albumins and the probes used. Carbonic anhydrase may lack the structural requirement for the binding of 1-anilinonaphthalene-8-sulfonate.

In contrast to the quenching of the native fluorescence of protein by the probes, no quenching was observed upon addition of the probes to tryptophan dissolved in buffer. This indicates that the driving force for the probe–protein binding is not mediated by direct interaction between the probe and tryptophan molecules, although tryptophan has been reported to have a binding affinity to various other organic molecules (24, 25). This observation also shows that the quenching of protein fluorescence by the probes was not due to concentration or inner filter effects.

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Drug–Biomolecule Interactions: Spin-Probe Study of Effects of Anesthetics on Membrane Lipids

K. W. BUTLER

Abstract □ The electron spin resonance spectra of probes were used to study the organization and motion of molecules in hydrated stacked bilayer or liposome model membrane systems. The same steroid structures required for reduction of membrane permeability were required to produce well-ordered films of brain lipid. Alcohols and anesthetic agents influence the structure of model membranes, with their order of efficacy paralleling their pharmacological effectiveness. Spin probes were also used to demonstrate effects of calcium and local anesthetics on the rate of penetration of ascorbate into lipid bilayers.

Keyphrases □ Anesthetics—effects on membrane lipids, electron spin resonance spectra □ Lipids, membrane—effects of anesthetics, electron spin resonance spectra □ Drug–biomolecule interactions—effects of anesthetics on membrane lipids, electron spin resonance spectra □ Interactions—drugs with biomolecules, symposium

Electron spin resonance spectroscopy (ESR) examines the spectra of free radicals in an applied magnetic field. In a filled electron orbital, there are two electrons whose magnetic moments cancel; but in a free radical, there is an unpaired electron with a net magnetic moment. This electron has two different energy states, which can be qualitatively described as having the magnetic moment parallel and antiparallel to an applied magnetic field. The energy difference between these states is equal to the energy of a quantum of microwave radiation (for the usual applied magnetic fields of 3–12 kilogauss). A single absorption line is observed in the case of an isolated electron. In a free radical, the magnetic environment of the electron can split this resonance into a number of lines.

In the spin-label method, a free radical with a suitably designed functional group is reacted with the system. In the spin-probe method, selected molecules with free radical moieties are intercalated into the

system. Details of the latter method, in particular with respect to the study of oriented lipid films, were discussed by Smith (1).

DISCUSSION

In this laboratory, two types of spin labels have been used primarily. One type (IV and V) is a steroid with a nitroxide group at the C_3 position. Another type (I and II) consists of a fatty acid or ester with a nitroxide group on the hydrocarbon chain. Probe III is also used occasionally as a monitor of head-group mobility.

Figure 1 describes how the spectra of a nitroxide depend upon the angle between the N—O bond and the magnetic field of the spectrometer. This bond is perpendicular to the long axis of the steroid nucleus (IV and V) and parallel to the long axis of the fatty acid (I and II). If probes IV and V orient themselves perpendicular to the plane of a lipid film and rotate rapidly about their long axes, their ESR spectra would consist of three lines separated by 6 and 19 gauss with the magnetic field perpendicular and parallel to the film, respectively.

When V is intercalated in brain lipid films, as the spectral anisotropy (difference between spectra run perpendicular and parallel to the magnetic field) decreases, the ratio of the heights of the peak 6–8 gauss downfield from the central peak and the central peak decreases when the spectra are observed with the film perpendicular to the magnetic field. This ratio, b/c, is a measure of the spectral anisotropy, varying from 1 for perfect order to 0 for a totally random array of spin probes. Under the same conditions, the separation of the three spectral lines of the spin-labeled fatty acid would be 32 and 6 gauss, respectively. For lower degrees of order, the spectral anisotropy is less.

Various theoretical models must be used to interpret these data (1–4). These spectral characteristics are observed with many lipid systems. The consistency of data obtained with both steroid and fatty acid spin probes demonstrates that the extrinsic probe is a faithful reporter of properties intrinsic to the lipid system.

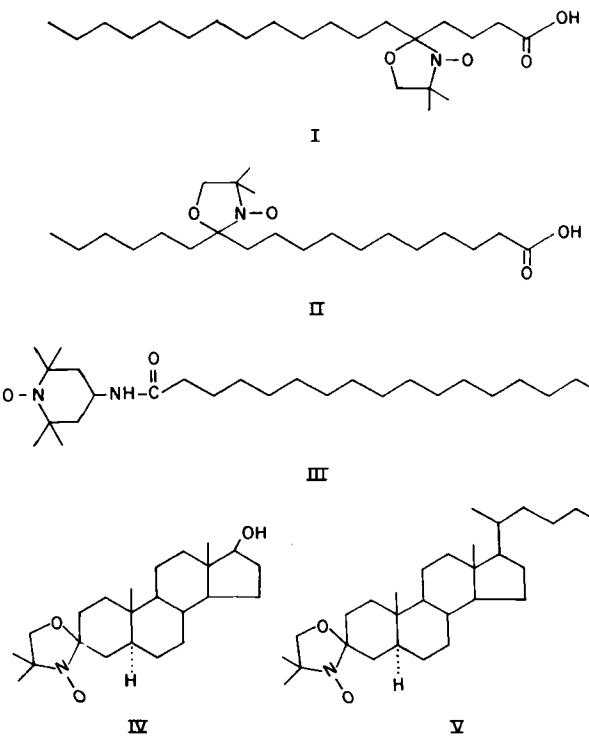
The detailed behavior of films depends upon their composition, the aqueous medium, and the temperature. The presence of ions in the aqueous medium is required for the integrity of films formed from charged lipids (2). Steroids have a marked effect; cholesterol is essential for a high degree of order in films of brain lipid (5) or egg lecithin (3) and decreases the rate and amplitude of motion and the probability of the *gauche*-conformation chains of the fatty acid in egg lecithin films (3, 4). Other steroids having a 3β-hydroxy group, a planar skeleton, and a nonpolar group at the C-17 position exert the same effect on brain lipids (5). These same factors were found by van Deenen (6) to be required for a reduction in membrane permeability.

Compounds in the aqueous phase can also influence the arrangement of molecules within the lipid phase. Proteins can be shown to exert various influences, either increasing or decreasing spectral anisotropy (1). The exact interpretation of the results is difficult, but it is certain that hydrophobic residues are involved in the disordering action. Polylysine increases order; Lys-Phe and Lys-Ala copolymers induce disorder, even in the presence of salt.

Many anesthetic agents interact with membranes. In a preliminary study, if butane or chloroform was passed in a stream of air over a hydrated lipid film, a large increase in the mobility of the spin probe occurred. This effect was dose dependent and reversible. Similar structural perturbations were observed with spin-labeled nerves and erythrocytes (7, 8).

EXPERIMENTAL

Alcohols—A dose–response study was made on the effects of a series of alcohols on human red cell lipids, lipids from the white matter of beef brain, and on egg lecithin using the cholestane spin probe (9). As the concentration of each alcohol increased, a point was reached where the separation between the lines of the spectra taken with the magnetic field of the spectrometer perpendicular to the film increased and the difference between the perpendicular and the parallel spectra decreased. At still higher alcohol concentrations, the film disintegrated. The concentration of alcohol required to cause a given change in the degree of anisotropy decreased as the chain length increased.



A plot of the concentration necessary to produce a 50% decrease in the height of the low field peak against the number of methylene groups in the alcohol is a straight line which parallels plots of the alcohol concentration necessary to inhibit tadpole reflexes, to bring about a decrease in the resistance of black lipid films, and to cause a 50% inhibition of red blood cell hemolysis. The displacement in the plots may be due to the varying amounts of disorder required to observe the various effects; more disorder is required for a 50% inhibition of red blood cell osmotic hemolysis than is required to inhibit tadpole reflexes, and even more is required to cause a resistance decrease in black lipid films. This disordering effect could cause anesthesia by directly affecting the permeability of the lipid layer, as seen with black lipid films, or by changing the lipid interaction with the membrane-bound proteins.

Local Anesthetics—Local anesthetics also increase the permeability of black lipid films (10), inhibit osmotic lysis of red cells (11, 12), and block nerve conduction (13–16). Unlike the effect of alcohols, the concentration of local anesthetic needed to cause a given response is pH dependent. This dependence has been rationalized by proposing different effects for the charged and uncharged forms of the drugs.

By using the cholestane spin probe (V), local anesthetics such as procaine, tetracaine, and butacaine affected films of brain white matter lipid in much the same manner as the alcohols. When the local anesthetic was added in a saline buffer to films that had been hydrated with the buffer alone, increasing concentrations of local anesthetic caused a decrease in the spectral anisotropy (hence order) of the films.

Unlike the effect of alcohols, the disordering effect increased with increasing pH at a fixed local anesthetic concentration. In the absence of local anesthetic, the amount of ESR spectral anisotropy was essentially constant over the pH range used. The concentrations necessary for marked disorder are high, in the toxic range according to studies performed on isolated nerves and red cells *in vitro*. It has been suggested that the uncharged form of local anesthetics penetrates membranes. The disordering effects observed may be due to disruption of the normal intermolecular forces between the phospholipids themselves or between phospholipids and cholesterol induced by high intramembrane concentrations of alcohols or local anesthetics.

As previously mentioned, films of brain lipid exhibit no ESR spectral anisotropy in the absence of cholesterol and only very slight anisotropy at 5 g % cholesterol. These films showed a very different response to the addition of local anesthetic. Whereas 1 m*M* tetracaine had no discernible effect on films containing the

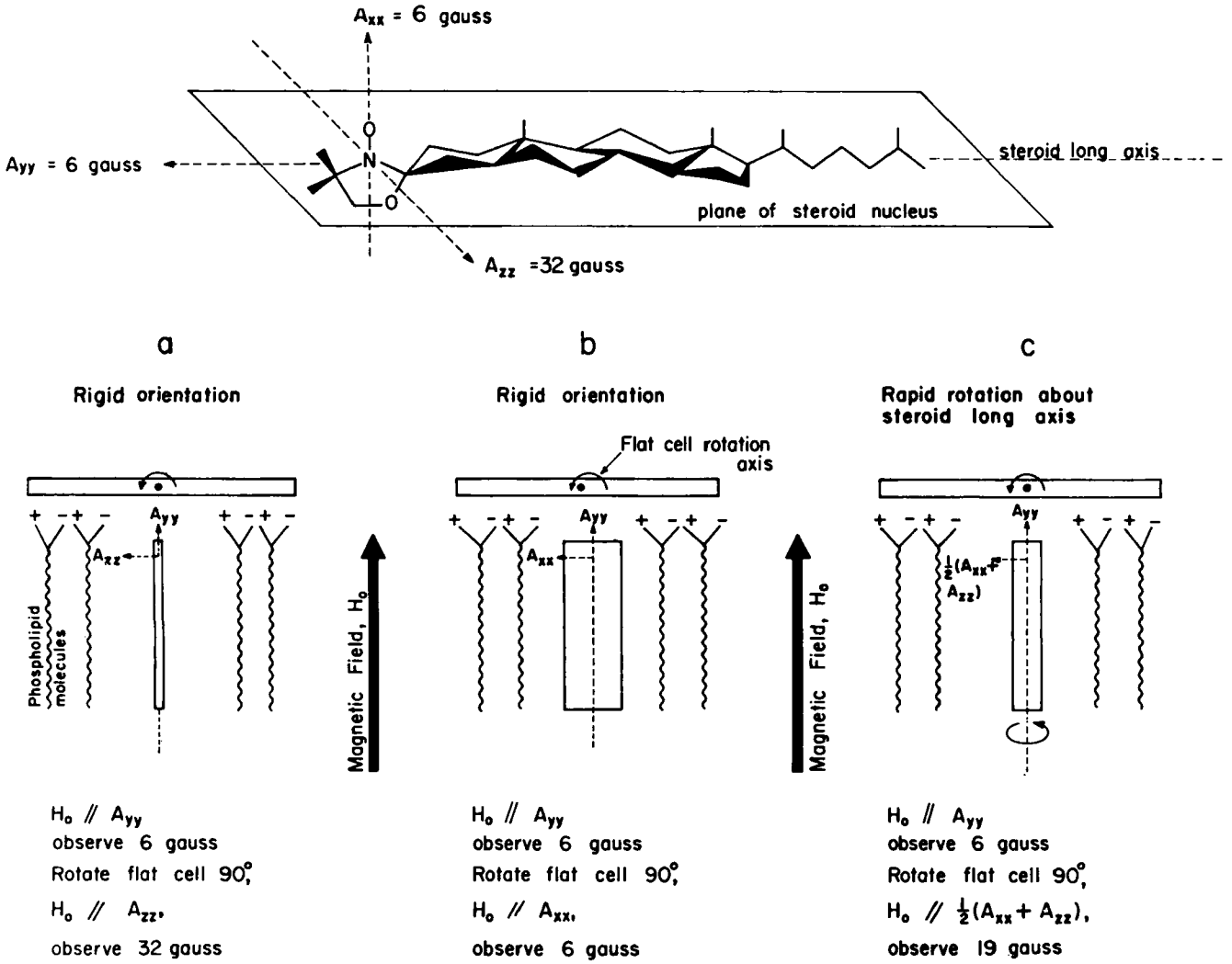


Figure 1—*Structure of V and the spectra that would be observed under various model conditions. (Reprinted, with permission, from Ref. 1.)*

normal amount of cholesterol, 1 m*M* tetracaine at pH 4.5 caused a massive increase in spectral anisotropy when applied to films containing 5% cholesterol.

Other local anesthetics also improved order when applied to films of brain lipid with 5% cholesterol at concentrations below those at which they disordered films of brain lipid with a normal cholesterol content. In addition, instead of efficacy increasing as a function of pH, each local anesthetic showed an optimal region of pH values for improving order. The optimum pH was different for each local anesthetic tested. Both uncharged and cationic forms of the local anesthetic apparently are required for this effect.

The effect of temperature on spectral anisotropy was measured with films of brain lipid. Figure 2 shows the effect of tetracaine and of cholesterol on the variation of spectral anisotropy with temperature. Both cholesterol and tetracaine caused the response to be greatly broadened.

The decrease in spectral anisotropy with a decrease in temperature is probably due to the formation of a gel phase which does not orient the spin probes; the decrease in spectral anisotropy with an increase in temperature can be ascribed to increased thermal motion of the acyl chains of the phospholipid. Cholesterol has been shown to increase the fluidity of the acyl chains below the transition temperature of the phospholipid and to decrease fluidity above the transition temperature (17). The results show that local anesthetics can exert much the same effect on brain lipid.

These results suggest two conclusions: (*a*) the response to local anesthetics of lipid films depends upon their composition, and (*b*) local anesthetics can exert a stabilizing effect on the organization of the lipids within the films. Since local anesthetics at anesthetic, nontoxic doses do not alter the resting potential of the nerve but rather block the transmission of impulses (18, 19), local anesthetics may act by stabilizing the membrane and preventing the conductance changes necessary for the propagation of action potentials.

Spin-Probe Reduction by Sodium Ascorbate—*Phosphatidyl Serine and Effect of Local Anesthetics*—The lipids used in the studies described here were extracted from biological material and consisted of a complex mixture of phospholipids, sulfatides, cerebrosides, and cholesterol. Other workers have investigated the effects of local anesthetics on the permeability, ion binding, and surface potential of pure systems or known mixtures (20). Tobias *et al.* (21) found that either cephalin (phosphatidyl serine plus phosphatidyl ethanolamine) alone or cephalin plus cholesterol showed a massive increase in resistance upon the addition of calcium. In this study, filters[1] were soaked in lipid to form the resistance barriers.

On the other hand, Papahadjopoulos (22) found that calcium increased the permeability of pure phosphatidyl serine vesicles to sodium. Local anesthetics also increased sodium permeability in the absence of calcium. At lower concentrations, these same local anesthetics opposed the effect of calcium; that is, in the presence of calcium, the addition of tetracaine or procaine decreased the sodium permeability of the liposomes.

The effects of calcium or local anesthetics on phosphatidyl serine films could not be studied since the films are unstable at pH values around neutrality, especially in the presence of calcium. Liposomes with an intercalated spin probe showed no significant spectral changes when local anesthetics, calcium, or both were

[1] Millipore.

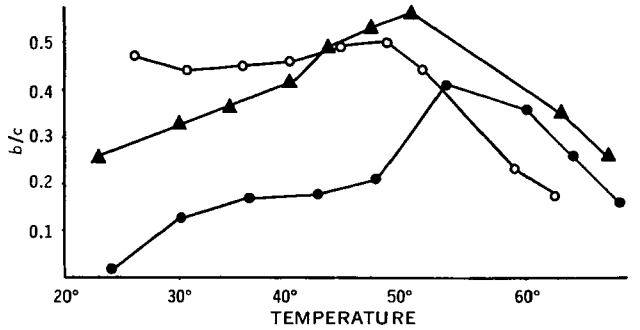


Figure 2—*Effects of tetracaine and cholesterol on the temperature response of brain lipid films. Key:* ●, *brain lipid and 5% cholesterol;* ▲, *brain lipid, 5% cholesterol, and 1 m*M *tetracaine; and* ○, *normal brain lipid.*

added. However, the permeability of these liposomes to sodium ascorbate was highly dependent upon the presence of calcium and of tetracaine. Metcalfe (23) reported that benzyl alcohol increased the rate of ascorbate penetration into liposomes. Therefore, the rate of reduction of the nitroxide moiety of the spin probe was used to monitor the local permeability of the lipid system.

With liposomes formed by water bath sonication of a mixture of phosphatidyl serine and spin probe I, the addition of 1 m*M* calcium caused a massive increase in the rate of reduction of the spin probe by ascorbate. The addition of 1 m*M* tetracaine caused a small increase in permeability, and 5 m*M* tetracaine caused a larger increase in permeability. When both 1 m*M* calcium and 1 m*M* tetracaine were present in the solution, the rate was less than with 1 m*M* calcium alone (Fig. 3).

The addition of lecithin to the system reduced the effect of calcium on the system to the point that pure lecithin liposomes showed no response to 10 m*M* calcium. As long as a calcium effect could be observed, tetracaine acted to oppose this effect. The addition of cholesterol up to a 1:1 mole ratio to the liposome did not inhibit the effect of calcium.

Other Charged Systems—Liposomes were also formed of egg lecithin and I to which either phosphatidic acid or dicetyl phosphate had been added to confer a negative charge. Both calcium and tetracaine at a concentration of 1 m*M* increased the rate of ascorbate reduction of I. Unlike the results with egg lecithin to which phosphatidyl serine had been added, the rate of reduction was greater when both calcium and tetracaine were added than with either alone (Fig. 4).

Brain Lipid Films—The results with brain lipids resembled those obtained with simple charged lipid systems. The rate of reduction of I incorporated in liposomes formed from the white matter lipid of beef brains increased upon the addition of either calcium or tetracaine at concentrations of 1 m*M*. Like the results obtained with egg lecithin plus either phosphatidic acid or dicetyl phosphate, the rate was greater with both calcium and tetracaine

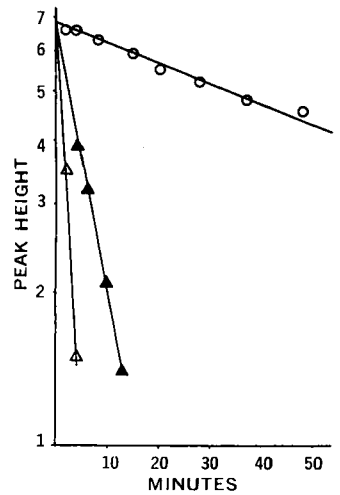


Figure 3—*Time course of the reduction of I in water bath-sonicated phosphatidyl serine vesicles by* 10^{-2} M *ascorbate at pH 7.2. Key:* ○, 7.5×10^{-3} M *ethylenediaminetetraacetate;* △, 1.0×10^{-3} M *calcium; and* ▲, 1.0×10^{-3} M *calcium* $+ 1 \times 10^{-3}$ M *tetracaine.*

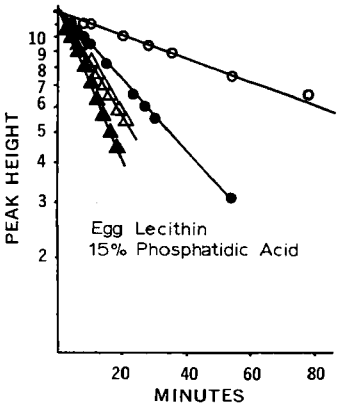


Figure 4—*Time course of the reduction of I in water bath-sonicated liposomes consisting of 85% egg lecithin and 15% phosphatidic acid by* 10^{-2} M *ascorbate at pH 7.4. Key:* ○, 7.5×10^{-3} M *ethylenediaminetetraacetate;* ●, 7.5×10^{-3} M *ethylenediaminetetraacetate* $+ 1 \times 10^{-3}$ M *tetracaine;* △, 1.0×10^{-3} M *calcium; and* ▲, 1.0×10^{-3} M *calcium* $+ 1 \times 10^{-3}$ M *tetracaine.*

than with either alone. When cholesterol was removed from the brain lipid mixture, the results, although not first order in the presence of calcium, more closely resembled the results obtained with systems containing phosphatidyl serine.

The results were qualitatively the same whether the liposomes were formed by simple hand shaking or by sonication with a probe or in a water bath. The direction of the effects of calcium and tetracaine was independent of liposome size.

Other spin probes, whose nitroxide groups lie at different depths within the lipid bilayer, were also used. The rate of reduction of the stearamide spin probe (III), in which the nitroxide group lies near the aqueous interface of the lipid bilayer, was six times as fast as the rate of reduction of I, in which the nitroxide group is buried within the lipid layer. This experiment was performed with large, hand-shaken liposomes. In water bath-sonicated (small) liposomes, V was reduced eight times as fast as was I.

Calcium and tetracaine had the same effects with these probes. Therefore, the accessibility of the interior of the lipid to the aqueous phase is concluded to be the major rate-limiting step.

CONCLUSION

It is apparent from these studies that the behavior of model systems when exposed to local anesthetics depends upon the lipid composition and upon the ions present in the aqueous environment. Phosphatidyl serine, a common constituent of nerves, responds to anesthetics in a manner very different from that of other phospholipids.

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Drug–Biomolecule Interactions: Mechanism of Ligand Interactions with Carbonic Anhydrase Studied by Magnetic Resonance Relaxation and Rapid Reaction Methods

PALMER TAYLOR

Abstract □ Kinetics of interaction between the metalloenzyme carbonic anhydrase and either monovalent anions or aromatic sulfonamides were examined by three distinct fast reaction techniques: stopped flow, equilibrium perturbation, and magnetic resonance relaxation. By correlating spectroscopic data on conformational and ionization equilibria of the complex and free species with the reaction kinetics, a relatively complete description of the mechanism can be presented. A proton-dependent equilibrium between two coordination forms of the free enzyme can be demonstrated spectroscopically. Anions selectively combine with the form predominating at low pH. For a series of carboxylate ligands, formate and substituted acetates, anion association is found to be three orders of magnitude greater than similar ligand substitution processes known in inorganic chemistry. For sulfonamide association, the attacking species are the form of carbonic anhydrase predominating at high pH and the neutral sulfonamide. Combination involving the neutral species is followed by loss of a proton to form the sulfonamido anion in the stabilized complex. This obligate proton transfer offers a probable explanation for the unique specificity of sulfonamides in inhibiting this enzyme. The anionic sulfonamido moiety can be shown to resemble closely a transition intermediate in the catalytic step of substrate hydration.

Keyphrases □ Ligand–carbonic anhydrase interactions—kinetics studied by magnetic resonance relaxation and rapid reaction methods □ Carbonic anhydrase–ligand interactions—kinetics studied by magnetic resonance relaxation and rapid reaction methods □ Drug–biomolecule interactions—mechanism of ligand interactions with carbonic anhydrase, magnetic resonance relaxation and rapid reaction methods □ Interactions—drugs with biomolecules, symposium

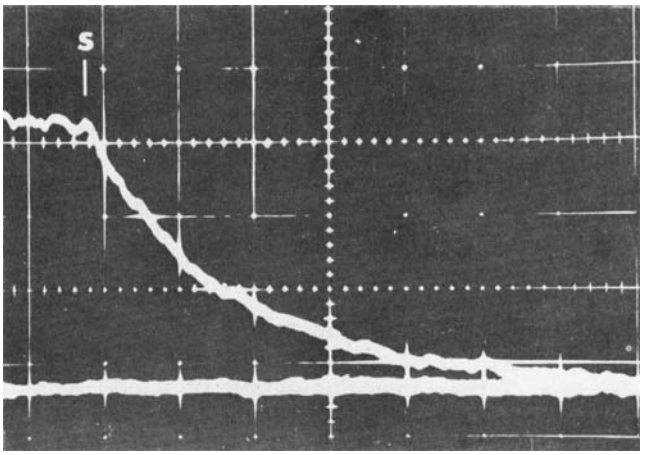

Figure 1—*Oscilloscope trace recording the change in carbonic anhydrase fluorescence for the reaction between 14 μM p-nitrobenzenesulfonamide and the human C isozyme. The oscilloscope was triggered with the initiation of flow so that the time dependence of fluorescence was measured during and after flow. Stoppage of flow occurs at s, and each large horizontal increment corresponds to 50 msec. The horizontal trace at the bottom of the screen was triggered ~10 sec later and corresponds with the fluorescence of the complex at equilibrium. Fluorescence was monitored at 345 nm; the excitation wavelength was 290 nm.*

The molecular basis of specificity in the formation of a ligand–macromolecule complex is of interest in many fields of the biological sciences, since complex formation represents the common initial event that leads to a divergence in response. The interaction of a macromolecule with a drug shows a capacity for selective recognition of ligand structure. In addition, receptors may exhibit specific capacity to translate an interaction with chemical mediators. The latter is generally considered to occur through ligand-sensitive conformational changes. Thus, in the study of specificity of drug–macromolecule interactions, both differential affinity and changes induced in the structure of the complex should be considered.

Complex formation between a drug and macromolecule usually involves an inherently rapid process(es). Examination of such interactions at a level where primary processes and transient species are detected requires methods applicable to following