

- Horwitz, J., and Heller, J. (1971), *Biochemistry* 10, 1402.
- Hubbard, R., and Kropf, A. (1958), *Proc. Nat. Acad. Sci. U. S. A.* 44, 130.
- Kägi, J. H. R., Li, T.-K., and Vallee, B. L. (1971), *Biochemistry* 10, 1007.
- Kirkwood, J. G. (1937), *J. Chem. Phys.* 5, 479.
- Kito, Y., Azuma, M., and Maeda, Y. (1968), *Biochim. Biophys. Acta* 154, 352.
- Matthews, R. G., Hubbard, R., Brown, P. K., and Wald, G. (1963), *J. Gen. Physiol.* 47, 215.
- Mommaerts, W. F. H. M. (1969), in *The Retina: Morphology, Function, and Clinical Characteristics*, Straatsma, B. R., Hall, M. O., Allen, R. A., and Crescitelli, F., Eds., Los Angeles, Calif., University of California Press, pp 225-234.
- Moscowitz, A. (1960), in *Optical Rotatory Dispersion*, Djerassi, C., Ed., New York, N. Y., McGraw-Hill.
- Schellman, J. A. (1968), *Accounts Chem. Res.* 1, 144.
- Shichi, H. (1970), *Biochemistry* 9, 1973.
- Takezaki, M., and Kito, Y. (1967), *Nature (London)* 215, 1197.
- Tomita, T. (1970), *Quart. Rev. Biophys.* 3, 179.
- Ulmer, D. D., and Vallee, B. L. (1965), *Advan. Enzymol.* 27, 37.
- Wald, G. (1968), *Nature (London)* 219, 800.
- Williams, T. P. (1966), *Vision Res.* 6, 293.
- Williams, T. P. (1970), *Vision Res.* 10, 525.

Solute Perturbation of Protein Fluorescence. The Quenching of the Tryptophyl Fluorescence of Model Compounds and of Lysozyme by Iodide Ion*

Sherwin S. Lehrer

ABSTRACT: The effect of iodide on the tryptophyl fluorescence of model compounds and of lysozyme was studied in order to evaluate the factors that determine the use of iodide as a selective quencher of the fluorescence of tryptophyl side chains of proteins exposed to solvent. The results with the model compounds indicate the involvement of a collisional quenching mechanism due to the agreement with the Stern-Volmer law and the proportionality of the quenching constant with T/η for indole-3-acetamide. Bimolecular rate constants, k_3 , calculated from measured quenching constants using available lifetime data are equal to, greater than, or less than $4-6 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$ for uncharged, positively charged, and nega-

tively charged tryptophyl compounds, respectively. A modified version of the Stern-Volmer law was calculated for a fluorophor population with different quantum yields and quenching constants. This formulation allows the calculation of the effective quenching constant from the intercept and the slope at low iodide concentration of a $F_0/\Delta F$ vs. $1/(I^-)$ plot. Data obtained for lysozyme indicate that for the native protein about one-half the tryptophyl fluorescence is accessible at pH 5.3 whereas all of the tryptophyl fluorescence is accessible in 6 M Gdn·HCl. Information regarding the presence of charged groups near tryptophyl side chains was obtained for lysozyme by studying the dependence of the quenching on pH.

In a preliminary study it was shown that a large fraction of the tryptophyl fluorescence of lysozyme in aqueous solution was quenched by low concentrations of iodide ion (Lehrer, 1967). It was concluded from a study of the magnitude of the quenching of fluorescence and the character of the difference fluorescence spectrum produced in the presence and absence of substrate that the fluorescence of tryptophyls exposed to solvent and located in the substrate binding site was preferentially quenched by iodide. It appeared that this technique, which can be called solute perturbation of protein fluorescence, could be used as a probe of fluorophor exposure in proteins in a manner analogous to the technique of solvent perturbation of protein absorption (Herskovits and Laskowski, 1960; Laskowski, 1966).

More recently, studies by other workers have used bromate (Winkler, 1969) and iodide (Arrio *et al.*, 1970) to quench extrinsic fluorescence (Teale and Badley, 1970). Oxygen has also been used as a quencher of pyrenebutyric acid bound to proteins (Vaughan and Weber, 1970). Burstein (1968a) has also independently studied the quenching of tryptophyl fluorescence in model compounds by iodide.

In order to learn more about the quenching mechanism and the factors which determine fluorophor exposure, various tryptophyl model compounds and a model protein, lysozyme, were used in the present study. The results of the model compound study provide evidence for a mechanism that follows the classical Stern-Volmer law (1919), predominantly involving collisional quenching, and illustrate the importance of local charge and solvent viscosity. The quenching of lysozyme fluorescence by iodide also appears to follow a similar mechanism because of the agreement obtained with a modified version of the Stern-Volmer law which was calculated for a heterogeneous distribution of fluorophors in a protein. Effective Stern-Volmer quenching constants and values for the fractional accessible fluorescence were obtained for lyso-

* From the Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114, and from the Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115. Received April 22, 1971. This work was supported by grants from the National Institutes of Health (AM 11677 and HE 05811) and the Massachusetts Heart Association (816).

zyme in 6 M Gdn·HCl,¹ 8 M urea, and in aqueous solution at different pH's using the modified Stern-Volmer law. Values obtained are consistent with information regarding accessibility obtained by other methods.

Experimental Section

Materials. The following high-purity compounds were used as obtained from Mann Research Laboratories, New York, N. Y.: indole-3-acetic acid, indole-3-propionic acid, indole-3-butyric acid, indole-3-acetamide, *N*-Ac-L-TrpNH₂, L-TrpOEt, Gdn·HCl, and urea. L-Trp (Cyclo Chemical Corp., Los Angeles), KI, Na₂S₂O₃, citric acid, and NaCl (Fisher Scientific Co., Freehold, N. J.) were all of high purity and used as obtained. Indole (Fisher) and skatole (3-methyl-indole) (Mann) were recrystallized from methanol containing Norit A (Matheson Coleman & Bell, Rutherford, N. J.). Hepes buffer was used as obtained from Calbiochem (Los Angeles). Poly(Glu⁹⁹Trp¹) and poly(Lys⁹⁷Trp³) were high molecular weight random sequence copolymers kindly supplied by Dr. G. Fasman. Lysozyme from two different sources were used (twice crystallized from Worthington Biochemical Corp., Freehold, N. J., and six-times crystallized from Miles Laboratories, Elkhart, Ind.). Both preparations gave similar results. Ac₃GlcN was kindly supplied by Dr. J. Rupley and glycol chitin was obtained from Miles Laboratories.

Methods. Quenching measurements at constant pH were made on five solutions of a given material containing increasing amounts of KI (0–0.2 M). These were prepared by diluting stock solutions of the model compound, of KI, of NaCl, and of buffer, into volumetric flasks. NaCl was used to keep the ionic strength constant. Stock solutions of the indole compounds were used within a few days of preparation and kept in the dark at 0–5° overnight. A small amount of S₂O₃²⁻ (ca. 10⁻⁴ M) was added to the iodide solution to prevent I₃⁻ formation. This was necessary because I₃⁻ absorbs in the wavelength region of tryptophyl fluorescence (filter effect) and because of possible chemical reaction. The solutions were equilibrated at 25° before the measurements. Stock solutions of lysozyme were routinely filtered through a Millipore filter (HAWP 0.45 μ) before use. pH titrations were performed in the absence and presence of iodide by adding small quantities of 0.5 M HCl to the solution in the cuvet, which contained 2 mM Hepes and 2 mM citrate, originally pH 8, then measuring the pH and fluorescence.

pH was measured with a Radiometer PHM4c meter standardized at pH 4 and 7. Fluorescence spectra and intensities were measured by exciting at 280 nm or longer. In most cases no corrections for iodide absorption were necessary. The fluorescence of a reference (usually the 0.2 M NaCl–0.0 M KI solution) was measured just before measuring the fluorescence of each solution in order to correct for any exciting lamp fluctuation.

Fluorescence measurements were made with either an Aminco-Bowman spectrofluorometer or an instrument that employs two Jarrell-Ash 0.25-m monochromators, an EMI 9601B photomultiplier, and either a high-pressure 200-W mercury lamp or a 150-W high-pressure xenon lamp. Low temperatures were obtained with a refrigerated water circulator attached to the sample housing. The temperature was measured by inserting a calibrated thermistor into the sample solution.

The activity of lysozyme was determined by the method of Hamaguchi *et al.* (1960). The decrease in viscosity with time caused by hydrolysis of glycol chitin (2 mg/ml) by lysozyme (0.02 mg/ml) in the presence of 0.2 M NaCl or 0.2 M KI in 2 mM citrate (pH 5.5) is the basis of this method. The specific viscosity of glycol chitin solutions in Cannon viscometers at 25° was measured with time after a small volume of lysozyme was added. The slope of the approximately linear viscosity decrease between 1 and 10 min was used as a measure of activity.

The optical rotatory dispersion and circular dichroism spectra of lysozyme (0.95 mg/ml) in 0.2 M NaCl or in 0.2 M KI, 2 mM citrate (pH 5.2) were measured in a 1-cm cell with a Jasco spectropolarimeter. The absorbance of I⁻ prevented measurements below 265 nm.

Difference spectra were either measured with a Cary 15 or a Beckman DK spectrophotometer using mixing cells (Pyrocell, Inc., N. Y.). The total absorption over the wavelengths scanned was always below 2.2. The low-temperature studies were performed with a Beckman DK using a refrigerated sample holder.

Calculations

Model Compounds. The following three processes with associated rate constants describe the deactivation of an excited indole fluorophore (T*) in the absence and presence of quencher (X)



In this scheme radiationless deactivation (process 2) and collisional quenching (process 3) compete with the fluorescence (process 1). With the usual steady-state approximation, the quantum yield in the absence of quencher, F_0 , is given by

$$F_0 = \frac{k_1}{k_1 + k_2} = k_1\tau_0 \quad (4)$$

where $\tau_0 = 1/(k_1 + k_2)$ is the fluorescence lifetime in the absence of quencher. In the presence of quencher the quantum yield, F , becomes

$$F = \frac{k_1}{k_1 + k_2 + k_3(X)} \quad (5)$$

Combination of eq 4 and 5 results in eq 6 embodying the familiar Stern-Volmer (1919) law.

$$F_0/F = 1 + K_Q(X) \quad (6)$$

with $K_Q = k_3\tau_0$.

Values of K_Q were calculated from the slope of F_0/F vs. (I⁻) plots, and bimolecular rate constants, k_3 , which are independent of quantum yield, were calculated from fluorescence lifetimes obtained by other workers. When experimental values were not available, lifetimes were calculated based on the assumption of proportionality of quantum yield with life-

¹ Abbreviations used are: Gdn·HCl, guanidine hydrochloride; Trp, tryptophyl or tryptophan; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Ac₃GlcN, tri-*N*-acetyl-D-glucosamine.

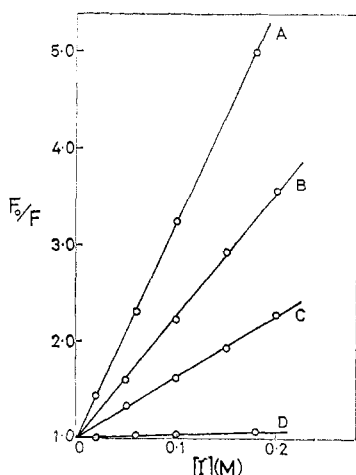


FIGURE 1: The quenching of tryptophyl fluorescence by iodide. (A) Poly(Lys⁹⁵Trp⁹), (B) L-Trp, (C) L-TrpOEt, and (D) poly(Glu⁹⁹-Trp¹). Ionic strength = 0.2; 10 mM Hepes, pH 7.5 (A and D); 2 mM Hepes, pH 7.5 (B); 2 mM citrate, pH 5.5 (C). $\lambda_{\text{excitation}} = 285$ nm (A and D) or 295 nm (B and C), 25°. $A(\lambda_{\text{excitation}}) \approx 0.1$ for all.

time, which appears to be true for the indole compounds studied (Weinryb and Steiner, 1968; E. Kirby and R. F. Steiner, 1970, personal communication; Badley and Teale, 1969; DeLauder and Wahl, 1970). The fluorescence lifetimes are listed in Table I with the results.

A similar law is obeyed by a mechanism of quenching that involves the formation of nonfluorescent complexes (static quenching) (Weber, 1948; Vaughan and Weber, 1970). A test of the dominance of collisional quenching over static quenching is the proportionality of the bimolecular quenching rate constant, k_q , with T/η (Vavilov, 1929; Vaughan and Weber, 1970), where T is the absolute temperature and η is the viscosity. Equivalently, it follows from eq 4 and 6 that a plot of K_q/F_0 vs. T/η should yield a straight line through the origin for collisional quenching since $F_0 \propto \tau_0$. Viscosity values for water were obtained from the Handbook of Chemistry and Physics (1970), and for urea and Gdn·HCl from the work of Kuwahara and Tanford (1966).

Quantum yields were calculated by comparing the integrated fluorescence intensity (measured with a planimeter) of the sample and a solution of L-Trp in H₂O corrected to the same absorption at the exciting wavelength and using the value 0.13 as the quantum yield of L-Trp (Chen, 1967).

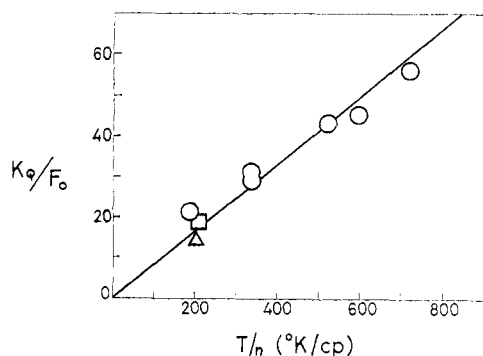


FIGURE 2: Proportionality of quenching rate constant with T/η for indole-3-acetamide. (○) H₂O at different temperatures (0–40°); (□) in 8 M urea; (Δ) in 6 M Gdn·HCl. 2 mM Hepes, pH 7.5. $\lambda_{\text{excitation}} = 280$ nm, $A_{280} \approx 0.1$.

Proteins. In the case of a protein containing many tryptophyl side chains in different environments, the simplest approximation is to consider independent, equally absorbing, fluorophors. Although quantum yields can vary, adherence to the Stern-Volmer law for each fluorophor is assumed. The protein fluorescence quantum yields, in the absence and presence of quencher, F_0 and F , respectively, are then given by

$$F_0 = \frac{\sum F_{0i}}{n}$$

$$F = \frac{\sum F_i}{n} = \frac{1}{n} \sum \frac{F_{0i}}{1 + K_{qi}(X)} \quad (7)$$

where the sums are taken over the n fluorophors of the molecule. The difference is

$$\Delta F = F_0 - F = \frac{1}{n} \left(F_{01} - \frac{F_{01}}{1 + K_{q1}(X)} + F_{02} - \frac{F_{02}}{1 + K_{q2}(X)} + \dots \right) \quad (8)$$

$$= \frac{1}{n} \sum \frac{F_{0i} K_{qi}(X)}{1 + K_{qi}(X)} \quad (9)$$

and

$$\frac{F_0}{\Delta F} = \left[\sum \frac{f_i K_{qi}(X)}{1 + K_{qi}(X)} \right]^{-1} \quad (10)$$

where $f_i = F_{0i}/F_0$. Equation 10 represents the effect of collisional quenching on the protein fluorescence quantum yield in terms of the fluorescence and quenching constants associated with the n fluorescent side chains. It is put in this form because it simplifies under certain experimentally attainable conditions. For example, if there are m accessible fluorophors with the same value of K_q , and $n - m$ inaccessible fluorophors ($K_q = 0$), eq 10 becomes

$$\frac{F_0}{\Delta F} = \frac{1}{(X)f_a K_q} + \frac{1}{f_a} \quad (11)$$

where $f_a = \sum f_i$ summed over m is the fractional maximum accessible protein fluorescence. A certain fraction of the protein fluorescence, f_b , is associated with the buried side chains inaccessible to quencher, and $f_a + f_b = 1$. From eq 11 a plot of $F_0/\Delta F$ vs. $1/(X)$ will yield a straight line of slope $(f_a K_q)^{-1}$ and intercept $1/f_a$, with $K_q = \text{intercept/slope}$. As will be shown below, the fluorescence quenching of lysozyme in 6 M Gdn·HCl appears to follow this behavior with $f_a = 1$.

In general, each exposed side chain may have a different value of K_q associated with the quenching of its fluorescence. A plot of $F_0/\Delta F$ vs. $1/(X)$ will yield a curve of increasing slope as $1/(X) \rightarrow 0$ which extrapolates to $F_0/\Delta F_{\text{max}} = 1/f_a$. The slope at low concentrations will mainly depend on the side chains with large f/K_q . At low quencher concentration eq 10 can be simplified by eliminating terms in $(X)^2$ and higher. For this case

$$\frac{F_0}{\Delta F} = \frac{1}{(X)\sum f_i K_{qi}} + \frac{\sum K_{qi}}{\sum f_i K_{qi}} \quad (12)$$

TABLE I: Quenching of Trp Model Compound Fluorescence by Iodide.

Compound	pH	Ionic Strength ^a	K_Q (M ⁻¹) ^b	Q^b	τ (nsec) ^c	$10^{-9}k_3$ (M ⁻¹ sec ⁻¹)	Mol Charge
Indole	7.4	0.18	34	0.23	4.1, ^d 4.0, ^e 2.7 ^f	8.3	0
	2.1	0.18	28	0.16	(2.9)	9.7	0
Skatole	7.5	0.18	12.0	0.32	9.1, ^d 9.4, ^e 6.4 ^f	1.32	0
	2.1	0.18	5.7	0.17	(4.8)	1.19	0
Indole-3-acetamide	7.5	0.18	29.6	0.23	5.0 ^e	5.9	0
	2.1	0.18	16.3	0.16	(3.5)	4.7	0
Indole-3-acetic acid	7.5	0.18	10.7	0.34	8.7, ^e 2.6 ^f	1.23	—
	2.0	0.18	10.4	0.10	(2.6)	4.0	0
Indole-3-propionic acid	7.5	0.18	11.3	0.34	(8.7)	1.30	—
	2.1	0.18	9.0	0.13	(3.3)	2.7	0
Indole-3-butyric acid	7.5	0.18	11.0	0.32	(8.2)	1.34	—
	2.0	0.18	7.1	0.12	(3.1)	2.3	0
L-Trp	7.4	0.18	11.6	0.13	2.8, ^e 2.6 ^f	4.1	0
	2.1	0.18	8.9	0.065	(1.4)	6.4	+
	11.2	0.18	10.6	0.31	(6.7)	1.58	—
N-Ac-L-TrpNH ₂	7.5	0.18	12.0	0.13	2.6 ^e	4.6	0
	2.1	0.18	8.9	0.10	(2.2)	4.1	0
N-Ac-L-TrpOEt	7.5	0.18	6.0	0.06	(1.3)	4.6	0
	2.1	0.18	5.8	0.05	(1.1)	5.2	0
L-TrpOEt	5.4	0.18	5.9	0.022	0.5 ^e	12	+
Poly(Lys ⁹⁷ Trp ³)	7.5	0.06	46	0.10	(2.2)	21	++
		0.18	22	0.10	(2.2)	10	++
		1.0		0.10	(2.2)		
	2.1	0.18	23	0.10	(2.2)	11	++
	2.1	1.0		0.064	(1.4)		++
	0	0.2		0.013	(0.3)		++
Poly(Glu ⁹⁹ Trp ¹)	7.5	0.06	0	0.09	(1.9)	0	--
		0.18	0.3	0.09	(1.9)	0.2	--
		0.66	0.8	0.09	(1.9)	0.4	--

^a Ionic strength kept constant by the addition of NaCl. ^b 10% uncertainty, $25 \pm 1^\circ$. ^c Italic values were used for the calculation of k_3 if more than one lifetime value available. Values in parentheses were calculated from measured lifetime values of similar components using $\tau \propto Q$; indole acid values calculated from indole-3-acetamide; Trp compound values calculated from L-Trp. ^d Walker *et al.* (1969). ^e E. Kirby and R. F. Steiner, 1969, private communication. ^f Chen *et al.* (1967). ^g Weinryb and Steiner (1968).

By analogy with eq 11, $1/\text{intercept} = \Sigma f_i K_{Qi} / \Sigma K_{Qi}$ can be considered an "effective" fractional maximum accessible fluorescence (f_a)_{eff}, and intercept/slope = ΣK_{Qi} can be considered as "effective" quenching constant (K_Q)_{eff}. As will be discussed below, the fluorescence quenching of lysozyme by iodide in the range 0–0.2 M appears to follow such a dependence with (f_a)_{eff} < 1.

For the lysozyme studies, plots of $F_0/\Delta F$ vs. $1/(I^-)$ were made to determine the effective constants. Relative fluorescence intensities at the wavelength of peak fluorescence were measured, using the fluorescence intensity of lysozyme in 0.2 M NaCl–2 mM Hepes (pH 7.5) as the reference. For this system, it is assumed that the quantum yield is proportional to the peak fluorescence, since the spectral shape does not vary significantly even though there are small wavelength shifts (Lehrer and Fasman, 1967). It is also assumed that changes in fluorescence intensity reflect changes in quantum yield rather than in absorbance, since changes in absorbance caused by changes in pH (Ogasahara and Hamaguchi, 1967; Donovan *et al.*, 1961) and by the presence of iodide were small compared to the observed fluorescence changes.

Results

Model Compounds. The variation of fluorescence intensity with iodide ion concentration at constant ionic strength followed the Stern–Volmer formulation (eq 6) without change in the spectral shape for all model compounds. This is illustrated in Figure 1 by the straight-line plots of F_0/F vs. (I^-) for a few model compounds with widely different values of quenching constants, K_Q . Evidence for a dynamic rather than static quenching process was obtained by studying the variation of the quenching constant for indole-3-acetamide in water as a function of temperature. The proportionality of K_Q/F_0 vs. T/η shown in Figure 2 indicates that the mechanism involves a predominantly collisional process, as discussed above. Bimolecular quenching rate constants, k_3 , were calculated from the K_Q values as outlined in the Calculation section. The pertinent parameters and calculated values are listed in Table I for all the model compounds studied.

CHARGE EFFECTS. From Table I it appears that there is a relationship between k_3 values and the molecular charge. For example, k_3 values calculated from reported directly measured

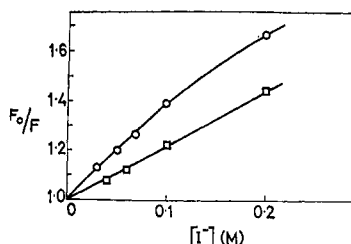


FIGURE 3: Stern-Volmer plot of the quenching of lysozyme fluorescence by iodide. (O) In 0.010 M HCl, ionic strength = 0.2, λ_{fl} = 340 nm; (□) in 6 M Gdn·HCl-2 mM Hepes, pH 7.3, λ_{fl} = 347 nm. Lysozyme concentration = 0.16 mg/ml, $\lambda_{excitation}$ = 295 nm, 25°.

lifetimes for substituted indole compounds with zero net charge (except skatole) are in the range of $4-6 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$. For negatively charged indole-3-acetate, $k_3 = 1.23 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$ and for positively charged L-tryptophan ethyl ester, $k_3 = 12 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$. Other compounds for which lifetimes were calculated by assuming proportionality of quantum yield to lifetime also resulted in k_3 values in the range illustrated above on the basis of their charge.

The dependence on whether the charge is positive or negative is dramatically illustrated by the results for the synthetic random sequence polypeptides poly(Lys⁹⁷Trp³) and poly(Glu⁹⁹Trp¹). At neutral pH the lysine and glutamate side chains are positively and negatively charged, respectively, resulting in a very high molecular charge. A comparison of the resulting iodide quenching of Trp fluorescence shown in Figure 1 (compare curves B and D) and in Table I indicates that the quenching constant is approximately 50 times greater for the positively charged polymer. For these charged polymers, the quenching constants were dependent upon ionic strength, as seen in Table I.

The fluorescence loss at low pH noted for uncharged indole compounds (in absence of I^-) appears to be due to quenching by protons (Weber, 1961). By analogy with the effect of molecular charge on the quenching by iodide, similar charge effects, although opposite in sign, were expected and, in fact, observed for the quenching by protons. Thus, although some quenching at low pH is noted for all uncharged compounds, no loss in fluorescence at low pH is observed for the positively charged lysine copolymer (Table I). The high positive charge presum-

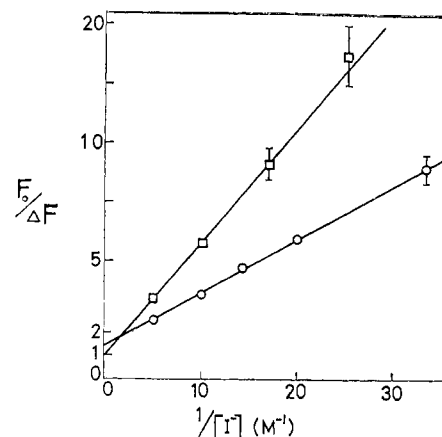


FIGURE 4: Modified Stern-Volmer plot of the quenching of lysozyme fluorescence by iodide. Same legends and data as for Figure 3.

ably reduces the accessibility of the proton. In agreement with this, loss of Trp fluorescence for poly(Lys⁹⁷Trp³) has been shown to occur only below pH 2 at an ionic strength of 0.2 (Fasman *et al.*, 1966). At pH 2, however, simply increasing the ionic strength from 0.18 to 1.0 caused a 36% decrease in fluorescence (Table I). It appears that increased shielding of the positively charged lysine polymer by the higher salt concentration resulted in an increase in accessibility of the tryptophyl side chains to collision with protons.

The effect of ionic strength on the fluorescence quenching by iodide of compounds with positive, negative, and zero net charge was investigated in a separate experiment. Large changes in k_3 with variation of ionic strength were observed for the charged compounds, but only a small effect was observed for zwitterionic L-Trp (Table II).

EFFECTS OF Gdn·HCl AND UREA. Some studies were done with indole-3-acetamide in 6 M Gdn·HCl and in 8 M urea as a model for proteins dissolved in these solvents. The quantum yield was 22% greater in 8 M urea and 25% smaller in 6 M Gdn·HCl than in water in the absence of iodide. The values of K_Q were 13.9 and 18.9 M^{-1} for indole-3-acetamide in 8 M urea and 6 M Gdn·HCl, respectively. When K_Q/F_0 was plotted against T/η for these systems, however, good agreement was obtained with the data in water as shown in Figure 2. This shows that the decrease in K_Q is most probably due to the increased viscosity of the medium.

Lysozyme. QUENCHING AT CONSTANT pH. The quenching of the tryptophyl fluorescence of native and denatured lysozyme by iodide is shown in Figures 3 and 4. Agreement with the simple Stern-Volmer relationship (eq 6) and the modified Stern-Volmer relationship (eq 10) is tested by plotting both F_0/F vs. (I^-) (Figure 3) and $F_0/\Delta F$ vs. $1/(\text{I}^-)$ (Figure 4), respectively. Figure 3 shows that a linear dependence obtains in 6 M Gdn·HCl where the protein is denatured (Ogasahara and Hamaguchi, 1967; Tanford *et al.*, 1966). This suggests that each of the six tryptophyl side chains is subject to a similar degree of fluorescence quenching under denaturing conditions.

The fluorescence of the native protein did not seem to follow the simple Stern-Volmer relationship as judged by the nonlinear plot of Figure 3. Plotting the data according to the modified relationship resulted in a linear dependence as seen in Figure 4. From the intercept a value of $(f_a)_{eff} = 0.66$ was obtained. Thus, about 66% of the tryptophyl fluorescence is accessible for quenching by iodide at pH 2, and the other 34% is not affected by iodide in the concentration range 0-0.2

TABLE II: Effect of Ionic Strength on Iodide Quenching of Charged Trp Model Compounds.^a

Compound	Mol Charge	Ionic Strength	K_Q (M^{-1})	Q	$10^{-9}k_3$ ($\text{M}^{-1} \text{ sec}^{-1}$)
L-Trp	0	0.06	12.8	0.13	4.6
		0.20	12.4	0.13	4.4
		2.0	10.8	0.13	3.9
Indole 3-acetate	—	0.06	9.4	0.34	1.1
		0.20	10.0	0.34	1.2
		2.0	14.1	0.34	1.6
L-TrpOEt	+	0.06	8.1	0.022	16
		0.20	6.3	0.022	13
		2.0	5.1	0.022	10

^a See footnotes a, b, and c of Table I.

TABLE III: The Quenching of the Tryptophyl Fluorescence of Lysozyme by Iodide.

Conditions ^a	Temp (°C)	(f_a) _{eff} ^b	(K_Q) _{eff} ^c (M ⁻¹)	λ_{\max} of Initial Spectrum (nm) ^d	λ_{\max} of Difference Spectrum (nm) ^d
Native, pH 7.5	25	0.38	7.5	337	346
Native, pH 5.2	25	0.55	4.4	337	345
Native, pH 2.0	25	0.66	6.9	336	344
Native, pH 5.3	4	0.53	3.0		
6 M Gdn·HCl, pH 7.3	25	1.0	2.1	347	347
8 M Urea, pH 5.4	25	0.42	3.6	337	348

^a Ionic strength = 0.18 except for Gdn·HCl. ^b ± 0.08 . ^c ± 0.6 . ^d ± 2 nm.

M. In contrast the fluorescence of lysozyme in 6 M Gdn·HCl is completely accessible to iodide quenching as seen in Figure 4 by the extrapolation to (f_a)_{eff} = 1.

A summary of the results obtained at different pH values and with different solvents is shown in Table III. In all cases straight lines were obtained when $F_0/\Delta F$ was plotted *vs.* $1/(I^-)$ over the range 0–0.2 M KI. Values of (K_Q)_{eff} = intercept/slope were calculated and are associated with the quenching of the fluorescence of the most accessible side chains (see Calculations). For the native protein it appears that the increase in quenching at lower pH values observed here, and noted earlier (Lehrer, 1967), is due to an increase in both (f_a)_{eff} and (K_Q)_{eff}. The decrease in quenching at lower temperatures is probably due to an increase in T/η and is further evidence for a diffusion controlled mechanism of quenching in lysozyme. In 6 M Gdn·HCl, (K_Q)_{eff} = 2.1 M⁻¹. This is a lower value than might be expected for totally accessible tryptophyls. It does not appear to be due to the presence of disulfide bonds since no change in parameters was observed when dithiothreitol was incubated with lysozyme in 6 M Gdn·HCl for 1 hr. The low value can be explained by the effect of the increased viscosity associated with 6 M Gdn·HCl on the quenching rate (see Discussion).

The effects of protein concentration and excitation wavelength were studied at pH 5.3. Within experimental error the same values for (f_a)_{eff} and (K_Q)_{eff} were obtained for lysozyme

concentrations of 0.05, 0.11, and 1.0 mg per ml for excitation wavelengths of 303, 295, and 285 nm.

In 8 M urea, at pH 5.4, the spectra and quenching results were hardly different from the results with the native protein, suggesting that 8 M urea at this pH has a limited effect on the conformation of lysozyme, in agreement with other studies (Edelhoch and Steiner, 1962).

SPECTRAL CHANGES. Further information regarding the environment of the accessible Trp side chains can be obtained from the fluorescence spectra, since the wavelength at maximum fluorescence of the indole fluorophor depends upon solvent polarity (Van Duuren, 1961). In 6 M Gdn·HCl, the fluorescence maximum of lysozyme is located at 348 nm and does not shift with increasing iodide concentration (Figure 5). Thus, for the denatured protein all of the Trp side chains are exposed to the aqueous environment, since λ_{\max} = 348 nm for L-Trp in H₂O (Teale and Weber, 1957). In contrast to the denatured protein spectrum, the spectrum of the native lysozyme at pH 5.3 has a peak at 335 nm and shifts to lower wavelength in the presence of iodide (Figure 6). The difference spectrum located at 345 nm, however, does not change position with increasing iodide concentration. Thus, perhaps not surprisingly, the accessible fluorescence is associated with the side chains exposed to the polar aqueous environment.

pH VARIATION OF IODIDE QUENCHING. The variation of peak fluorescence intensity as a function of pH in the absence and presence of 0.2 M iodide is shown in Figure 7 for native lysozyme. In the absence of iodide there is a small drop in fluores-

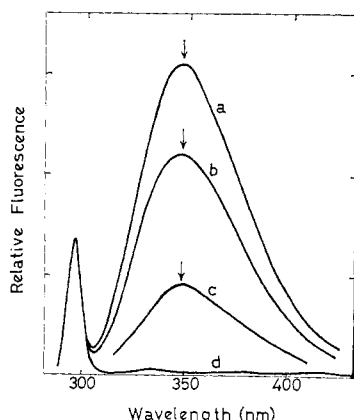


FIGURE 5: Fluorescence spectra of denatured lysozyme (a) in 6 M Gdn·HCl, (b) in 0.2 M KI-6 M Gdn·HCl, (c) difference spectrum a - b, and (d) 6 M Gdn·HCl-2 mM Hepes blank, pH 7.5. $\lambda_{\text{excitation}}$ = 295 nm, 25°. Lysozyme concentration = 0.16 mg/ml. Arrows indicate fluorescence peaks.

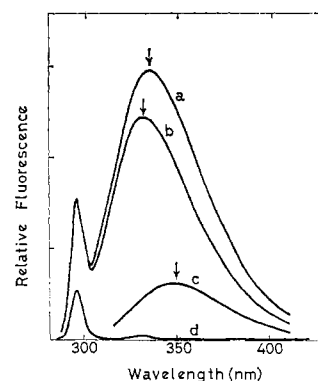


FIGURE 6: Fluorescence spectra of native lysozyme (a) in 0.2 M NaCl, (b) in 0.2 M KI, (c) difference spectrum a - b, and (d) 0.2 M KI blank. 2 mM citrate buffer, pH 5.2. $\lambda_{\text{excitation}}$ = 295 nm, 25°. Lysozyme concentration = 0.16 mg/ml. Arrows indicate fluorescence peaks.

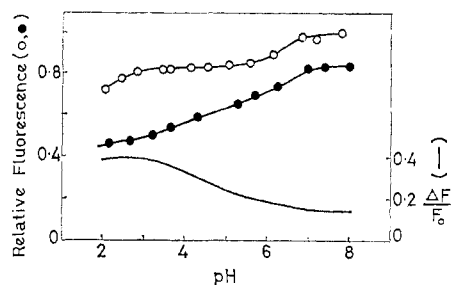


FIGURE 7: Fluorescence pH titration of native lysozyme (○) in 0.2 M NaCl and (●) in 0.2 M KI. Lysozyme concentration = 0.18 mg/ml in 2 mM Hepes and 2 mM citrate. $\lambda_{\text{excitation}} = 295$ nm, $\lambda_{\text{fl}} = 335$ nm, 25°. (—) $\Delta F/F_0$ calculated from above data.

cence between pH 7 and 5, presumably associated with the protonation of a group with $pK_a \approx 6$. The slightly greater drop than that first observed (Lehrer and Fasman, 1967) may be due to differences in excitation wavelength. In the presence of 0.2 M iodide a pH-dependent decrease in fluorescence is observed. Two quenching zones can be seen in the plot of $\Delta F/F_0$ vs. pH. A major increase in quenching appears to depend on the protonation of certain groups in the pH range of 5–3 and a small quenching increase in the pH range 7–5.

Similar titrations in 6 M Gdn·HCl and 8 M urea are shown in Figure 8. The fluorescence intensities are normalized relative to lysozyme at pH 8. In 6 M Gdn·HCl the variation in $\Delta F/F_0$ can be interpreted as a variation in $(K_Q)_{\text{eff}}$ since all Trp side chains are accessible. It can be seen that there is very little change from pH 3 to 8. In 8 M urea, the initial fluorescence is slightly less than in water, in agreement with other studies (Steiner *et al.*, 1964), and the pH dependence of $\Delta F/F_0$ from pH 8 to 4 is very similar to that of the native protein. Below pH 4, however, the fluorescence begins to increase and the spectrum shifts to longer wavelength in a time dependent manner. The observations are in agreement with studies indicating that, although lysozyme is stable at neutral pH in 8 M urea, it denatures as the pH decreases below pH 4 (Edelhoc and Steiner, 1962).

ABSORPTION DIFFERENCE SPECTRA. Iodide may be expected

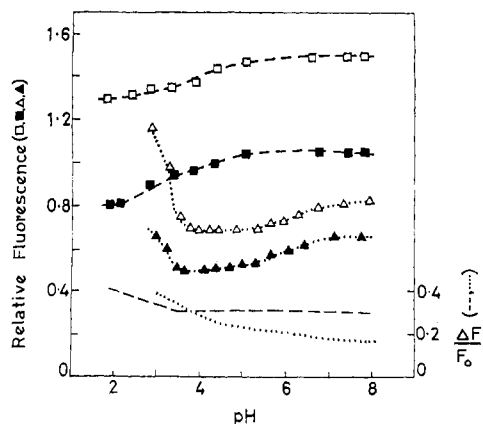


FIGURE 8: Fluorescence pH titration of lysozyme in 6 M Gdn·HCl and 8 M urea in the absence and presence of 0.2 M KI. (□) In 6 M Gdn·HCl; (■) in 6 M Gdn·HCl and 0.2 M KI; (△) in 8 M urea and 0.2 M NaCl; (▲) in 8 M urea and 0.2 M KI. Lysozyme concentration = 0.16 mg/ml in 2 mM Hepes and 2 mM citrate; $\lambda_{\text{excitation}} = 295$ nm, $\lambda_{\text{fl}} = 347$ nm (Gdn·HCl) or 335 nm (urea), 25°. $\Delta F/F_0$ calculated from the above data. (---) In 6 M Gdn·HCl; (···) in 8 M urea.

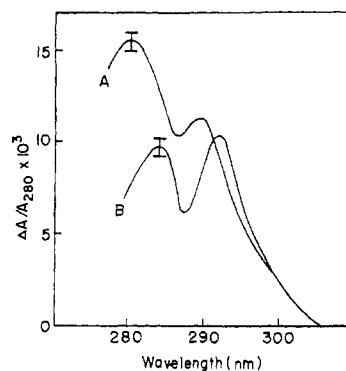


FIGURE 9: Difference absorption spectra of lysozyme and indole-3-acetamide produced by 0.2 M iodide ion. Reference is 0.2 M NaCl in each case. (A) Indole-3-acetamide in H_2O , 25°, $A_{280} = 2.18$; (B) lysozyme in 2 mM citrate, pH 5.4, 25°, $A_{280} = 1.82$.

to bind to lysozyme in view of the known binding of chloride (Carr, 1953), and fluorescence quenching may result if the binding site is close to Trp side chains. To obtain information about possible iodide–Trp interactions, ultraviolet difference spectra in the presence of iodide were studied for lysozyme at pH 5.3 and indole-3-acetamide under the same conditions of constant ionic strength used for the fluorescence measurements. As seen in Figure 9, positive difference spectra with two peaks separated by 8–10 nm were obtained in the two cases, indicating perturbation of Trp absorption. Although the model compound difference spectrum can be explained by a solvent refractive index change (Bigelow and Geschwind, 1960; Hamaguchi *et al.*, 1963) the lysozyme difference spectrum is more complex because of the different spectral shape and magnitude: $\Delta\epsilon_{282}$ (lysozyme) = 360 whereas $\Delta\epsilon_{290}$ (indole-3-acetamide) = 60. As discussed below, these observations suggest the possibility that iodide binds near one or more Trp side chains of lysozyme.

AUXILIARY MEASUREMENTS. Some further studies were done to verify the lack of an effect of iodide ion on the conformation of lysozyme. Optical rotatory dispersion and circular dichroism spectra (from 340 to 265 nm) of lysozyme at pH 5.2 in 0.2 M KI and 0.2 M NaCl were identical. The enzymatic activity at pH 5.4, as measured by the rate of viscosity change associated with the hydrolysis by lysozyme of glycol chitin, was the same in 0.2 M NaCl and 0.2 M KI.

Discussion

Factors Influencing Quenching. From the model compound study it appears that the magnitude of the quenching rate constant, k_3 , can be used as a measure of the accessibility of a protein fluorophor to a collisional quencher. For L-Trp, derivatives of L-Trp, and certain indoles substituted in the 3 position, $k_3 = 4\text{--}6 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$. Values of k_3 for indole and skatole are greater and smaller, respectively. These differences may be due to steric or inductive effects altering the ability to form the excited collisional complex. The accessibility depends upon the molecular charge of ionizable groups attached to the indole. This would influence the collision rate with the negatively charged iodide and has been observed in another system (Holmstrom and Tegner, 1966). For a protein in 0.2 M NaCl, it is expected that local charges will similarly affect accessibility. The lower values of K_Q obtained in 6 M Gdn·HCl and 8 M urea for indole-3-acetamide appear to be due to a viscosity effect. Similar effects are expected for pro-

teins dissolved in viscous liquids and can be corrected for in the manner described above. For a protein, the maximum possible accessibility is determined by the degree of solvent exposure that is imposed on a given tryptophyl side chain by the three-dimensional folding. The degree of exposure of hydrophobic side chains has been shown to vary from completely buried to completely exposed in a series of proteins whose structure has been determined by X-ray studies (Klotz, 1970). This is discussed further in the case of lysozyme in the next section.

There are two additional factors which must be considered in interpreting the quenching of protein fluorescence by iodide in terms of the results with model compounds. The smaller diffusion coefficient of a protein as compared to a low molecular weight model compound will cause a reduction in the quenching rate constant, since the quenching rate constant is proportional to the sum of the diffusion coefficients of the quencher and the fluorophor (Förster, 1951). For iodide as the quencher and L-Trp as the model compound, the diffusion coefficients are $2.0 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$ (Harned and Owen, 1958) and $0.55 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$ calculated with the nomogram of Othmer and Thakar (1953). The sum of the diffusion coefficients is, then, $2.55 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$. The diffusion coefficient of lysozyme must be at least an order of magnitude smaller than the value for iodide (Tanford, 1961) and can, therefore, be neglected. The quenching rate constant for a protein-bound exposed Trp will thus be reduced by a factor of $2.0/2.55 = 0.78$ compared to L-Trp.

Many proteins are known to bind anions in general, and iodide in particular (Steinhardt and Reynolds, 1969). If the iodide binding site is located near a Trp, efficient collisional quenching can occur by a mechanism that does not involve free diffusion. In this case the fluorescence dependence on the concentration of iodide will be influenced by the binding. This has been observed for human serum albumin (Lehrer, 1971). An indication of iodide binding near Trp side chains can be obtained by a study of the ultraviolet difference spectrum. Lysozyme difference spectra obtained in the presence of high concentrations of LiCl, LiBr, and NaBr at pH 7 have been interpreted as being due to a change in refractive index on two to three exposed Trp side chains rather than specific binding (Hamaguchi and Kurono, 1963). In the presence of iodide at pH 5.3, difference spectra of a different shape, and a two to three times greater $\Delta\epsilon_{292}$ value, were obtained for lysozyme as compared to indole-3-acetamide (Figure 9). It appears that an additional effect may be operating to increase the lysozyme difference spectrum near 292 nm relative to the 283-nm peak. Perturbation of Trp absorption by a negative vicinal charge can produce such an effect (Donovan, 1969; Donovan *et al.*, 1961). It appears, however, that appreciable fluorescence quenching by bound iodide does not occur because agreement with the Stern-Volmer law was obtained and because the quenching was less at lower temperature. It is possible that the Trp side chains located near the iodide binding site do not appreciably contribute to the protein fluorescence.

Selective Quenching and Accessibility in Lysozyme. Studies on tryptophyl model compounds have shown that the interaction between their excited states and the polar solvent causes a red shift of the fluorescence spectrum (Van Duuren, 1961; Walker *et al.*, 1967). The polarity of the solvent determines the extent of the shift at temperatures above which solvent reorientation can occur (Longworth, 1969; Eisinger and Navon, 1969). Thus the wide range of λ_{max} observed for tryptophyl fluorescence in different proteins, which shift to a common wavelength (*ca.* 350 nm) when the proteins are de-

natured (Teale, 1960), suggests the existence of different tryptophans in different environments (Konev, 1967; Lehrer and Fasman, 1967). Selective perturbation of tryptophyl fluorescence has been observed in some systems (Konev, 1967; Lehrer and Fasman, 1967; Lehrer, 1967; Elkana, 1968) suggesting a broad classification of tryptophyl groups into two classes, buried and exposed. Heterogeneity of emission was also observed in studies of the phosphorescence of lysozyme (Churchich, 1966) and more recently in studies of protein phosphorescence (Purkey and Galley, 1970) and in fluorescence studies of pepsin and pepsinogen (Teale and Badley, 1970).

A study of the fluorescence difference spectrum produced by iodide quenching provides information about the polarity of the environment of the accessible fluorophors. Values of $(K_Q)_{\text{eff}}$ and $(f_a)_{\text{eff}}$ associated with these accessible fluorophors are obtained from the slope at low iodide concentration and the extrapolated intercept of the modified Stern-Volmer plot. For native lysozyme a linear dependence of $F_0/\Delta F$ on $1/(I^-)$ was obtained over an iodide concentration of 0–0.2 M. The lack of curvature of this plot suggests that the fluorescence of only one class of tryptophyls was selectively affected. This interpretation is supported by the fact that the difference spectrum is constant in shape over the same range of iodide concentration. The location of λ_{max} of the difference spectrum at 344–346 nm shows that these accessible tryptophyls are largely exposed to the aqueous solvent.

This selective quenching would not have been observed if there had been efficient energy transfer of fluorescence from buried to exposed side chains. In such a case uniform quenching of the total protein fluorescence would occur. The lack of uniform quenching observed for lysozyme must mean that distances and orientations between at least some of the buried and exposed fluorophors are not optimal for this long-range energy transfer.

In 6 M Gdn·HCl all tryptophyls are exposed to solvent and accessible for I^- since $\lambda_{\text{max}} = 348 \text{ nm}$ and the initial and final spectrum is the same, and $f_a = 1$. In 8 M urea (pH 5.4) the values of $(K_Q)_{\text{eff}}$ and $(f_a)_{\text{eff}}$ are not significantly different from the native protein. This result is in agreement with other studies which show that higher concentrations of urea are necessary to denature lysozyme (Edelhoc and Steiner, 1962; Hamaguchi and Kurono, 1963).

Approximate values of k_2 for the accessible side chains can be calculated from the K_Q values listed in Table III using eq 4, 6, and 11. Combining these equations leads to

$$(k_3)_{\text{eff}} = \frac{(K_Q)_{\text{eff}} k_1 n_a}{n_t F_0 (f_a)_{\text{eff}}} \quad (13)$$

where K_Q and F_0 have been defined earlier, n_a is the number of tryptophyl side chains per molecule with fractional maximum accessible fluorescence, $(f_a)_{\text{eff}}$, and n_t is the total number of Trp side chains. For lysozyme in 6 M Gdn·HCl, $(K_Q)_{\text{eff}} = 2.1 \text{ M}^{-1}$, $(f_a)_{\text{eff}} = 1$, $n_a = n_t = 6$, and $F_0 = 1.5 \times 0.047$ where 1.5 is the ratio of fluorescence of lysozyme in 6 M Gdn·HCl to that of lysozyme in aqueous solution at pH 8, and 0.047 is the quantum yield of lysozyme at pH 8 (Lehrer and Fasman, 1967) (corrected to $Q_{\text{trp}} = 0.13$). Assuming $F_0 = k_1 \tau_0$ for all Trp compounds (Weinryb and Steiner, 1968; Badley and Teale, 1969; DeLauder and Wahl, 1970), a value of k_1 can be estimated from values for L-Trp, *i.e.*, $k_1 = (0.13/2.8) \times 10^9 \text{ sec}^{-1} = 4.6 \times 10^7 \text{ sec}^{-1}$. Putting these values in eq 13, $(k_3)_{\text{eff}} = 1.4 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$. Correcting for the higher viscosity of 6 M Gdn·HCl from Figure 2 and for the diffusion coefficient

effect described above, $(k_3)_{\text{eff}}(\text{cor}) = 4 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$. Thus, k_3 is the range of values observed for model compounds with no effect of local charge. This is further evidence for complete accessibility of the tryptophyl side chains of lysozyme in 6 M Gdn·HCl.

For native lysozyme at pH 5.4, a similar calculation using eq 13 yields $(k_3)_{\text{eff}} = n_n (2 \times 10^9) \text{ M}^{-1} \text{ sec}^{-1}$. A reasonable value for $(k_3)_{\text{eff}}$ is thus obtained for $n_n = 1$ or 2, although at this level of approximation it is not possible to rule out the involvement of more groups if the local charge is sufficiently positive. It may be possible to make a reasonable guess concerning the tryptophyl side chains that are selectively quenched. At pH 5.4 where there is optimum binding of Ac₃Gln (Lehrer and Fasman, 1966; Rupley *et al.*, 1967) and where the fluorescence quantum yields of lysozyme and lysozyme-Ac₃Gln complex are about the same, the iodide quenching is three to four times less in the complex (Lehrer, 1967). This has been verified in this study. In addition, the λ_{max} of the iodide-produced difference spectrum of the complex indicates a relatively nonpolar environment for the side chains whose fluorescence was slightly quenched. Thus, it appears that most of the exposed and accessible side chains of lysozyme are in the Ac₃Gln binding site. From the X-ray data three tryptophyls are known to be in the binding site, 62, 63, and 108 (Blake *et al.*, 1967), with all three at least partially exposed (Browne *et al.*, 1969). Trp-108 appears to be the most accessible to chemical oxidation by I⁻, although at higher concentrations Trp-62 is also affected (Hartdegen and Rupley, 1964; Blake, 1967). Recent studies on the fluorescence of selectively oxidized lysozyme have suggested that Trp-108 contributes 56% of the fluorescence of lysozyme at pH 5.6 (Teichberg and Sharon, 1970). It is possible that Trp-108 is the residue that is predominantly quenched by iodide. Support for this idea includes the agreement between $(f_n)_{\text{eff}} = 0.53$ (Table III) and the fluorescence contribution of Trp-108 (56%) noted above. In addition, the fact that Trp-108 is selectively accessible to I₃⁻ for chemical modification suggests that it may be similarly accessible to I⁻ for collisional quenching. Quenching studies on selectively oxidized lysozyme will decide which of the three tryptophyls are primarily involved.

Influence of Local Charge. Some indication about the influence of local charge was obtained from the pH dependence observed in Figure 7. Since lysozyme does not appear to undergo conformational change in the pH range studied here (Yang and Foster, 1955; Jirgensons, 1958; Sophianopoulos and Van Holde, 1964; Williams *et al.*, 1965; Lehrer and Fasman, 1967), the increase in quenching by iodide with decreasing pH is most probably due to loss of negative charge associated with the protonation of carboxylate groups of glutamate and aspartate located near the tryptophyl side chains. In particular, Glu-35, which has a pK of 6.3 (Rupley *et al.*, 1967), is close to Trp-108 and becomes closer on binding Ac₃Gln (L. Johnson, 1967, personal communication). Since Trp absorption (Donovan *et al.*, 1961) and fluorescence (Lehrer and Fasman, 1967) are affected by the protonation of this group it might be expected that a large change in iodide quenching might be associated with the loss of the negative charge between pH 7 and 5. Only a 5–10% increase in quenching by iodide occurs (Figure 7). The lack of a large increase may be due to the fact that Trp-108 is not sufficiently exposed to solvent, or because the charge effect is small or because the fluorescence of Trp-108 has already been largely quenched by iodide at pH 8. The latter seems most likely in view of the results of Teichberg and Sharon (1970).

The large increase in iodide quenching in the pH 5–3 region

appears to be due to protonation of carboxylate groups of Glu or Asp with approximately normal pK_a's located near Trp-62 and/or Trp-63. The X-ray data do show a few acid groups within several angstroms of these tryptophyls (Blake *et al.*, 1967). In 6 M Gdn·HCl almost no change in iodide quenching was observed from pH 8 to 3, showing that a general increase in net positive charge does not increase the iodide quenching. In 8 M urea at pH values below *ca.* 3.5 the fluorescence increases in a time-dependent manner approaching values for 6 M Gdn·HCl, suggesting denaturation in agreement with other observations (Edelhoch and Steiner, 1962).

Comparison to Solvent Perturbation. Due to the qualitative similarity of the technique of solute perturbation of protein fluorescence with solvent perturbation of protein absorption, it may be worthwhile to compare the two techniques. The fractional change in absorbance of a chromophore by a given solvent perturbation is linearly related to the degree of exposure through the difference molar extinction coefficient, which is assumed to be the same for all protein chromophores of the same type (Herskovits, 1967). The average degree of exposure can then be used to obtain the average number of exposed chromophores. For solute perturbation of fluorescence the fractional fluorescence change depends on the quantum yield of the fluorophor as well as the accessibility (measured by k_3) through the Stern-Volmer law. For a native protein with a heterogeneous distribution of fluorophors it is generally not possible to obtain a value for the average number of accessible fluorophors since fluorescence quantum yields can vary a great deal, in contrast to absorption extinction coefficients. It may be possible, however, to obtain effective values of k_3 and associated quantum yields of the most accessible fluorophors from the slope at low concentrations through the use of the modified Stern-Volmer law, as was done for lysozyme. Although the study of certain special cases might yield a great deal of quantitative information, it appears that the principal use of solute perturbation will be as a probe of accessibility change accompanying a significant protein interaction. The dependence of the quenching with pH may also provide information regarding energy transfer (Teale and Badley, 1970) and denaturation mechanisms may possibly also be obtained by this technique.

While this work was in progress, I became aware of the studies of Burstein (1968a). His results for iodide quenching of Trp model compounds do not agree with the results presented here primarily because his data exhibited curvature when plotted according to the Stern-Volmer law over the same range of iodide concentration. It is not clear why the discrepancy exists. His study of iodide quenching of ribonuclease fluorescence (1968b), however, clearly shows the selective quenching of the fluorescence of accessible tyrosyl side chains.

Acknowledgments

The author thanks Mrs. G. Kerwar and Miss M. Ho for their excellent technical assistance, Dr. B. Nagy for aid in the circular dichroism and optical rotatory dispersion measurements, and Drs. E. Kirby and R. F. Steiner for their unpublished fluorescence lifetime data.

References

- Arrio, B., Rodier, F., Boisson, C., and Vernotte, C. (1970), *Biochem. Biophys. Res. Commun.* 39, 589.
- Badley, R. A., and Teale, F. W. J. (1969), *J. Mol. Biol.* 44, 71.

- Bigelow, C. C., and Geschwind, I. I. (1960), *C. R. Trav. Lab. Carlsberg* 31, 283.
- Blake, C. C. F. (1967), *Proc. Roy. Soc., Ser. B* 167, 435.
- Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1967), *Proc. Roy. Soc., Ser. B* 167, 365.
- Browne, W. J., North, A. C. J., Phillips, D. C., Brew, K., Vanaman, T. C., and Hill, R. L. (1969), *J. Mol. Biol.* 42, 65.
- Burstein, E. A. (1968a), *Biofizika* 13, 433.
- Burstein, E. A. (1968b), *Biofizika* 13, 718.
- Carr, C. W. (1953), *Arch. Biochem. Biophys.* 46, 417.
- Chen, R. F. (1967), *Anal. Lett.* 1, 35.
- Chen, R. F., Vurek, G. G., and Alexander, N. (1967), *Science* 156, 949.
- Churchich, J. E. (1966), *Biochim. Biophys. Acta* 120, 406.
- DeLauder, W. B., and Wahl, D. H. (1970), *Biochemistry* 9, 2750.
- Donovan, J. W. (1969), in *Physical Principles and Techniques of Protein Chemistry*, Part A, Leach, S., Ed., New York, N. Y., Academic Press.
- Donovan, J. W., Laskowski, M. J., Jr., and Sheraga, H. A. (1961), *J. Amer. Chem. Soc.* 83, 2686.
- Edelhoc, H., and Steiner, R. F. (1962), *Biochim. Biophys. Acta* 60, 365.
- Eisinger, J., and Navon, G. (1969), *J. Chem. Phys.* 50, 2069.
- Elkana, Y. (1968), *J. Phys. Chem.* 72, 3654.
- Fasman, G., Bodenheimer, E., and Pesce, A. (1966), *J. Biol. Chem.* 241, 916.
- Förster, Th. (1951), *Fluorescence Organischer Verbindungen*, Göttingen, Vandenhoeck and Ruprecht.
- Hamaguchi, K., and Kuroki, A. (1963), *J. Biochem. (Tokyo)* 54, 111.
- Hamaguchi, K., Rokkaku, K., Funatsu, M., and Hayashi, K. (1960), *J. Biochem. (Tokyo)* 48, 351.
- Harned, H. S., and Owen, B. B. (1958), *The Physical Chemistry of Electrolytic Solutions*, New York, N. Y., Reinhold Publishing Co.
- Hartdeggen, F. J., and Rupley, J. (1964), *Biochim. Biophys. Acta* 92, 625.
- Herskovits, T. T. (1967), *Methods Enzymol.* 11, 748.
- Herskovits, T. T., and Laskowski, M., Jr. (1960), *J. Biol. Chem.* 235, PC57.
- Holmström, B., and Tegner, L. (1966), *Photochem. Photobiol.* 5, 207.
- Jirgensons, B. (1958), *Arch. Biochem. Biophys.* 74, 70.
- Klotz, M. (1970), *Arch. Biochem. Biophys.* 138, 704.
- Konev, S. V. (1967), *Fluorescence and Phosphorescence of Proteins and Nucleic Acids*, New York, N. Y., Plenum Press.
- Kuwahara, K., and Tanford, C. (1966), *J. Biol. Chem.* 241, 3228.
- Laskowski, M., Jr. (1966), *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* 25, 20.
- Lehrer, S. S. (1967), *Biochem. Biophys. Res. Commun.* 29, 767.
- Lehrer, S. S. (1971), *Abstr. Biophys. Soc. Meeting*, New Orleans, La.
- Lehrer, S. S., and Fasman, G. D. (1966), *Biochem. Biophys. Res. Commun.* 23, 133.
- Lehrer, S. S., and Fasman, G. D. (1967), *J. Biol. Chem.* 242, 4644.
- Longworth, J. W. (1969), *Biophys. J.* 9, A217.
- Ogasahara, K., and Hamaguchi, K. (1967), *J. Biochem. (Tokyo)* 61, 199.
- Othmer, D. F., and Thakar, M. S. (1953), *Ind. Eng. Chem.* 45, 589.
- Purkey, R. M., and Galley, W. C. (1970), *Biochemistry* 9, 3569.
- Rupley, J., Butler, L., Gering, M., Hartdeggen, F. J., and Pecoraro, R. (1967), *Proc. Nat. Acad. Sci. U. S.* 57, 1088.
- Sophianopoulos, A. J., and Van Holde, K. E. (1964), *J. Biol. Chem.* 239, 2516.
- Steiner, R. F., Lippoldt, R. E., Edelhoc, H., and Frattali, V. (1964), *Biopolym. Symp.* 1, 355.
- Steinhardt, J., and Reynolds, J. A. (1969), *Multiple Equilibria in Proteins*, New York, N. Y., Academic Press.
- Stern, O., and Volmer, M. (1919), *Phys. Z.* 20, 183.
- Tanford, C. (1961), *Physical Chemistry of Macromolecules*, New York, N. Y., Wiley.
- Tanford, C., Pain, R., and Otchin, N. (1966), *J. Mol. Biol.* 15, 489.
- Teale, F. W. J. (1960), *Biochem. J.* 76, 381.
- Teale, F. W. J., and Badley, R. A. (1970), *Biochem. J.* 116, 341.
- Teale, F. W. J., and Weber, G. (1957), *Biochem. J.* 65, 467.
- Teichberg, V. I., and Sharon, N. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 7, 171.
- Van Duuren, B. L. (1961), *J. Org. Chem.* 26, 2954.
- Vaughan, W. M., and Weber, G. (1970), *Biochemistry* 9, 464.
- Vavilov, S. I. (1929), *Z. Phys.* 53, 665.
- Walker, M. S., Bednar, T. W., and Lumry, R. (1967), *J. Chem. Phys.* 47, 1020.
- Weast, R. C., Ed. (1970), *Handbook of Chemistry and Physics*, Cleveland, Ohio, Chemical Rubber Publishing Co.
- Weber, G. (1948), *Trans. Faraday Soc.* 44, 185.
- Weber, G. (1961), in *Light and Life*, McElroy, W., and Glass, B., Ed., Baltimore, Johns Hopkins University Press, p 82.
- Weinryb, I., and Steiner, R. F. (1968), *Biochemistry* 7, 2488.
- Williams, E. J., Herskovits, T. T., and Laskowski, M., Jr. (1965), *J. Biol. Chem.* 240, 3574.
- Winkler, M. H. (1969), *Biochemistry* 8, 2587.
- Yang, J. T., and Foster, J. F. (1955), *J. Amer. Chem. Soc.* 77, 2374.