ideally have a large number of very small holes. The flow divider is required to prevent the occurrence of the layering phenomenon mentioned above. Further break up of the DMSO solution packets is accomplished by the turbulence produced in the remainder of the mixer, in which final diffusional mixing is also occurring.

Since this mixing device is quite simple to build and may have a general applicability in chemistry, we include detailed plans for its construction. Fig. 1 shows an overall

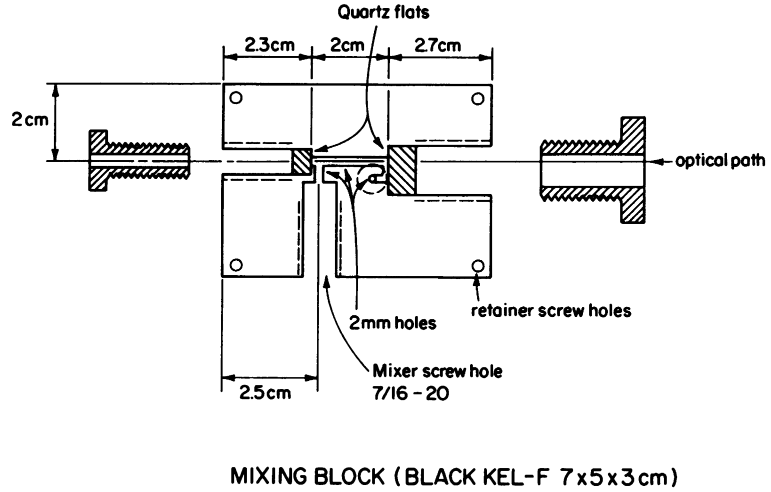


FIGURE 4 Detail of mixing block. Fluid exit part is out of the plane and is directed to a stop syringe by stainless steel connectors similar to the plastic devices described by Ballou (8).

schematic, indicating the liquid flow pattern from left to right. Fluid 1, from the small syringe, is forced into the flow divider (Fig. 2). Fluid 2 enters a chamber around the flow divider, where it encounters fluid 1 in the form of a group of fine jets emanating from the tip of the flow divider. There follow one or two four-jet tangential mixers only 0.06″ in thickness (Fig. 3), separated by a thin spacer having a 1-mm hole at its center. In each of these the heterogeneous fluid is divided into four streams on

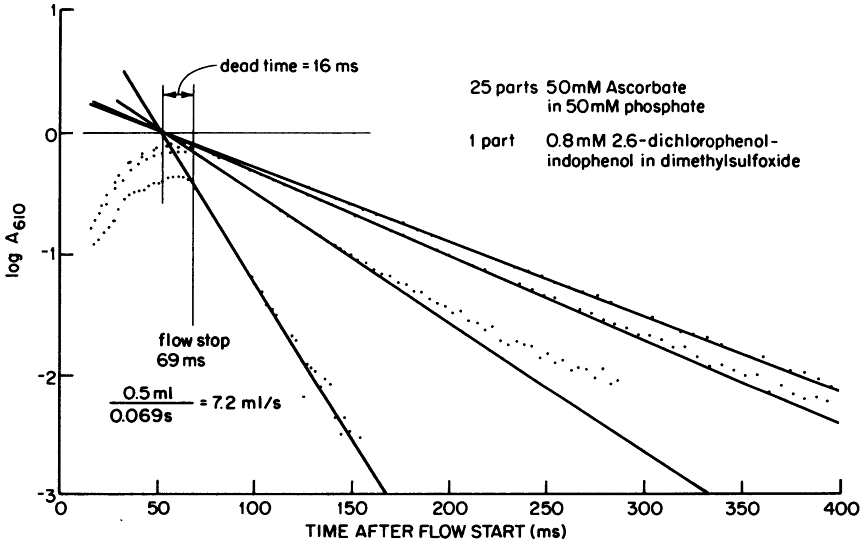


FIGURE 5 Typical performance test of the mixing device. The pH of the buffer was adjusted from 6.4 through 8.4 to obtain the different rates of reaction.

one side and re-mixed on the other side. Finally the mixed solution enters the observation chamber, indicated by the passage of light, and into the stop-syringe. Fig. 4 shows the plastic block in which the various parts are assembled.

Methods for testing stopped-flow mixers have been described (1, 8). One involves observation of optical absorption changes of acid-base indicators upon changing the pH in the mixing process. The protonation and deprotonation reaction of acid-base indicators are very fast and therefore, any absorbance change observed after flow stop is due to the kinetics of mixing and not of the chemical reaction (7). In tests of our mixing system, we observe less than 2% of the expected absorbance change to occur after flow stop. Another test method involves the extrapolation of absorbance versus time data from an observed first-order reaction back to the known initial absorbance at the point of mixing to obtain a measure of the dead time of the mixer. For our mixer this varies from 10 to 20 ms, though it may be reduced by increasing the flow rate of the reacting solutions above that used for this test (Fig. 5), which was about 7 ml/s (i.e., a maximal linear flow velocity of about 9 m/s).

This mixing method has been applied to the study of spontaneous and protein-catalyzed superoxide dismutation (9, 10) and the reactions of O_2^- with Fe (II) and Fe(III) chelated by ethylenediaminetetraacetic acid (EDTA) (11).

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FLUID MECHANICS OF RAPID MIXING

Jerome H. Milgram, *Massachusetts Institute of Technology,*
Cambridge, Massachusetts 02139 U. S. A.

Theoretical and experimental studies and analysis of rapid mixing processes will result in an increased understanding of the mechanics that may lead to the development of