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# Indole Fluorescence Quenching Studies on Proteins and Model Systems: Use of the Inefficient Quencher Succinimide†

Maurice R. Eftink* and Camillo A. Ghiron

ABSTRACT: We have compared the quenching of the fluorescence of proteins by acrylamide and succinimide, two chemically similar quenchers. We find that the ratio of the apparent rate constants for succinimide and acrylamide quenching, $\gamma_{S/A}$, ranges from ~0.1 to ~0.7. Proteins having relatively buried tryptophan residues, such as ribonuclease $T_1$, cod parvalbumin, and aldolase, are found to have small values of $\gamma_{S/A}$ (i.e., succinimide quenches with a much smaller rate constant than acrylamide); proteins with relatively solvent-exposed tryptophan residues, such as glucagon and adrenocorticotropin, are found to have larger values of $\gamma_{S/A}$. We interpret this range of $\gamma_{S/A}$ values as being due to either (a) a critical size dependence of the dynamic penetration of quencher through a protein matrix (succinimide being larger than acrylamide) and/or (b) an inherent dependence of the succinimide quenching reaction on the microenvironment of the indole ring. The latter interpretation is supported by studies of the solvent dependence of the quenching of the fluorescence of indole and 5-methoxyindole by succinimide and acrylamide. These studies show that whereas acrylamide is an efficient quencher in all solvents investigated, succinimide is a relatively inefficient quencher in aprotic solvents. Thus, both of the above molecular bases for poor quenching of interior tryptophan residues in proteins by succinimide (i.e., a critical size dependence of a microenvironment dependence) are consistent with the fluorescence quenching process occurring within the globular structure of proteins by a dynamic penetration mechanism, as opposed to an unfolding mechanism by which interior residues would be periodically exposed to the solvent.

Studies of indole fluorescence quenching by added solutes have, in recent years, provided valuable information regarding the structure and dynamics of proteins in solution (Lehrer, 1971; Lehrer & Leavis, 1978; Lakowicz & Weber, 1973; Lakowicz et al., 1983; Eftink & Ghiron, 1976a, 1977, 1981). The most significant finding with this technique is that even tryptophan (Trp) residues that are presumably deeply buried within globular proteins are able to be quenched by the uncharged quenchers oxygen and acrylamide with quenching rate constants on the order of $10^9$ $M^{-1}$ $s^{-1}$. These results have been interpreted as indicating that the quenchers are able to penetrate into the matrix of globular proteins, with this penetration

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being facilitated by small-amplitude fluctuations in the protein's structure occurring on the nanosecond time scale (Lakowicz & Weber, 1973; Eftink & Ghiron, 1976a).

In a recent phosphorescence and fluorescence quenching study, Calhoun et al. (1983) have emphasized another possible interpretation of the quenching of buried Trp residues. They have argued for a mechanism involving the local unfolding of a protein to expose internal residues to the solvent, followed by quenching of the transiently exposed residues by quencher.

Essentially the difference between these two models is whether quenching occurs by penetration of the quencher into the protein, facilitated by rapid, small-amplitude structural fluctuations in the protein, or whether quenching occurs in an aqueous environment, facilitated by relatively large-amplitude unfolding transitions in the protein. A possible experimental way to distinguish between these mechanisms would seem to be to compare quenchers of different molecular size. With increasing quencher size, one would expect the rate of penetration into a globular protein to decrease dramatically due to the increase in the amplitude of the required fluctuations (Richards, 1979). If quenching occurs by a local unfolding mechanism, a much smaller dependence on the size of the quencher would be expected (Calhoun et al., 1983).

In this paper, we will report studies of the quencher size dependence of the quenching of the fluorescence of several proteins using acrylamide and succinimide as quenching probes. In such a comparative study, it is desirable to select two or more quenchers which are chemically similar. Both acrylamide and succinimide are polar, neutral compounds, with the latter being roughly 20% larger than the former, as calculated from van der Waals atomic increments (Edward, 1970). Acrylamide has been used extensively as a quencher of protein fluorescence (Eftink & Ghiron, 1976a,b); succinimide has been used only on occasion for this purpose (Badley, 1975; Eftink & Ghiron, 1981).

Part of our report involves the further characterization of succinimide as a quencher of indole fluorescence. One problem with succinimide, as we will show below, is that is is not a completely efficient quencher. The analysis of quenching data can be complicated when an inefficient quencher is used (Eftink & Ghiron, 1981). One of the objectives of this work, in addition to our goal of using acrylamide and succinimide quenching to test the above-mentioned mechanisms for the quenching of buried Trp residues in proteins, is to characterize the nature of succinimide's inefficiency in order to delineate what useful information can be obtained in applications of succinimide quenching to proteins.

## Theory

The concept of efficiency in a diffusion-controlled reaction has been described by Weller (1961). An analogous and more general treatment, applied to fluorescence quenching, has been given recently (Eftink & Ghiron, 1981), and only relevant aspects will be recalled here.

A general mechanism for the quenching of an excited, luminescent state (A*) by quencher (Q) is

$$A^* + Q \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} A^*\cdots Q \xrightarrow{k_i} A + Q + \Delta$$

The apparent rate constant for the quenching reaction, $k_q$, will be given as $k_q = \gamma k_d$, where $k_d$ is the diffusion-limited rate constant for formation of the encounter complex (A*···Q), and $\gamma$, the quenching efficiency, is (at low [Q] and assuming that the radiative rate constant, $k_f$, is the same for A* and A*···Q)

$$\gamma = \frac{k_i}{k_{-d} + k_i + k_f} \qquad (1)$$

$k_i$ is the rate constant for the radiationless deactivation of the A*···Q encounter complex. When $k_i > k_{-d} + k_f$, $\gamma$ approaches unity and $k_q$ approaches the diffusion-limited value, $k_d$. On the other hand, when $k_i \approx k_{-d} + k_f$, $\gamma$ will be less than unity, $k_q$ will be less than $k_d$, and the quenching process will be inefficient (i.e., not all encounter complexes undergo the quenching reactions; some dissociate to A* and Q, and some radiate a photon). When the quencher is inefficient, the dependence of $k_q$ on such variables as temperature, solvent viscosity, and solvent polarity may be complicated due to the effect of these variables not only on $k_d$, but also on $k_i$, $k_{-d}$, and $k_f$. [See Eftink & Ghiron (1981) for further discussion.]

In order to determine $\gamma$ for a quencher, $k_q$ values can be experimentally determined by a Stern–Volmer analysis of quenching data. The following is a modified form of the Stern–Volmer equation which adequately describes solute quenching in condensed phases (Eftink & Ghiron, 1976b):

$$\frac{F_0}{F \exp(V[Q])} = 1 + K_{sv}[Q] = \frac{\tau_0}{\tau} \qquad (2)$$

$F_0$, $F$ and $\tau_0$, $\tau$ are the fluorescence intensities and lifetimes in the absence and presence of quencher, respectively. $K_{sv}$, the Stern–Volmer constant for the dynamic quenching process, is equal to $k_q\tau_0$. $V$ is a static quenching constant, which is often found to be required to adequately describe the entire quenching process. Using eq 2, we can analyze either steady-state fluorescence data or fluorescence lifetime data to obtain $k_q$. By comparing $k_q$ to a value of $k_d$ calculated via the Smulochowski equation for diffusion [eq 4 in Eftink & Ghiron (1976b)], we can calculate $\gamma$.

In the work presented below, we will find it convenient to express the efficiency of succinimide quenching as the ratio of the rate constant for succinimide quenching ($k_q^S$) of a particular sample to the rate constant for acrylamide quenching ($k_q^A$) of the same sample. That is, $\gamma_{S/A} = k_q^S/k_q^A$. Since we will show that acrylamide is a unitary efficiency quencher under all conditions studied, the value of $\gamma_{S/A}$ actually represents the efficiency of succinimide quenching. This relative efficiency term can also be conveniently calculated as the ratio of Stern–Volmer constants (i.e., $\gamma_{S/A} = K_{sv}^S/K_{sv}^A$), thus making it unnecessary to know fluorescence lifetimes. Also in studies with proteins, the relative efficiency, $\gamma_{S/A}$, provides a direct comparison of the effectiveness of these two quenchers to quench Trp residues.

## Materials and Methods

*Materials.* The source of all proteins is described elsewhere (Eftink, 1983). Indole was recrystallized 3 times from cyclohexane and stored in an amber bottle. L-Tryptophan, L-tryptamine, 3-methylindole, and 2-methylindole were obtained from Sigma Chemical Co. 5-Methoxyindole and 1-methylindole were obtained from Aldrich Chemical Co. *N*-Acetyl-L-tryptophanamide was obtained from Fox Chemical Co. *N*-Acetyl-L-tryptophan and *N*-methylsuccinimide were obtained from Pfaltz and Bauer Research Chemicals. Acrylamide was recrystallized from ethyl acetate, succinimide was recrystallized from ethanol, and *N*-methylsuccinimide was recrystallized from cyclohexane. The melting points of acrylamide, succinimide, and *N*-methylsuccinimide were determined to be 86–87, 123–125, and 70–73 °C, respectively. Organic solvents were spectral grade in almost all cases (ethylene glycol was reagent grade). Formamide, *N*-methylformamide, and 1-methylindole were vacuum distilled. Water was distilled and deionized.

*Methods.* Steady-state fluorescence measurements were made with either a Perkin-Elmer MPF 44 or an SLM 4800

Table I: Acrylamide and Succinimide Quenching of Indole Derivatives and Other Fluorophores[a]

| | | acrylamide | | succinimide | | |
|---|---|---|---|---|---|---|
| | $\tau_0$ (ns) | $k_q$ ($\times 10^{-9}$ $M^{-1}$ $s^{-1}$) | $V$ ($M^{-1}$) | $k_q$ ($\times 10^{-9}$ $M^{-1}$ $s^{-1}$) | $V$ ($M^{-1}$) | $\gamma_{S/A}$ |
| indole | 4.1[b] | 7.7 (7.2) | 3.0 | 5.1 (4.8) | 2.5 | 0.67 (0.67) |
| 1-methylindole | 8.5[c] | 7.6 | 1.6 | 5.2 | 2.0 | 0.68 |
| 3-methylindole | 8.5[b] | 7.0 (7.3) | 3.0 | 4.9 (4.6) | 2.0 | 0.70 (0.64) |
| 5-methoxyindole | 4.1[b] | 7.0 (7.1) | 3.3 | 4.9 (4.6) | 2.0 | 0.82 (0.81) |
| L-tryptophan | 3.0[b] | 5.9 | 1.3 | 3.5 | 1.5 | 0.60 |
| *N*-acetyl-L-tryptophanamide | 2.8[b] | 5.8 | 1.5 | 3.9 | 1.3 | 0.68 |
| *N*-acetyl-L-tryptophan | 4.2[b] | 7.0 | 2.3 | 4.4 | 1.0 | 6.3 |
| phenol | 3.2[b] | 7.0 (7.7) | 3.0 | 5.3 (5.5) | 2.0 | 0.75 (0.68) |
| naphthalene | 34.1[b] | 5.6 (4.7) | 4.0 | 0.44 (0.40) | *d* | 0.08 (0.08) |

[a] Experiments were performed at 25 °C. Values in parentheses were obtained from fluorescence lifetime quenching data. Other values were obtained from steady-state quenching data (see eq 2). [b] Fluorescence lifetimes were measured with a phase-modulation fluorometer operating at 18 MHz; values were calculated from the phase lag. Lifetimes calculated from the degree of modulation were with 0.2 ns of the phase lifetime for all compounds except *N*-acetyltryptophan and naphthalene. [c] Lifetime from Walker et al. (1969). [d] Stern–Volmer plot is downward curving.

Table II: Acrylamide (A) and Succinimide (S) Quenching of the Fluorescence of Indole and 5-Methoxyindole in Various Solvents[a]

| | indole | | | | 5-methoxyindole | | | |
|---|---|---|---|---|---|---|---|---|
| solvent | $\tau_0$ (ns) | $k_q^A$ ($\times 10^{-9}$ $M^{-1}$ $s^{-1}$) | $k_q^S$ ($\times 10^{-9}$ $M^{-1}$ $s^{-1}$) | $\gamma_{S/A}$ | $\tau_0$ (ns) | $k_q^A$ ($\times 10^{-9}$ $M^{-1}$ $s^{-1}$) | $k_q^S$ ($\times 10^{-9}$ $M^{-1}$ $s^{-1}$) | $\gamma_{S/A}$ |
| dioxane | 4.3 | 8.3 (6.1) | 1.2 (1.1) | 0.15 (0.18) | 5.4 | 5.6 (4.9) | 0.17 (0.17) | 0.03 (0.035) |
| dimethyl sulfoxide | 4.8 | 4.4 (3.3) | 0.16 (0.17) | 0.04 (0.05) | 5.7 | 3.4 (2.8) | 0.017 (0.014) | 0.005 (0.005) |
| acetonitrile | 3.6 | 18.6 (17.5) | 1.8 (1.7) | 0.10 (0.10) | | | | |
| dimethylformamide | 2.9 | 7.3 (6.6) | 0.07 (0.24) | 0.01 (0.036) | 5.3 | 5.5 (4.8) | 0.04 | 0.01 |
| *N*-methylformamide | 2.4 | 5.4 (4.2) | 1.3 (1.3) | 0.25 (0.29) | 2.5 | 5.0 (4.8) | 1.0 (0.7) | 0.20 (0.19) |
| formamide[b] | | | | | 1.7 | 4.4 (3.4) | 1.5 (1.4) | 0.34 (0.41) |
| methanol | 3.3 | 10.5 (10.2) | 5.2 (5.7) | 0.50 (0.56) | 4.6 | 11.4 (11.1) | 5.2 (5.3) | 0.46 (0.48) |
| ethanol | 3.6 | 6.9 (6.1) | 4.1 (3.4) | 0.59 (0.56) | | | | |
| 2-propanol | 3.9 | 4.3 (3.6) | 2.8 (2.5) | 0.64 (0.70) | 5.0 | 4.7 (4.1) | 2.8 (2.5) | 0.61 (0.61) |
| *tert*-butyl alcohol | 4.3 | 2.2 (1.7) | 1.5 (1.1) | 0.68 (0.65) | | | | |
| ethylene glycol | 3.2 | 1.3 (0.95) | 0.95 (0.62) | 0.67 (0.65) | | | | |
| water | 4.0 | 7.7 (7.2) | 5.2 (4.8) | 0.67 (0.67) | 4.4 | 7.0 (7.1) | 5.6 (5.7) | 0.82 (0.81) |

[a] Experiments were performed at 25 °C. Fluorescence lifetimes were determined by phase-modulation fluorometry at 18 MHz; values were calculated from the phase lag. Lifetimes calculated from the degree of modulation were with 0.2 ns of the phase lifetime in all solvents, except ethylene glycol where there was a 0.5-ns difference. Rate constants and $\gamma_{S/A}$ in parentheses were determined from lifetime measurements. Other quenching data were obtained from steady-state measurements. For the latter data, rate constants were obtained from the initial slope of Stern–Volmer plots [i.e., from $K_{sv}(\text{eff}) \approx k_q\tau_0$]. In most solvents, the Stern–Volmer plots were upward curving, but only for water was analysis for $V$ included. [b] Due to the large absorbance of formamide below 300 nm, experiments were not done with indole.

spectrophotofluorometer. Unless stated otherwise, fluorescence was measured at 25 °C with excitation at 295 nm and emission monitored at the wavelength of maximum fluorescence of the sample. Quenching was observed by adding aliquots of a stock quencher solution (made in the solvent under investigation) to the sample by means of a microliter pipet or precision syringe. Corrections due to absorptive screening were made when necessary as described elsewhere (Eftink & Ghiron, 1976a). Such corrections are usually required for studies with acrylamide ($\epsilon_{295} = 0.25$ $M^{-1}$ $cm^{-1}$ in water, and larger values were found for dioxane, acetonitrile, dimethyl sulfoxide, dimethylformamide, and *N*-methylformamide) but are small or negligible with succinimide ($\epsilon_{295} = 0.02$ $M^{-1}$ $cm^{-1}$ in water, and values at least this small were found in all organic solvents). Quenching data were analyzed to obtain $k_q$ (and in some cases $V$) by use of eq 2 as described elsewhere (Eftink & Ghiron, 1976a). In our studies in organic solvents, only the effective Stern–Volmer constant, $K_{sv}(\text{eff})$, was determined (as the initial slope of a plot of $F_0/F$ vs. [Q]); no attempt was made to analyze the data to determine $V$ values.

Fluorescence lifetime measurements were made with an SLM 4800 phase fluorometer using 18 MHz as the modulation frequency (Spencer & Weber, 1969). The lifetimes reported were determined from the phase shift method. *p*-Terphenyl in ethanol was used as a reference in place of a scattering solution, as described by Lakowicz et al. (1981). Again, quenching was achieved by adding aliquots from a stock quencher solution to the sample in a fluorescence cuvette, and lifetime data were analyzed with eq 2 to obtain $k_q$ values. Solutions were not degassed.

pH measurements were made by using a London PHM 64 pH meter. The viscosity of organic solvents was measured, relative to the value of $\eta = 0.89$ cP for water at 25 °C, with an Ostwald viscometer in a temperature-controlled bath.

## Results

*Comparison of the Acrylamide and Succinimide Quenching of Indole Fluorescence. (A) Indole Derivatives.* In Table I are presented data for the acrylamide and succinimide quenching of a series of indole derivatives in water at 25 °C. $\gamma_{S/A}$, the ratio of $k_q^S$ to $k_q^A$, is found to range from 0.60 to 0.82, with an average of 0.67. The quenching of indole fluorescence by *N*-methylsuccinimide was also studied, and a ratio of $k_q^{\text{methyl-S}}$ to $k_q^A$ of 0.67 was found. In additional studies, $\gamma_{S/A}$ values for phenol and naphthalene were determined to be 0.68 and 0.08, respectively.

*(B) Solvent Dependence.* The acrylamide and succinimide quenching of indole and 5-methoxyindole has been studied in the various solvents listed in Table II. These two fluorophores were selected as representative of exiplex and nonexiplex emitters (Hershberger & Lumry, 1976). Additionally, 5-methoxyindole was selected because its red-shifted absorption spectrum (relative to indole) enables excitation at wavelength above 295 nm, thus minimizing the magnitude of absorptive screening corrections made due to acrylamide. Quite a difference is seen in the solvent dependence of the quenching by

Table III: Acrylamide and Succinimide Quenching of Single Trp Containing Proteins[a]

| | $\tau_0$ (ns) | acrylamide $k_q$ ($\times 10^{-9}$ $M^{-1}$ $s^{-1}$) | acrylamide $V$ ($M^{-1}$) | succinimide $k_q$ ($\times 10^{-9}$ $M^{-1}$ $s^{-1}$) | succinimide $V$ ($M^{-1}$) | $\gamma_{S/A}$ |
|---|---|---|---|---|---|---|
| parvalbumin[b] | 3.3 | 0.21 (0.16) | | 0.03 (0.02) | | 0.16 (0.14) |
| RNase $T_1$[b] | 3.5 | 0.22 | | 0.04 | | 0.17 |
| nuclease[d] | 5.2 | 0.77 | 0.2 | 0.22 | | 0.29 |
| fd phage[c] | 3.7 | 0.64 | 0.3 | 0.27 | | 0.42 |
| monellin[d] | 2.6 | 1.65 | 0.15 | 0.94 | | 0.57 |
| melittin, tetramer[b] | 2.1 | 2.0 (1.3) | 0.5 | 0.52 (0.48) | 0.3 | 0.26 (0.37) |
| melittin, monomer[b] | 3.1 | 3.4 (3.2) | 1.0 | 2.4 (1.9) | 0.8 | 0.73 (0.60) |
| phospholipase $A_2$[b] | 3.2 | 2.2 | 0.1 | 1.4 | | 0.62 |
| chorionic gonadotropin[e] | 3.0 | 1.9 | | 1.2 | | 0.63 |
| adrenocorticotropin[b] | 2.7 | 3.6 (3.5) | 1.0 | 2.4 (1.8) | 0.7 | 0.69 (0.53) |
| glucagon[b] | 2.7 | 3.5 (3.2) | 0.8 | 2.4 (1.7) | 0.5 | 0.67 (0.54) |

[a] Experiments were performed at 25 °C in the following buffers: 0.033 M sodium phosphate, pH 7.0, parvalbumin, ribonuclease $T_1$, monellin, phospholipase $A_2$, adrenocorticotropin, and chorionic gonadotropin; 0.01 M Tris, pH 7.0, fd phage, and melittin monomer; 0.45 M sodium phosphate, pH 7.0, melittin tetramer; 0.01 M Tris with 0.01 M $CaCl_2$, pH 7, nuclease; 0.1 M NaCl, pH 3, glucagon. Quenching data in parentheses were obtained from lifetime measurements; all other quenching data were obtained from steady-state measurements. When no $V$ values are given, the Stern–Volmer plots are linear or slightly downward curving. [b] Fluorescence lifetime determined by phase-modulation fluorometry at 18 MHz; value is calculated from the phase lag. Lifetimes are not necessarily single-exponential decays. [c] Fluorescence lifetimes determined by phase-modulation fluorometry at 30 MHz; value given is the average from that calculated by the phage lag and degree of modulation. [d] Lifetimes taken from Eftink & Ghiron (1976); values are from single-component fits of impulse–decay data. [e] Lifetime taken from Lakowicz et al. (1983). Data obtained with human serum albumin were anomolous in that more quenching was observed with succinimide than with acrylamide ($\gamma_{S/A} \approx 2$). We suspect that this is due to some type of interaction between succinimide and this protein which enhances the quenching process, whether it be static or dynamic. We note that a relatively large static quenching constant has been found for the acrylamide quenching of human serum albumin as well (Eftink & Ghiron, 1976a).

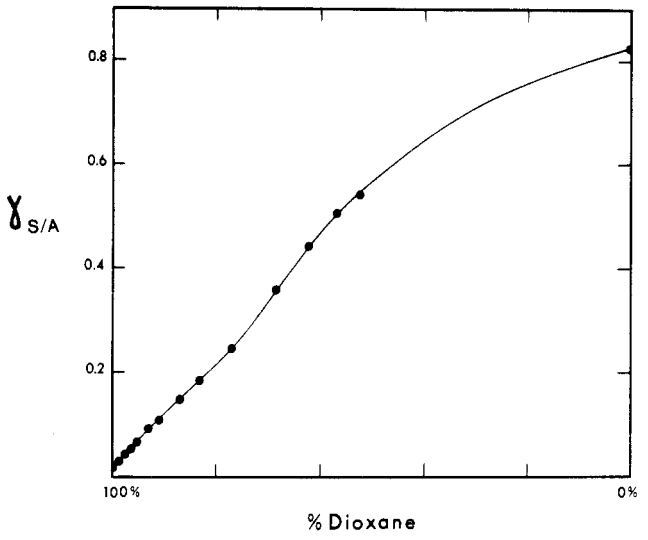


FIGURE 1: Dependence of $\gamma_{S/A}$ for succinimide and acrylamide quenching of 5-methoxyindole on the solvent composition of dioxane–water mixtures (plotted as volume percent).

the two quenchers. $\gamma_{S/A}$ is found to vary from ~0 to ~0.8. To provide further information concerning the nature of this solvent dependence, we also studied the quenching of 5-methoxyindole fluorescence in water/dioxane mixtures. As shown in Figure 1, there is a monotonic increase in $\gamma_{S/A}$ from 0.02 to 0.82 as the volume percent of water increases from 0 to 1.0.

*Acrylamide and Succinimide Quenching of Proteins. (A) Single Tryptophan Containing Proteins.* The acrylamide and succinimide quenching of the fluorescence of a number of single tryptophan containing proteins has been studied, and respective quenching rate constants and fluorescence lifetimes are listed in Table III. These proteins range from ribonuclease $T_1$ and cod parvalbumin, whose tryptophan residues are relatively buried (Longworth, 1968; Eftink & Ghiron, 1976a; Permyakov et al., 1980; Horie & Vanderkooi, 1982), to glucagon, adrenocorticotropin, and melittin (monomeric form), whose tryptophan residues are solvent exposed (Edelhoch & Lippolt, 1969; Lauterwein et al., 1980; Georghiou et al., 1982).

Table IV: Acrylamide (A) and Succinimide (S) Quenching of Multi-Trp-Containing Proteins[a]

| protein | $\tau_0$ (ns) | $k_q^A$ ($\times 10^{-9}$ $M^{-1}$ $s^{-1}$) | $k_q^S$ ($\times 10^{-9}$ $M^{-1}$ $s^{-1}$) | $\gamma_{S/A}$ |
|---|---|---|---|---|
| aldolase[b] | 2.3 | 0.10 | 0.01 | 0.10 |
| chymotrypsin[b] | 2.1 | 0.50 | 0.12 | 0.25 |
| β-trypsin[b] | 2.3 | 1.0 | 0.27 | 0.27 |
| β-lactoglobulin[c] | 1.9 | 0.53 | 0.15 | 0.28 |
| pepsin[b] | 6.0 | 1.3 | 0.53 | 0.41 |
| lysozyme[d] | 1.9 | 1.8 | 1.1 | 0.64 |

[a] Experiments were performed at 25 °C in the following buffers: 0.033 M phosphate buffer, pH 7.0, aldolase, chymotrypsin, β-trypsin, pepsin, and lysozyme; 0.1 M sodium acetate buffer, pH 4.5, β-lactoglobulin. All quenching data were from steady-state measurements. Stern–Volmer plots are either linear or downward curving for these proteins [see Eftink & Ghiron (1976a)]. [b] Fluorescence lifetimes from single-component fits of impulse–decay data; see Eftink & Ghiron (1976a). [c] Lifetime determined by phase-modulation fluorometry at 30 MHz; value given is the average from the phase lag and degree of modulation methods. [d] Lifetime taken from Formoso & Forster (1975).

The Stern–Volmer $F_0/F$ plots for the acrylamide and succinimide quenching of these proteins were found to be linear or slightly upward curving [such upward curvature being indicative of static quenching; see Eftink & Ghiron (1976a)]. We have attempted to analyze for the static and dynamic components, as described elsewhere (Eftink & Ghiron, 1976a). The static constants, when significant, are given in Table III. For some proteins, including parvalbumin, melittin monomer and tetramer, and adrenocorticotropin, we have also obtained quenching constants from fluorescence lifetime data ($\tau_0/\tau$ plots), which yield only the dynamic quenching component. These data are also given in Table III.

We find the $k_q^A$ for acrylamide quenching of the single Trp proteins to range from $0.2 \times 10^9$ to $4.0 \times 10^9$ $M^{-1}$ $s^{-1}$, a 20-fold span of rate constants. The range of $k_q^S$ for succinimide quenching is found to be even larger, being from $0.03 \times 10^9$ to $2.5 \times 10^9$ $M^{-1}$ $s^{-1}$. In other words, $\gamma_{S/A}$ is found to range from ~0.1 to ~0.7 for this set of proteins. Obviously succinimide appears to be a more discriminating quencher of Trp fluorescence in proteins than acrylamide. It appears that Trp residues that are relatively buried in acrylamide (low $k_q^A$) are

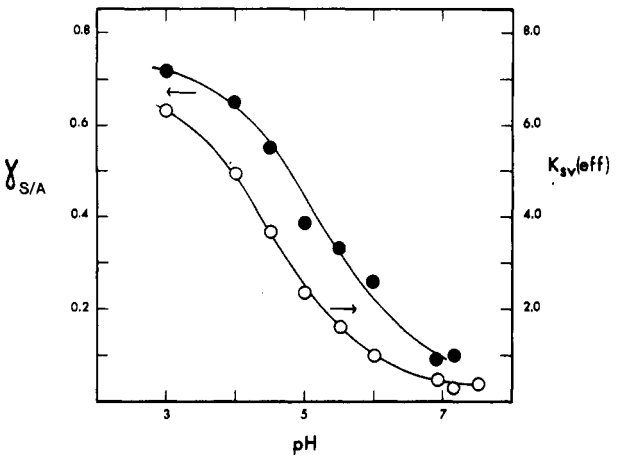


FIGURE 2: pH dependence of $\gamma_{S/A}$ and the effective $K_{sv}$ for acrylamide quenching of aldolase at 25 °C in a buffer containing 0.003 M sodium phosphate and 0.1 M NaCl.

even less accessible to succinimide.

*(B) Multi-Tryptophan-Containing Proteins.* We have also studied several multi-Trp-containing proteins, the data for which are presented in Table IV. Again $\gamma_{S/A}$ is found to range from as low as 0.1 for aldolase to 0.6 for lysozyme.

The fluorescence of these proteins is expected to be heterogeneous. In quenching studies, a heterogeneity in Trp accessibility is also to be expected due to variation in both the quenching rate constant ($k_{qi}$) and the fluorescence lifetime ($\tau_i$) for the individual Trp residues. For the proteins pepsin, β-lactoglobulin, and aldolase, however, the difference in Trp accessibility among the constituent residues apparently is not great, since approximately linear Stern–Volmer plots are obtained for these proteins. The Stern–Volmer constants determined from such plots are effective values (Eftink & Ghiron, 1976a), and when an average fluorescence lifetime is used for such proteins, an effective quenching rate constant can be calculated, as given in Table IV.

For lysozyme and chymotrypsin, downward-curving Stern–Volmer plots are obtained, indicating a high degree of heterogeneity in the accessibility of the Trp residues in these proteins. For these cases, we report in Table IV an effective quenching rate constant derived from the initial $K_{sv}$(eff) of the Stern–Volmer plot. Such a $k_q$(eff) corresponds to the quenching of the more solvent-exposed Trp residues in the protein (Eftink & Ghiron, 1976a).

Despite the complications due to heterogeneity in these proteins, the $\gamma_{S/A}$ values fall in the same range as that found for single Trp proteins.

Aldolase is a protein of particular interest since the Trp residues in this protein are deeply buried and its $\gamma_{S/A}$ value is extremely low. Aldolase is known to undergo subunit dissociation below pH 4 (Stellwagen & Schachman, 1962), and we have studied its acrylamide and succinimide quenching as a function of pH. As shown in Figure 2, we find that not only the effective $K_{sv}$ for acrylamide quenching increases as the subunits of aldolase dissociate but also the value of $\gamma_{S/A}$ increases from 0.1 at pH 7 to 0.7 at pH 3.0. The increase in both $\gamma_{S/A}$ and $K_{sv}$(eff) with decreasing pH argues that the change in $K_{sv}$(eff) is due to a change in the degree of solvent exposure of the tryptophan residues (i.e., a change in $k_q$ with pH), rather than just a change in fluorescence lifetime with pH.

## Discussion

As mentioned in the introduction, our purpose in comparing succinimide and acrylamide quenching data for proteins was to investigate the possible dependence of the quenching process on the size of the quencher. Our early results with proteins (see Tables III and IV) seemed to reveal such a quencher size dependence by showing succinimide to be a poorer quencher than acrylamide, especially for buried Trp residues. The prima facie interpretation of this size dependence was that it supports a kinetic mechanism in which the quenching of buried tryptophan residues occurs by the dynamic, stepwise penetration of the quencher through the protein matrix. As we continued our studies with succinimide, particularly with the investigation of the solvent dependence of the quenching reaction, we obtained information that requires us to modify the above interpretation. As we will argue below, the fact that succinimide is a poorer quencher than acrylamide for certain Trp residues in proteins not only may be due to a critical size dependence for a penetration process but also may be due to the effect of the local microenvironment of a tryptophan residue on the efficiency of the quenching by succinimide.

*Model System Studies.* The $k_q^A$ values for acrylamide quenching given in Tables I and II indicates that acrylamide is an efficient quencher of indole fluorescence under all the circumstances investigated. The $k_q^A$ values for various indole derivatives in water are all found to be approximately the value expected for a diffusion-controlled reaction. As we have discussed elsewhere (Eftink & Ghiron, 1976b), using the Smoluchowski equation one can calculate a value of $7.4 \times 10^9$ $M^{-1}$ $s^{-1}$ for the diffusion-limited rate constant ($k_d$) for collision between two molecules the size of indole and acrylamide in water at 298 K. The calculated $k_d$ value agrees excellently with the observed $k_q$ value, indicating that the efficiency, $\gamma$, of acrylamide quenching is unity (i.e., $\gamma = k_q/k_d$). Other evidence that acrylamide is a unitary efficient quencher has been given elsewhere (Eftink & Ghiron, 1976b).

In acrylamide quenching studies in different solvents (Table II), $k_q^A$ is also found to be approximately equal to the diffusion-limited value, when one considers the viscosity differences among the solvents. That is, the ratio $k_q^A/\eta$, where $\eta$ is the bulk viscosity of the solvent, is $(7.2 \pm 1.6) \times 10^9$ $M^{-1}$ $s^{-1}$ $cP^{-1}$ for all the solvents studied. Figure 3 illustrates the direct dependence of $k_q^A$ on $1/\eta$ for these solvents. We are led to conclude that $\gamma = 1$ for the acrylamide quenching of indole fluorescence in all solvents.

Quenching of indole fluorescence in water by succinimide is found not to be completely efficient, however. The $k_q^S$ values of $\sim 5 \times 10^9$ $M^{-1}$ $s^{-1}$ in Table I are less than the value of $7.4 \times 10^9$ $M^{-1}$ $s^{-1}$ calculated for $k_d$ by the Smoluchowski equation for the frequency of collision between molecules the size of indole and succinimide. (Note that although succinimide is approximately 20% larger in size than acrylamide, its larger encounter radius cancels its smaller diffusion coefficient to give a value of $k_d$ that is almost the same as that for acrylamide.) Succinimide quenching in water is thus only about a 65–70% efficient process [$\gamma = k_q^S(\text{indole})/k_d$]. Since, as argued above, acrylamide is a completely efficient quencher under all conditions studied, a convenient expression for the quenching efficiency of succinimide is $\gamma_{S/A} = k_q^S/k_q^A$. As listed in Table I, $\gamma_{S/A}$ is found to be 0.60–0.85 for all indole derivatives in aqueous solution. (For other types of fluorophores, such as phenol and naphthalene, $\gamma_{S/A}$ has different values, as shown in Table I.)

The slight inefficiency of sucinimide quenching in aqueous solution is, itself, a minor factor to consider in interpreting studies with proteins. That is, one would expect succinimide quenching rate constants to be only 70% as large as the value for acrylamide, even for solvent-exposed Trp residues. How-

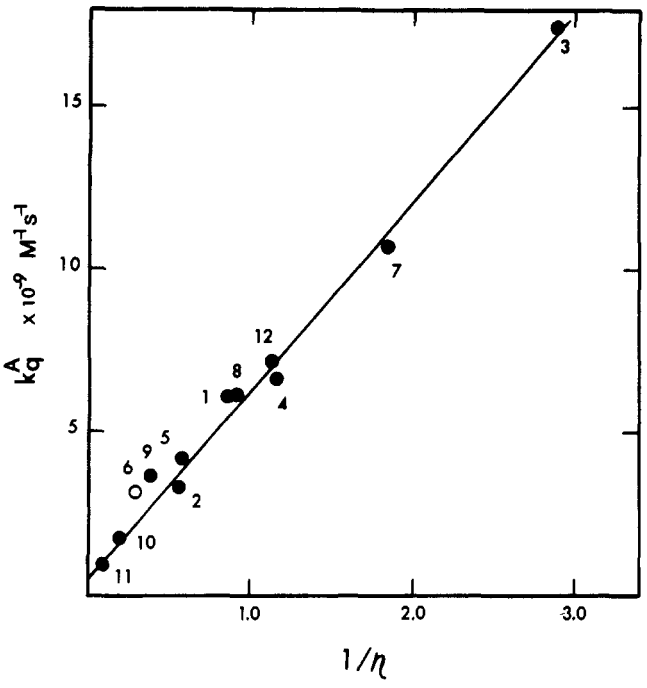


FIGURE 3: Dependence of the rate constant for acrylamide quenching of indole fluorescence on solvent viscosity. The solvents used and their viscosity (in centipoise) are as follows: (1) dioxane, 1.17; (2) dimethyl sulfoxide, 1.76; (3) acetonitrile, 0.345; (4) dimethylformamide, 0.85; (5) *N*-methylformamide, 1.70; (6) formamide, 3.42; (7) methanol, 0.55; (8) ethanol, 1.10; (9) 2-propanol, 2.63; (10) *tert*-butyl alcohol, 5.22; (11) ethylene glycol, 10.8; (12) water, 0.89. The point for formamide was measured with 5-methoxyindole as the fluorophore, due to the strong absorbance of this solvent below 300 nm. Temperature, 25 °C.

ever, our solvent dependence studies revealed that the inefficiency of succinimide quenching is of greater consequence.

Quenching of indole fluorescence by succinimide was found to be much more dependent on the nature of the solvent than quenching by acrylamide. (See the $\gamma_{S/A}$ values in Table II.) This solvent dependence is complicated in that there is not a direct dependence of $\gamma_{S/A}$ on such bulk solvent properties as the dielectric constant, $\epsilon$, viscosity, or polarity, as described by such polarity scales as the Z scale (Kosower, 1968), the $E_T(30)$ scale (Reichardt & Dimroth, 1968), the $\alpha$ scale (Taft & Kamlet, 1976), or the $\pi$ scale (Kamlet et al., 1977). To mention a few examples, the $\gamma_{S/A}$ values for the solvents dioxane, dimethyl sulfoxide ($Me_2SO$), acetonitrile, and dimethylformamide are all between 0 and 0.2, even though the dielectric constant of the latter three solvents is much greater than that of dioxane. Also, the $\gamma_{S/A}$ values for *N*-methylformamide is relatively small, despite the fact that the dielectric constant for this solvent is very large.

The solvent dependence of $\gamma_{S/A}$ can best be rationalized as a dependence on whether the solvent is protic or aprotic. All aprotic solvents (dioxane, $Me_2SO$, DMF, and acetonitrile) are found to have $\gamma_{S/A} < 0.2$. All protic solvents are found to have $\gamma_{S/A} > 0.2$. For the several alcohols studied, $\gamma_{S/A}$ values fall in the range of 0.5–0.7. Of particular note is the solvent series formamide, *N*-methylformamide, and *N*,*N*-dimethylformamide where $\gamma_{S/A}$ goes from ~0.4 to ~0 (for quenching of 5-methoxyindole) as the solvent becomes more aprotic.

We can only speculate as to the reason for this difference in the solvent dependence of acrylamide and succinimide quenching. In general, one can argue from eq 1 that the solvent independence of $k_q/\eta$ for acrylamide indicates that $k_i$ must be much larger than $k_{-d} + k_f$ in all solvents (i.e., so that $k_q = k_d$). For succinimide, $k_i$ apparently is not significantly larger than $k_{-d} + k_f$, and any solvent dependence of $k_i$, the rate constant for the deactivation of the excited-state encounter complex, will show up in the $\gamma$ value for succinimide quenching (and hence will also be seen in the $\gamma_{S/A}$ values). The question then becomes what is the mechanism of the deactivation process and why is it solvent dependent for succinimide. (Note that $k_i$ may also be solvent dependent for acrylamide, but so long as $k_i > k_{-d} + k_f$, no solvent dependence will be observed.)

The mechanism of quenching by most quenchers is really not known for certain. Acrylamide, like many quenchers, is believed to quench indole fluorescence by a mechanism involving some degree of electron transfer between the excited state and the quencher. Indirect evidence for this mechanism comes from the correlation noted by Steiner & Kirby (1969) between the rate constant for quenching by several quenchers, including acrylamide, and the rate constant for the scavenging of hydrated electrons by these quenchers. Succinimide and *N*-methylsuccinimide have also been reported to be excellent scavengers of hydrated electrons (Simic & Hayon, 1973), thus suggesting that they also quench by an electron transfer mechanism. For such a mechanism, it is not unreasonable that the favorable solvation of the transient charge-transfer complex would facilitate its formation. There have been several studies of the solvent dependence of fluorescence quenching reactions (Okada et al., 1970; Selinger & McDonald, 1972; O'Connor & Ware, 1979; Ahmad & Durocher, 1981). The present solvent dependence studies of acrylamide and succinimide quenching are not unusual, even though we cannot claim at this time to fully understand the basis for the observed solvent dependence. For our present purpose of applying acrylamide and succinimide quenching to studies with proteins, however, we can phenomenologically state that *the fluorescence quenching of indole by succinimide is much more solvent dependent than quenching by acrylamide* and that, in particular, *the efficiency of succinimide quenching decreases as the solvent is changed from being protic to aprotic.*

*Studies with Proteins.* Let us now examine the fluorescence quenching studies with proteins in light of the above solvent dependence studies. In Tables III and IV, we find $\gamma_{S/A}$ to range from 0.1 for proteins containing relatively buried Trp residues to 0.7 for proteins with solvent-exposed residues. As we have discussed previously (Eftink & Ghiron, 1976a), $k_q^A$ for acrylamide quenching can be used as a measurement of the kinetic exposure of Trp residues in proteins. In Figure 4, we have plotted $\gamma_{S/A}$ vs. $k_q^A$ for all the proteins in Tables III and IV to illustrate the correlation between $\gamma_{S/A}$ and residue exposure as measured by $k_q^A$. A smooth curve can be drawn through the points. Some scatter is observed, which is not surprising in view of the widely different protein structures involved (i.e., random coils, small globular proteins, and oligomeric proteins) and the possibility of having occasional specific protein–quencher interactions (see the legend of Table III for a comment on human serum albumin). In general, however, the body of data clearly shows a correlation between $\gamma_{S/A}$ and $k_q^A$.

In view of our solvent dependence studies, we now realize that there are two possible explanations for the decrease in $\gamma_{S/A}$ with decreasing Trp exposure. The decrease in $\gamma_{S/A}$ may be a result of (a) steric limitation of the diffusion of succinimide due to its larger size than acrylamide or (b) the effect of the local microenvironment around a Trp residue on the efficiency of succinimide quenching. This dichotomous interpretation may at first glance seem disappointing, but if we restate the reasons for low $\gamma_{S/A}$ values in another way, we see that important insights can be gained from these studies. *For*

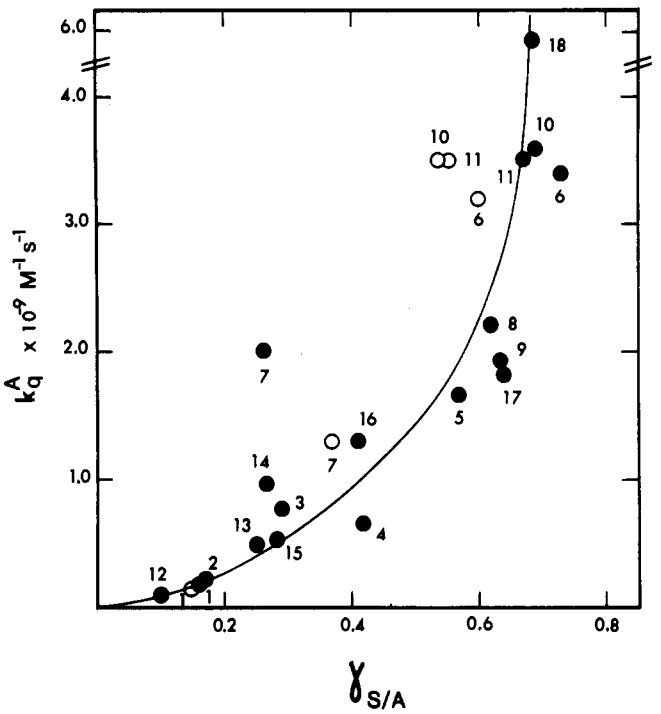


FIGURE 4: Plot of the apparent rate constant for acrylamide quenching of the fluorescence of several single and multi-tryptophan-containing proteins vs. $\gamma_{S/A}$, the relative efficiency of succinimide and acrylamide for the quenching of the protein. Closed circles were obtained from fluorescence intensity data; open circles were from fluorescence lifetime data. The proteins studied are as follows: (1) cod parvalbumin; (2) ribonuclease $T_1$; (3) nuclease; (4) fd phage; (5) monellin; (6) melittin (monomer, in 0.01 M Tris buffer, pH 7.0); (7) melittin (tetramer, in 0.45 M sodium phosphate buffer, pH 7.0); (8) phospholipase $A_2$; (9) chorionic gonadotropin; (10) adrenocorticotropin; (11) glucagon; (12) aldolase; (13) chymotrypsin; (14) $\beta$-trypsin; (15) $\beta$-lactoglobulin; (16) pepsin; (17) lysozyme; (18) *N*-acetyltryptophanamide. See Tables III and IV for conditions. The curve drawn has no theoretical basis.

*proteins in which $\gamma_{S/A}$ is low, the quenching process must be occurring in a nonaqueous microenvironment, within the globular protein structure.* This general conclusion encompases both of the aforementioned explanations for low $\gamma_{S/A}$ values. If a low $\gamma_{S/A}$ value reflects the quencher size dependence of the penetration of quenchers into a protein (see the introduction), then quenching is occurring within the protein. Alternately, low $\gamma_{S/A}$ values may be due to the fact that quenching is taking place in the relatively apolar interior of a protein. Actually, both reasons may be partially responsible for low $\gamma_{S/A}$ values. We note that the lowest $\gamma_{S/A}$ values measured, for aldolase, ribonuclease $T_1$, and parvalbumin, are intermediate between the $\gamma_{S/A}$ values found for the quenching of indole in dioxane ($\epsilon$ = 2.2, aprotic) and *N*-methylformamide ($\epsilon$ = 182, monoprotic), so that the interior of such proteins cannot be said to be completely aprotic.

The succinimide/acrylamide studies also allow us to discount the quenching mechanism involving local unfolding of the protein, at least for the proteins studied in this work. According to this mechanism (Calhoun et al., 1983), the protein (or parts of the protein) is considered to be in equilibrium with "open" conformations in which the Trp residues are solvent exposed (the opening equilibrium constant, $K_{op}$, being $k_{op}/k_{cl}$ as defined by the following equation):

$$P^*_{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} P^*_{open} \xrightarrow{[Q]k_d} P_{open} + Q + \Delta \qquad (3)$$

The fluorescence of Trp residues in the open conformations is then quenched with a diffusion-limited rate constant, $k_d$ (or, in general, $\gamma k_d$ for cases in which the quencher is inefficient, where $\gamma$ is the efficiency for quenching the solvent-exposed residue). The apparent bimolecular quenching rate constant for such a mechanism is

$$k_q = \frac{\gamma k_d k_{op}}{k_{cl} + k_d[Q]} \qquad (4)$$

Further, as pointed out by Calhoun et al. (1983), since quenching reactions generally appear to be first order in [Q] (or, in other words, $k_q$ is not dependent on [Q]), $k_{cl}$ must be greater than $k_d$[Q], so that

$$k_q = \gamma k_d K_{op} \qquad (5)$$

According to this mechanism, the apparent rate constant for quenching a buried Trp residue by a particular quencher will depend on the population of the open conformation (given by $K_{op}$) and on the efficiency of quenching a solvent-exposed Trp residue by the quencher. This mechanism predicts, therefore, that when the quenching of a buried Trp residue by a pair of quenchers such as succinimide and acrylamide is compared, the ratio of $k_q$ values will be just equal to the ratio of efficiencies for the two quenchers for the quenching of an aqueous indole solution.

Clearly the ratio $k_q^S/k_q^A$ ($=\gamma_{S/A}$) we have found for the proteins studied in this work is much lower, in many cases, than that found for quenching aqueous indole. The low values of $\gamma_{S/A}$ we see for aldolase, ribonuclease $T_1$, parvalbumin, etc. cannot be explained by the local unfolding model, unless one postulates that the unfolding events leave the Trp residues surrounded by apolar amino acid side chains, in which case such a view of an unfolding process would approach what is generally considered to be the penetration mechanism.

The present discussion of unfolding vs. penetration mechanisms for fluorescence quenching is, of course, reminiscent of the debate concerning the mechanism of isotope exchange of buried hydrogens in proteins (Englander et al., 1972, 1980; Woodward et al. 1979, 1982). The comparison of an inefficient and solvent dependent quencher (succinimide) with an efficient quencher (acrylamide) is analogous to studies of the pH dependence of the isotope exchange kinetics in proteins in that both provide evidence that the reaction being investigated takes place primarily in the nonaqueous interior of a protein.

As argued above, the $\gamma_{S/A}$ values we find are consistent with a penetration mechanism. These studies thus provide additional support, to go along with studies showing the $k_q^A$ for acrylamide quenching to be relatively independent of bulk viscosity and to have an activation enthalpy much different from that for diffusion through the bulk solvent, for the quencher penetration mechanism for certain proteins (Eftink & Ghiron, 1977).

Calhoun et al. (1983) have recently published phosphorescence quenching data with alcohol dehydrogenase and alkaline phosphatase, using various polar quenchers including acrylamide, which they claim to be more consistent with an unfolding mechanism than with a penetration mechanism. The basis for their argument is that the apparent rate constant for phosphorescence quenching of the buried residues by these quenchers is about the same for most of the quenchers studied and is much smaller than the anticipated value of $k_d$. They conclude that these findings are more consistent with an unfolding than a penetration model. We acknowledge that there may be some deeply buried Trp residues in certain proteins which may not be accessible to small polar quenchers by a penetration mechanism and that an unfolding mechanism may be required to quench such residues. Most of the single

Trp-containing proteins that have been studied are relatively small which may limit the degree to which their Trp residues can be dimensionally and energetically buried. Azurin from *Pseudomonas fluorescens* (Eftink & Ghiron, 1976a) and asparaginase from *Escherichia coli* (M. R. Eftink, unpublished results) are worth noting as proteins whose fluorescence cannot be appreciably quenched by acrylamide at room temperature. Deeply bured Trp residues in large multi-Trp proteins may escape investigation due to the heterogeneity of the protein's fluorescence.

We must offer one counterpoint to the arguments of Calhoun et al. The apparent quenching rate constants they measured are actually equal to $\gamma k'_d$ (where $k'_d$) is for diffusion through the protein) or, according to the unfolding model, $\gamma k_d K_{op}$. That is, the efficiency of phosphorescence quenching by the set of quenchers used must be considered. In their interpretation, Calhoun et al. apparently assumed the efficiency of indole phosphorescence quenching to be unity for all quenchers for all indole environments. Their conclusions rest heavily on this assumption. For example, if the efficiency of acrylamide quenching of the indole triplet state is found to be very low in apolar solvents, one could argue that the low rate constant reported by Calhoun et al. for the phosphorescence quenching of alcohol dehydrogenase and alkaline phosphatase by acrylamide is still consistent with a penetration model, with the low rate constant reflecting the relatively apolar microenvironment about the Trp residue in the protein's interior. We note in regard to this point that Pepmiller et al. (1983) have recently reported a rate constant of $2.8 \times 10^9$ $M^{-1}$ $s^{-1}$ for the acrylamide quenching of the triplet state of 1-methylindole in water at room temperature. This rate constant is only about 40% of the diffusion-controlled value, indicating that acrylamide quenching of the triplet state of this indole derivative is not completely efficient in water. Additional information is needed on the quenching of the indole triplet state in other solvents before one can unambiguously interpret phosphorescence quenching studies with proteins.

Finally, we note that succinimide should be a useful quencher in studies with proteins which contain tyrosine and no tryptophan (class A proteins). The lower absorbance of succinimide, as compared to acrylamide, in the range of 290 nm should minimize screening corrections.

## Acknowledgments

We thank Karen Hagaman, Richard Keeling, and Dr. Roger Mallinson for technical assistance. Also, we thank Dr. F. Walz, Kent State University, for supplying a sample of ribonuclease $T_1$ and Dr. R. Webster, Duke University, for supplying a sample of fd phage.

**Registry No.** Acrylamide, 79-06-1; succinimide, 123-56-8; ribonuclease $T_1$, 9026-12-4; aldolase, 9024-52-6; glucagon, 9007-92-5; adrenocorticotropin, 9002-60-2; indole, 120-72-9; 5-methoxyindole, 1006-94-6; 1-methylindole, 603-76-9; 3-methylindole, 83-34-1; L-tryptophan, 73-22-3; *N*-acetyl-L-tryptophanamide, 2382-79-8; *N*-acetyl-L-tryptophan, 1218-34-4; phenol, 108-95-2; naphthalene, 91-20-3; nuclease, 9026-81-7; melittin, 37231-28-0; phospholipase $A_2$, 9001-84-7; chorionic gonadotropin, 9002-61-3; chymotrypsin, 9004-07-3; β-trypsin, 9002-07-7; pepsin, 9001-75-6; lysozyme, 9001-63-2.

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# Cross-Linking Agents Induce Rapid Calcium Release from Skeletal Muscle Sarcoplasmic Reticulum†

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ABSTRACT: The passive permeability of skeletal muscle sarcoplasmic reticulum vesicles to $Ca^{2+}$ ions is drastically increased upon addition of the oxidizing agent cupric phenanthroline. The permeability change, which occurs very rapidly, is partially reversed by reducing agents and cannot be explained by a direct effect of cupric phenanthroline on the lipid moiety of the membranes. The rapid efflux phenomenon is due to protein cross-linking induced by the cupric phenanthroline catalyzed oxidation of SH groups to disulfide bridges. Similar effects are also induced by cross-linking sarcoplasmic reticulum proteins with dithiodipropionic acid disuccinimido ester. The rapid $Ca^{2+}$ efflux is inhibited by micromolar concentrations of lanthanum and by labeling the $Ca^{2+}$-ATPase with dicyclohexylcarbodiimide. These observations suggest that $Ca^{2+}$ channels are formed by chemical modification of the ATPase. The $Ca^{2+}$ permeability rate of sarcoplasmic reticulum obtained after cross-linking is compatible with the requirements of $Ca^{2+}$ release in vivo. The possibility that $Ca^{2+}$-ATPase oligomers might mediate the release process is discussed.

The fast regulation of the cytosolic free $Ca^{2+}$ concentration in fast skeletal muscle cells is assured mainly by the action of a highly specialized membrane system, the sarcoplasmic reticulum (SR).[1] Fast removal of $Ca^{2+}$ ions from the cytosol, which induces relaxation, occurs via an efficient $Ca^{2+}$-pumping ATPase which is the major protein component of SR membranes. Many details of the mechanism of action of this ATPase have been clarified [for a review, see Tada et al. (1978) and deMeis (1981)]. The enzyme has now became a model of reference for the study of energy conversion by other ion-motive ATPases. However, the phenomenon which precedes contraction of the myofilaments, i.e., the factors which induce the rapid $Ca^{2+}$ outflow from the intracellular $Ca^{2+}$ stores [mainly from the terminal cisternae of the SR (Huxley & Taylor, 1958)], is still poorly understood. It seems plausible that the action potential at the sarcolemma and its invaginations (T system) is transmitted either via electrical or by chemical coupling to the SR cisternae, which respond by opening $Ca^{2+}$ channels. Experiments with both isolated skinned fibers and fragmented SR vesicles have shown that a multitude of manipulations can induce contractures which are correlated to a massive release of $Ca^{2+}$ ions from the SR network [for a review, see Endo (1977)]. The physiological significance of these experiments, however, is still a matter of speculation. An interesting hypothesis was formulated by Vanderkooi et al. (1977), who suggested that a reversible transient association of several ATPase molecules could provide the structural basis for the rapid $Ca^{2+}$ release occurring during excitation.

In this paper, we will present evidence that, indeed, accompanying the formation of ATPase oligomers by means of covalent cross-linking a dramatic increase of the passive permeability of SR vesicles occurs. The possibility of a noncasual link between the $Ca^{2+}$-release phenomenon and the presence of ATPase oligomers will be discussed.

## Materials and Methods

*Materials.* *N,N'*-[$^{14}$C]Dicyclohexylcarbodiimide (DCCD) was obtained from the Commisariat à l'Energie Atomique (CEA, France); 3,3'-dithiodipropionic acid disuccinimido ester (DSP) was obtained from Fluka, AG Buchs (Switzerland). All other reagents were of the highest grade commercially available. SR vesicles were isolated from white muscles of the hind legs of rabbits according to Eletr & Inesi (1972). The preparation obtained, which consisted of fragments derived from both the longitudinal and cisternal systems, was used in most of the experiments. When required, SR vesicles were subfractionated on a linear sucrose gradient (0.85-1.6 M) after centrifugation in a swinging bucket rotor (Beckman SW 27) at 25 000 rpm for 16 h (Myiamoto & Racker, 1981) in order to separate the light fraction (0.9-1.05 M) from the heavy vesicles (1.2-1.4 M sucrose). Cross-linking of SR proteins was induced by either cupric phenanthroline (CuPh) or DSP. SR vesicles (usually 1 mg/mL) were incubated at room temperature in 100 mM NaCl (or KCl), 20 mM MOPS, pH 7, 20 $\mu$M $CaCl_2$, and various amounts of 1:3 $CuSO_4$:1,10-orthophenanthroline. At various time intervals, oxidation was either stopped in SDS sample buffer supplemented with 10 mM EDTA and 50 mM *N*-ethylmaleimide and samples were analyzed by gel electrophoresis or stopped by dilution in an ice-cold medium (100 mM NaCl, 20 mM MOPS, pH 7, and

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[1] Abbreviations: SR, sarcoplasmic reticulum; MOPS, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CuPh, cupric phenanthroline; DTT, 1,4-dithiothreitol; DSP, 3,3'-dithiodipropionic acid disuccinimido ester; DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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