Brown, J. R., and Hartley, B. S. (1963), *Biochem. J. 89*, 59P.
Brown, J. R., and Hartley, B. S. (1966), *Biochem. J. 101*, 214.
Canfield, R. E., and Liu, A. K. (1965), *J. Biol. Chem. 240*, 1997.
Cautrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 636.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Givas, J., Centeno, E. R., Manning, M., and Sehon, A. H. (1968), *Immunochemistry 5*, 314.
Gray, W. R., and Hartley, B. S. (1963), *Biochem. J. 89*, 59P.
Habeeb, A. F. S. A., and Atassi, M. Z. (1969), *Immunochemistry 6*, 555.
Haber, E. (1968), *Annu. Rev. Biochem. 37*, 497.
Haber, E. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 66.
Haimovich, J., Hurwitz, E., Novik, N., and Sela, M. (1970a), *Biochim. Biophys. Acta 207*, 115.
Haimovich, J., Hurwitz, E., Novik, N., and Sela, M. (1970b), *Biochim. Biophys. Acta 207*, 125.
Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 197.
Hirs, C. H. W., Moore, S., and Stein, W. H. (1960), *J. Biol. Chem. 235*, 633.
Imanishi, M., Miyagawa, N., Fujio, H., and Amano, T. (1969), *Biken J. 12*, 85.
Isagholain, L. B., and Brown, R. K. (1970), *Immunochemistry 7*, 167.
Krause, R. M. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 59.
Mamet-Bratley, M. D. (1966), *Immunochemistry 3*, 155.
McFarlane, A. S. (1958), *Nature (London) 182*, 53.
Neumann, H., Steinberg, I. Z., Brown, J. R., Goldberger, R. F., and Sela, M. (1967), *Eur. J. Biochem. 3*, 171.
Porath, J., Axen, R., and Ernback, S. (1967), *Nature (London) 215*, 1491.
Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.
Richards, F. F., and Haber, E. (1967), *Biochim. Biophys. Acta 140*, 558.
Richards, F. F., Pincus, J. A., Broch, J. K., Barnes, W. T., and Haber, E. (1969), *Biochemistry 8*, 1377.
Roholt, O. A., Seon, B. K., and Pressman, D. (1970), *Immunochemistry 7*, 329.
Sela, M., Schechter, B., Schechter, I., and Borek, F. (1967), *Cold Spring Harbor Symp. Quant. Biol. 32*, 537.
Shinka, S., Imanishi, M., Miyagawa, N., Amano, T., Inouye, M., and Tsugita, A. (1967), *Biken J. 10*, 89.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 301*, 1190.
Strosberg, A. D., and Kanarek, L. (1970), *Eur. J. Biochem. 14*, 161.
Wrigley, C. W. (1968), *J. Chromatog. 36*, 362.

Varying Luminescence Behavior of the Different Tryptophan Residues of Papain*

R. F. Steiner†

ABSTRACT: The fluorescence of papain at 25° is dominated by a tryptophan residue with unusually high quantum yield and excited lifetime. This residue is largely shielded from the action of external perturbants or quenchers but can be oxidized by *N*-bromosuccinimide. The dominant tryptophan may be quenched by protonation of a histidine or carboxyl group in its vicinity, or by the Hg atom of mercuripapain. The residual tryptophans have quite different properties, including a lower average quantum yield and lifetime and a different spectral distribution of fluorescence.

In an earlier publication the luminescence of papain was examined as a function of conditions (Weinryb and Steiner, 1970). Preparations of unactivated commercial crystalline papain were used in this study. (The term "unactivated papain" refers to the enzymically inert material lacking a free sulfhydryl group; treatment with a sulfhydryl reagent produces the activated form which contains a sulfhydryl group and possesses enzymic activity.) The principal conclusions may be briefly summarized as follows.

It was found that, at 25° in aqueous solution, the fluorescence emission spectrum was essentially characteristic of tryptophan, with little or no indication of any significant contribution from the tyrosine residues of papain. The implication was that the tyrosine emission is largely or entirely abolished either by quenching or as a consequence of radiationless energy transfer to tryptophan. Under conditions where the organized three-dimensional structure of papain is eliminated, as in 6 M guanidine hydrochloride, a tyrosine emission band appears.

The fluorescence of unactivated papain was partially quenched at pH values acid to 8, the midpoint of the quenching process being about pH 6.6 in agreement with Barel and Glazer (1969). In the pH range 3–5, where the fluorescence intensity was constant, direct evidence for energy transfer from tyrosine to tryptophan was obtained, the quantum yield based upon tryptophan absorption increasing for excitation wavelengths within the absorption band of tyrosine.

Recently, the picture has been complicated by the finding of Sluyterman and Wijdenes (1970) that crystalline papain may be resolved into two components, one of which (PI) cannot

* From the Naval Medical Research Institute, Bethesda, Maryland. *Received August 14, 1970.*

† Present address: Department of Chemistry, University of Maryland, Baltimore County, Baltimore, Md.

be activated, while the other (PII) can be converted into an active enzyme with a free sulfhydryl group (HSP) by treatment with a sulfhydryl reagent. Species PII can be isolated as the mercury complex Hg–P.

In view of the X-ray crystallographic work of Drenth *et al.* (1968), which was done for unfractionated papain, it is likely that PI and PII are similar in structure, although this conclusion must remain tentative pending comparative studies upon the individual species.

Sluyterman and De Graaf (1970) have examined the pH profiles of fluorescence intensity for PI, PII, HgP, and HSP. PI and PII were quenched at acid pH, the midpoints of the quenching regions being pH 6.6 and 4, respectively. HSP was quenched in a more alkaline range, the midpoint being at about pH 8. The fluorescence intensity of Hg–P was almost invariant between pH 4 and pH 9.

It is the purpose of the present paper to compare the luminescence properties of the various forms of papain in order to confirm the conclusions reached earlier for unfractionated papain and to present further data upon the varying emission characteristics of the different tryptophans of papain.

Experimental Section

Methods. Fluorescence and phosphorescence measurements. Determination of fluorescence and phosphorescence emission spectra were made with an American Instrument Company spectrofluorometer, which was equipped with a spectral compensation unit (provided by the same company) to correct for the varying wavelength response of the photomultiplier. In this way corrected plots of fluorescence intensity as a function of emission wavelength were recorded. The integrated areas under these curves are proportional to the energy yield rather than the quantum yield and it is the former quantity which is compared here. In determining relative energy yields, a base line for the appropriate solvent was recorded and subtracted from the observed emission spectrum. A correction was also made for the "inner-filter" effect in the case of solutions with significant absorbance, as described elsewhere (Weinryb and Steiner, 1968, 1970).

In determining the variation of energy yield with excitation wavelength, each papain was compared to a standard tryptophan solution which was measured under exactly the same conditions for each excitation wavelength. The ratio of the corrected areas under the fluorescence emission curves, divided by the ratio of the absorbances at the particular excitation wavelength, gives the relative energy yield. This quantity, divided by the fractional absorbance of the tryptophan residues of papain at the given excitation wavelength, gives the relative yield based on tryptophan absorption alone (Weinryb and Steiner, 1970).

Fluorescence and phosphorescence spectra at liquid nitrogen temperatures were obtained with the use of a bronze "cold finger" partially immersed in a secondary liquid nitrogen reservoir and in thermal contact with the cell, which was a 4-cm length of 3-mm diameter quartz electron spin resonance tubing sealed at one end. Frosting was prevented by a stream of dry nitrogen gas (Weinryb and Steiner, 1968).

Fluorescence lifetimes at room temperature were measured using the TRW system, which has been adequately described elsewhere (Chen *et al.*, 1967). A 295-mμ interference filter was used for excitation and a Corning 5970 filter intercepted the fluorescence beam.

Phosphorescence decay curves were determined with the American Instrument Company spectrofluorometer described above, using the technique described in the previous paper. Intensities were recorded at 0.2-sec intervals with a Digital PDP/8 computer, using a data acquisition program developed by Dr. Edward Kirby.

Reaction with *N*-bromosuccinimide. Oxidation of tryptophans with 0.01 M *N*-bromosuccinimide was normally carried out in 0.1 M KOAc, pH 5.0, at 25°. The concentration of papain was 0.3–0.5 mg/ml. The reaction was monitored spectrally from the drop in absorbance at 280 mμ, using a Gilford spectrophotometer. The number of tryptophans oxidized per mole (n) was computed using eq 1 (Witkop, 1961),

$$n = 1.3\Delta A_{280}/5500m \quad (1)$$

where ΔA_{280} is the drop in absorbancy at 280 mμ, and m is the molar concentration of papain.

The NBS[1] reaction is not completely specific for tryptophan; tyrosine and histidine may also be attacked (Witkop, 1961). In addition there exists the possibility of an indirectly induced conformational change. Both of these may give rise to a significant change in absorbancy which introduces error into the use of eq 1. For this reason values of n computed from eq 1 should be regarded as only approximate. This does not affect the argument to be presented here.

Materials. Crystalline papain was purchased from the Worthington Biochemical Corporation. Purified samples of PI and of the mercury derivative (Hg–P) of active papain were obtained through the generosity of Dr. L. A. E. Sluyterman.

Hg–P was converted into active papain (HSP) by treatment with 0.01 M cysteine in 0.1 M KOAc–10^{-4} M EDTA, pH 5.0, for 2 hr. For some applications, cysteine was removed by passage through a Sephadex G-25 column, eluting with water.

Carboxymethylated papain (CM-P) was prepared by first treating Hg–P with 0.01 M cysteine in 0.1 M KOAc–10^{-4} M EDTA, pH 5.0, for 2 hr and then adding an excess (0.05 M) of sodium iodoacetate. After 30 min the mixture was passed through a Sephadex G-25 column, eluting with water.

The guanidine hydrochloride used was the ultrapure preparation of Mann Chemical Co. Tryptophan was obtained from the Sigma Chemical Co. All other reagents were analytical grade. Glass-redistilled water was used for the preparation of all solutions.

Results

pH Dependence of Fluorescence. The pH profiles of fluorescence intensity at 350 mμ for PI and unfractionated papain are quite similar (Figure 1). Two regions of constant intensity at pH values 3–4.5 and 8–8.5 are separated by a zone in which quenching occurs with decreasing pH, the midpoint of the process occurring at about pH 6.6.

The pH profile for CM-P likewise displayed a region of quenching, which was displaced considerably to the acid of that for PI, the midpoint being close to pH 5.5 (Figure 1). The curve for CM-P lies significantly to the alkaline of that for the derivative PII studied by Sluyterman and De Graaf (1970), in which the sulfhydryl group was blocked by disulfide formation with an unchanged C_2H_5SH group. Apparently the presence of the negatively charged carboxylate group in CM-P serves to raise the pK of the quenching group.

The activated form of papain, HSP, shows quite different properties. Extensive quenching occurs at pH values acid

[1] The abbreviations used are: NBS, *N*-bromosuccinimide; CM-P, carboxymethylated papain.

TABLE I: Relative Energy Yields and Excited Lifetimes of Papain Species.[a]

Species	pH	Energy Yield Relative to Tryptophan[b]: Based on Total Absorbance[c]	Energy Yield Relative to Tryptophan[b]: Based on Trp Absorbance[d]	Excited Lifetime (nsec)
Unfractionated	5.0	0.53	0.96	3.0
	7.0	0.73	1.32	
PI	5.0	0.435	0.79	3.0
	7.8	0.835	1.52	5.2
Hg–P	5.0	0.39	0.71	3.0
HSP	5.0	0.39	0.71	2.8
CM-P	4.6	0.53	0.96	3.0
	7.6	1.10	2.0	6.2
NBS–CMP	7.6	0.25	0.45	3.1
Trp	5.0	1.00	1.00	

[a] The excitation wavelength is 280 mμ for the fluorescence yield and 295 mμ for the excited lifetime. The buffer is 0.1 M KOAc at pH 5.0 and 0.1 M PO_4 at pH 7.6. [b] The ratio of the energy yield to that of tryptophan for the same conditions. [c] Based on the total absorbance of papain at 280 mμ. [d] Based on the tryptophan absorbance of papain at 280 mμ, being equal to the yield based on total absorbance divided by 0.55.

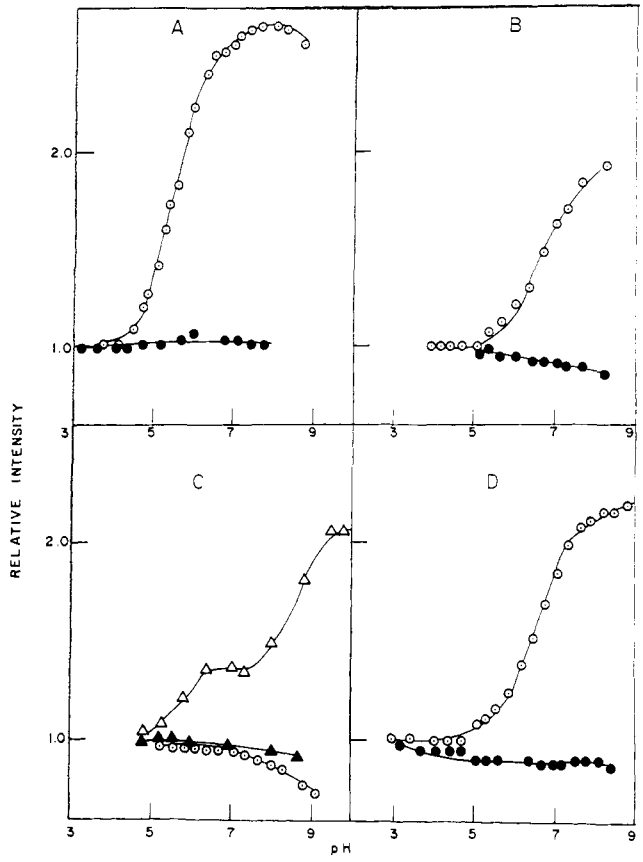

FIGURE 1: pH dependence of relative fluorescence intensity for the native (○ or △) and NBS-oxidized (● or ▲) forms of four papain species. The excitation and emission wavelengths are 280 mμ and 350 mμ, respectively. The solvent is 0.01 M KOAc–0.01 M PO_4 at 25°. The relative intensity is normalized with respect to the value at the lowest pH in each case. (The absolute intensity for the NBS-oxidized form is actually much less than that of the native form in each case.) (A) (○) CM-P, (●) NBS oxidized; (B) (○) PI, (●) NBS oxidized; (C) (△) HSP, (▲) NBS oxidized (see text), (○) Hg–P; (D) (○) unfractionated papain, (●) NBS oxidized.

to pH 9, the midpoint being close to pH 8. There is some indication of an inflection near pH 7. The pH zone of quenching corresponds to the expected pK for the sulfhydryl group. Cysteine has been shown to quench indole derivatives (Steiner and Kirby, 1969).

In agreement with Sluyterman and De Graaf (1970), it was found that the Hg derivative of HSP showed no quenching at acid pH (Figure 1), its intensity remaining roughly at the level of the quenched forms of the other derivatives between pH 4 and pH 9.

At a pH which lies within the zone of constant intensity on the acid side of the quenching region, PI, Hg–P, HSP, CM-P, and unfractionated papain do not differ greatly in emission properties (Table I). The yields, excited lifetimes, and positions of the emission maxima are fairly similar, although quantitative differences exist.

In the cases of PI, HSP, CM-P, and unfractionated papain the quenched form has a yield which is lower by a factor of two or more than that of the unquenched form, for excitation at 295 mμ (Table II). The drop in yield is in each case accompanied by a major decrease in lifetime and by a shift in the wavelength of the fluorescence maximum to lower values (Figure 2 and Table I). This effect has already been reported for unfractionated papain (Weinryb and Steiner, 1970).

The results cited above can be fitted into the following general picture. The pH dependence of tryptophan emission for each of the forms of papain results from the quenching of a particular tryptophan group (or possibly groups) which may be quenched by either the un-ionized form of the sulfhydryl group of cysteine or by a second ionizable site with a pK in the region 5 to 7. This tryptophan may also be quenched by the Hg derivative of the cysteine. The emission properties of the quenched form of each papain species are dominated by the residual, unquenched tryptophans and are rather similar for all species. There is no evidence for a conformational change in the pH region 4–8 (Barel and Glazer, 1969).

Dependence of Emission Properties upon Excitation Wavelength. It has already been reported for the case of unfractionated papain at pH 5.0 that both the apparent energy yield, based on tryptophan absorption,[2] and the shape of the emission spectrum depend upon the excitation wavelength (Weinryb and Steiner, 1970). For excitation wavelengths below 290 mμ, where tyrosine contributes a major fraction of the absorption, the tryptophan-based apparent yield increases and λ_{max} is displaced to lower wavelengths, as compared with longer excitation wavelengths, where the absorption of tyrosine is almost nil (Figure 3). Hg–P and the quenched forms of PI and CM-P show similar behavior (Figure 3).

At pH 8, on the alkaline side of the quenching zone, this effect is greatly diminished for unfractionated papain (Figure

[2] A spectral shift of the emission band affects the relationship between energy yield and quantum yield. For the relative yields reported here the maximum deviation between relative energy yield and relative quantum yield is never greater than 10%, which is of the same order as the experimental uncertainty. For the purposes of the argument it is thus unnecessary to differentiate between energy yields and quantum yields.

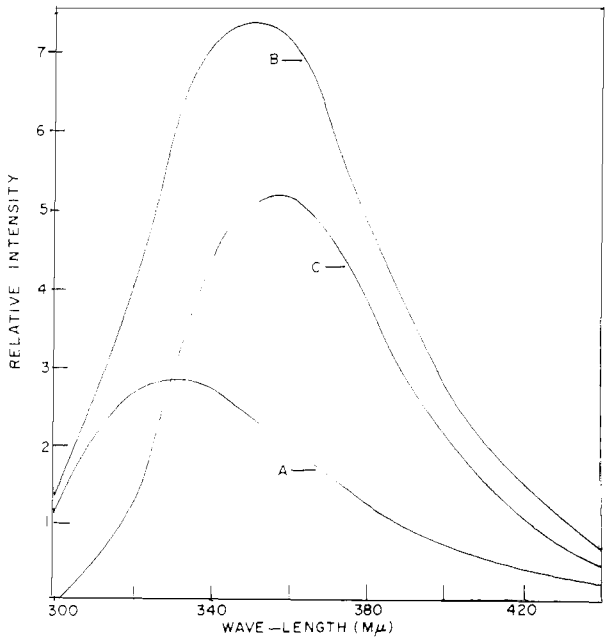

FIGURE 2: Fluorescence spectrum of CM-P at pH 3.75 (curve A) and pH 8.63 (curve B) in 0.01 M KOAc–0.01 M PO_4 at 25°. Curve C is the difference between curves B and A and corresponds to the spectrum of the tryptophan quenched at acid pH. The excitation wavelength is 280 mμ.

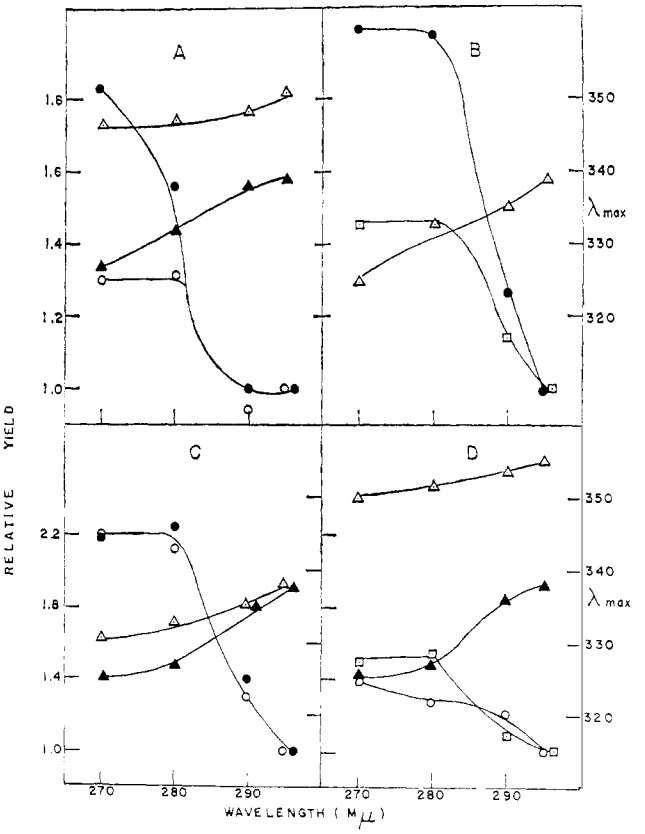

FIGURE 3: Dependence of relative energy yield and of the wavelength of maximum fluorescence intensity (λ_{max}) upon excitation wavelength for four papain species. The papain yields are based on tryptophan absorption. The relative energy yields are normalized to a value of unity at 295 mμ. (A) (●) *Relative* values of $(\text{energy yield})_{papain}/(\text{energy yield})_{tryptophan}$ for unfractionated papain in 0.1 M KOAc, pH 5.0; (○) *relative* values of $(\text{energy yield})_{papain}/(\text{energy yield})_{tryptophan}$ for unfractionated papain in 0.1 M PO_4, pH 7.9; (▲) λ_{max} for unfractionated papain in 0.1 M KOAc, pH 5.0; (△) same, 0.1 M PO_4, pH 7.9. (B) (□) *Relative* values of $(\text{energy yield})_{NBS\ oxidized}/(\text{energy yield})_{native}$ for PI in 0.1 M PO_4, pH 7.6; (●) *relative* values of $(\text{energy yield})_{PI}/(\text{energy yield})_{tryptophan}$ for PI in 0.1 M KOAc, pH 5.0; (△) λ_{max} for PI in 0.1 M KOAc, pH 5.0. (C) (○) *Relative* values of $(\text{energy yield})_{Hg-P}/(\text{energy yield})_{tryptophan}$ for Hg–P in 0.1 M KOAc, pH 5.0; (●) *relative* values of $(\text{energy yield})_{HSP}/(\text{energy yield})_{tryptophan}$ for HSP in 0.1 M KOAc, pH 5.0; (△) λ_{max} for Hg–P in 0.1 M KOAc, pH 5.0; (▲) λ_{max} for HSP in 0.1 M KOAc, pH 5.0. (D) (○) *Relative* values of $(\text{energy yield})_{Ph\ 3.75}/(\text{energy yield})_{Ph\ 8.6}$ for CM-P in 0.01 M KOAc–0.01 M PO_4; (□) *relative* values of $(\text{energy yield})_{NBS\ oxidized}/(\text{energy yield})_{native}$ for CM-P in 0.1 M PO_4, pH 7.6; (△) λ_{max} for CM-P in 0.01 M KOAc–0.01 M PO_4, pH 8.6; (▲) same, pH 3.75.

3). Both λ_{max} and the tryptophan-based quantum yield show much less variation with excitation wavelength than at pH 5. As a consequence, the energy yield at pH 5, relative to that at pH 8, increases with decreasing excitation wavelength (Figure 3). PI and CM-P show a parallel effect, if the pH values compared are chosen to fall within the constant regions on either side of the quenching zone (Figure 3).

It is difficult to explain the dependence of energy yield upon excitation wavelength except in terms of radiationless energy transfer from tyrosine to tryptophan at the singlet level (Konev, 1967). This process, whose occurrence in proteins was long the subject of considerable controversy, has been demonstrated to occur in ribonuclease (Longworth, 1968), but only to a small extent in pepsin (Teale and Badley, 1969).

One important possible complicating factor deserves comment. Purkey and Galley (1970) have found that the excitation spectra of the different tryptophans within several proteins are significantly different and depend on their microenvironments, so that the observed emission spectra depend on the excitation wavelength. It is impossible to assess the importance of this factor quantitatively in the present case, since we do not know the individual absorption spectrum of each tryptophan. However, the results of Purkey and Galley were obtained for excitation wavelengths at the long-wavelength edge of the tryptophan spectrum (>295 mμ) where the effects of spectral shifts are magnified. It seems unlikely that these effects would be as important for wavelengths well within the tryptophan band. For this reason, and because the wavelength dependence of the effects observed in the present study parallels the absorption spectrum of tyrosine, the present results have been interpreted in terms of energy transfer from tyrosine to tryptophan.

Two further implications follow from this result. The occurrence of significant transfer from tyrosine indicates that the tyrosines of papain are not entirely quenched by process other than energy transfer. Moreover, the tryptophan

TABLE II: Relative Energy Yields of Papain for Quenched and Unquenched States.[a]

Species	λ_{ex}	pH (Quenched)	pH (Unquenched)	Relative Yield (Quenched/Unquenched)
Unfractionated	295	5.0	7.9	0.51
PI	295	5.0	7.6	0.47
CM-P	295	3.7	8.7	0.31
HSP	295	4.8	9.7	0.44

[a] The solvent is 0.01 M KOAc–0.01 M PO_4 at 25°.

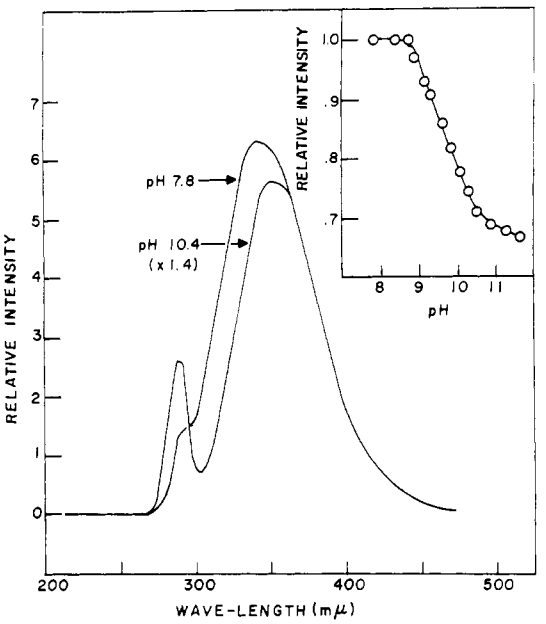

FIGURE 4: Emission spectra for PI in 0.1 M lysine–0.05 M PO_4, at pH 7.8 and at pH 10.4 (multiplied by 1.4). The excitation wavelength is 280 mμ. (Inset) Ratio of fluorescence intensity at 350 mμ of PI at given pH to that for pH 7.8. The excitation wavelength is 280 mμ. The solvent is 0.1 M lysine–0.05 M PO_4.

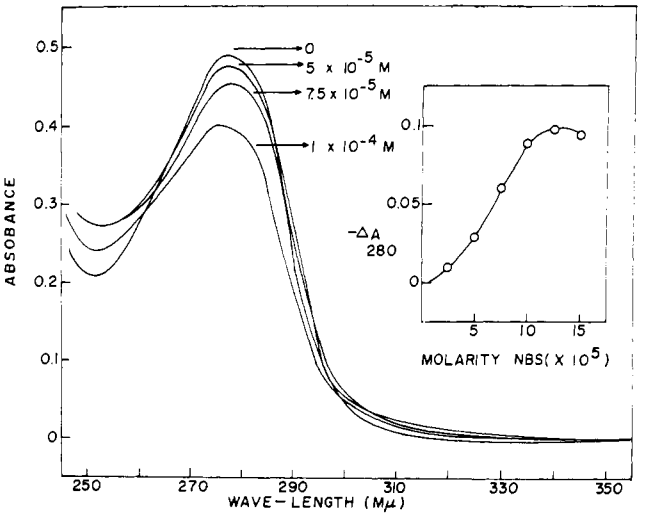

FIGURE 5: Absorption spectra of Hg–P (0.20 mg/ml) in 0.1 M KOAc, pH 5.0, in the presence of increasing molarities of NBS. Inset: drop in absorbance at 280 mμ with increasing molarity of NBS for above system.

of high-quantum yield, which is responsible for the pH dependence of quantum yield, appears to be involved in energy transfer from tyrosine to a significantly less extent than the remaining tryptophans. This may be a consequence of the competitive quenching of the tyrosines nearest to this residue by other processes.

If the pH is increased beyond pH 9 the fluorescence yield decreases and the shape of the fluorescence spectrum is altered. This is illustrated for the case of PI in Figure 4, but the behavior of the other species is qualitatively similar. The fluorescence intensity at low wavelengths is selectively reduced, presumably as a consequence of radiationless energy transfer to ionized tyrosine. The implication is that radiationless transfer from the dominant tryptophan to ionized tyrosine is less important than for the remaining tryptophans, whose contribution is more important at the lower wavelengths.

NBS Oxidation. The addition of successive aliquots of NBS to each of the forms of papain in 0.1 M KOAc, pH 5.0, results in a monotonic decrease in absorbancy at 280 mμ, followed by a constant region and then by a gradual rise in absorbance (Figure 5). If eq 1 is used to compute the apparent number of tryptophans oxidized, it is found that approximately 2 react in the cases of Hg–P, and PI, the values ranging from 1.9 to 2.5. Because of the shortcomings of the spectral approach to computing the number of reacting tryptophans, the figure of 2 should probably be regarded as a lower limit.

Treatment with NBS results in a major drop in quantum yield and excited lifetime at pH 8 for unfractionated papain, CM-P, and PI and in a shift of λ_{max} to lower wavelengths (Figure 6 and Table I). Moreover, the quantum yield relative to the native form increases with decreasing wavelength (Figure 3). The behavior of NBS-oxidized papain qualitatively resembles that of the quenched form of native papain at acid pH.

NBS-treated papain does not show the pronounced drop in fluorescence yield at acid pH which is characteristic of the native species (Figure 1).

If Hg–P is oxidized with NBS and then converted into the sulfhydryl form by treatment with 10^{-2} M cysteine, it is found that the pH dependence of the fluorescence intensity of HSP is likewise almost abolished (Figure 1). The emission properties of the product resemble those of the oxidized forms of PI and CM-P.

It can be concluded from these results that the tryptophan (or tryptophans) responsible for the pH dependence of the fluorescence yield of PI, CM-P, and HSP, is susceptible to NBS oxidation and that this residue dominates the fluorescence spectrum at pH values alkaline to the quenching zone.

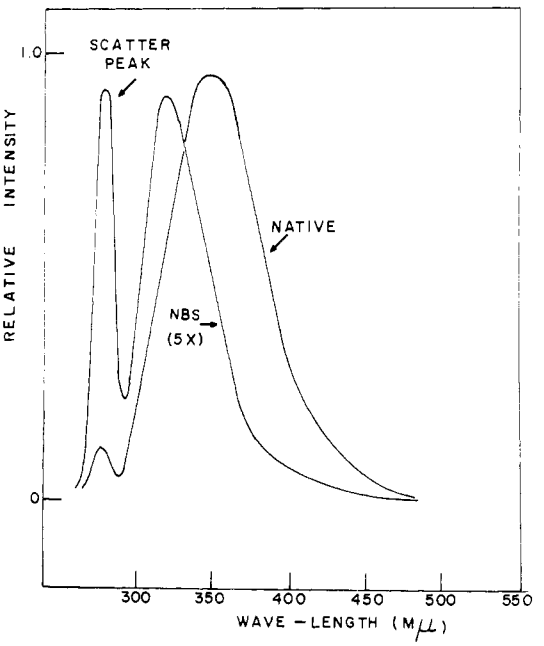

FIGURE 6: Fluorescence emission spectra for CM-P and its NBS-oxidized derivative in 0.1 M PO_4, pH 7.6. The excitation wavelength is 280 mμ. The concentration of the NBS-oxidized material is 0.2 mg/ml, while that of the native CM-P is 0.05 mg/ml.

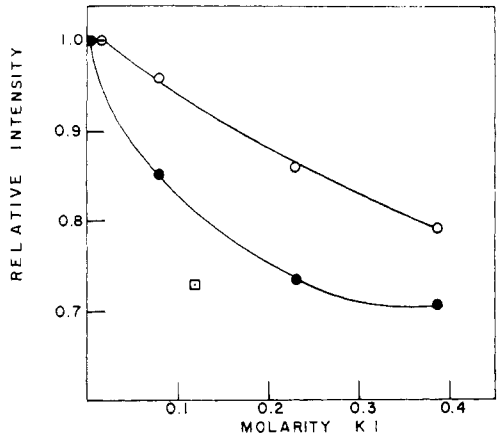

FIGURE 7: Quenching of CM-P by KI. The excitation wavelength is 280 mμ; (●) 0.1 M KOAc, pH 4.52. The emission wavelength is 335 mμ; (○) 0.1 M PO_4, pH 8.0. The emission wavelength is 350 mμ; (□) 6 M guanidine hydrochloride is 0.1 M PO_4, pH 7.6. The emission wavelength is 350 mμ.

Not all of the decrease in fluorescence yield resulting from NBS oxidation can be attributed to loss of tryptophans. The absorbance at wavelengths above 300 mμ increases significantly (Figure 5). Since the absorption appearing in this region overlaps the emission band of tryptophan, the possibility arises that part of the drop in quantum yield could arise from radiationless energy transfer from intact to oxidized tryptophans.

Nevertheless, it is probably safe to conclude that the residual tryptophans have an emission spectrum which is quite different from that of the dominant tryptophan, the maximum being shifted to lower wavelengths by over 30 mμ. This shift is in the direction expected if the residual tryptophans were in a relatively nonpolar environment compared to the dominant tryptophan.

Since the dependence of energy yield on excitation wavelength is greater for the residual tryptophans than for the dominant tryptophan, it appears that radiationless energy transfer from the tyrosines is more important for the former.

Perturbation and Quenching. The fluorescence of CM-P is quenched by KI at both pH 4.5, where the dominant tryptophan is quenched, and at pH 8.0, where it is unquenched (Figure 7). The quenching efficiency at pH 8.0 is actually substantially less than at pH 4.5, indicating that the dominant tryptophan is less susceptible to KI quenching than the residual tryptophans. Quenching at both pH values is substantially less than for CM-P in 5 M guanidine hydrochloride, in which the protein is expected to be substantially unfolded, with all tryptophan groups accessible to solvent.

Fluorescence perturbation studies of CM-P in 20% ethylene glycol (Table III) indicated almost no enhancement of fluorescence intensity at λ_{max} at pH 9.0 and a small increase at pH 4.2. Thus the dominant tryptophan appears to be less susceptible to ethylene glycol perturbation than the residual tryptophans.

Low-Temperature Behavior. Figure 8 shows the fluorescence and phosphorescence emission spectrum of Hg–P at liquid nitrogen temperatures. It is similar to that already reported for unfractionated papain under the same conditions and, in particular, displays at the highest resolution a significant splitting of the fluorescence band with detectible maxima at about 317 and 325 mμ (Weinryb and Steiner, 1970). At lower resolutions the 317-mμ peak appears as a shoulder. In 6 M guanidine hydrochloride–50% ethylene glycol the splitting disappears and a single peak at about 315–317 mμ is present—as reported earlier (Weinryb and Steiner, 1970).

TABLE III: Perturbation of Papain Fluorescence by Ethylene Glycol.[a]

Species	pH	Ethylene Glycol	Intensity Relative to that in Absence of Ethylene Glycol
Hg–P	5.0	20	1.08
CM-P	4.2	20	1.08
CM-P	9.0	20	1.00

[a] The excitation wavelength is 280 mμ. The emission wavelength is 350 mμ at pH 9.0 and 340 mμ at pH 4.2 and 5.0. The solvent is 0.01 M KOAc–0.01 M Tris.

The total emission spectrum for HSP is similar, except for an increase in intensity in the 350- to 400-mμ region. This suggests that tyrosine phosphorescence is making a contribution. This conclusion is confirmed by an examination of the phosphorescence spectrum, using a rotating shutter (Figure 8). For Hg–P the intensity in the 350- to 400-mμ region is quite low even when excited at 270 mμ. The contribution of tyrosine to the phosphorescence is less than 5% of the total. However, for HSP the tyrosine band appears as a distinct shoulder to the tryptophan band and accounts for about 15% of the total phosphorescence when the excitation wavelength is 270 mμ. When excitation is made at 295 mμ, which is outside the tyrosine absorption band, the emission below 400 mμ almost disappears (Figure 8).

It thus appears that some fraction of the tyrosine groups of papain which are "dark" at 25° in HSP emit at low temperature and that these are quenched by the Hg atom of Hg–P.

For HSP excited at 270 mμ the phosphorescence at 370 mμ and at 450 mμ displays exponential decay. The decay time at 450 mμ is 5.2 sec, which is characteristic of tryptophan, while that at 370 mμ is 2.4 sec, which is in the expected range for tyrosine.

Discussion

It is clear from the results reported here that the tryptophan residues of papain are far from equivalent in their luminescence properties. The behavior of each of the forms examined is consistent with the presence of one or more tryptophan residues of high-quantum yield which dominates the luminescence behavior of the protein when it is in the unquenched state.

It is, of course, very difficult to establish with certainty the number of tryptophans which are quenched at acid pH. However, the bandwidth of the tryptophan emission which is quenched, as obtained by difference (Figure 2), is relatively narrow and comparable to that of tryptophan itself. If more than one tryptophan is involved, their emission properties must be very similar. This seems unlikely in view of the anomalous properties of the quenched tryptophan, which would be unlikely to recur among a set of tryptophans of such differing microenvironments. Moreover, both would have to be within the quenching radius of the Hg atom of Hg–P. While two tryptophans (26 and 177) are in fact close to the Hg atom,

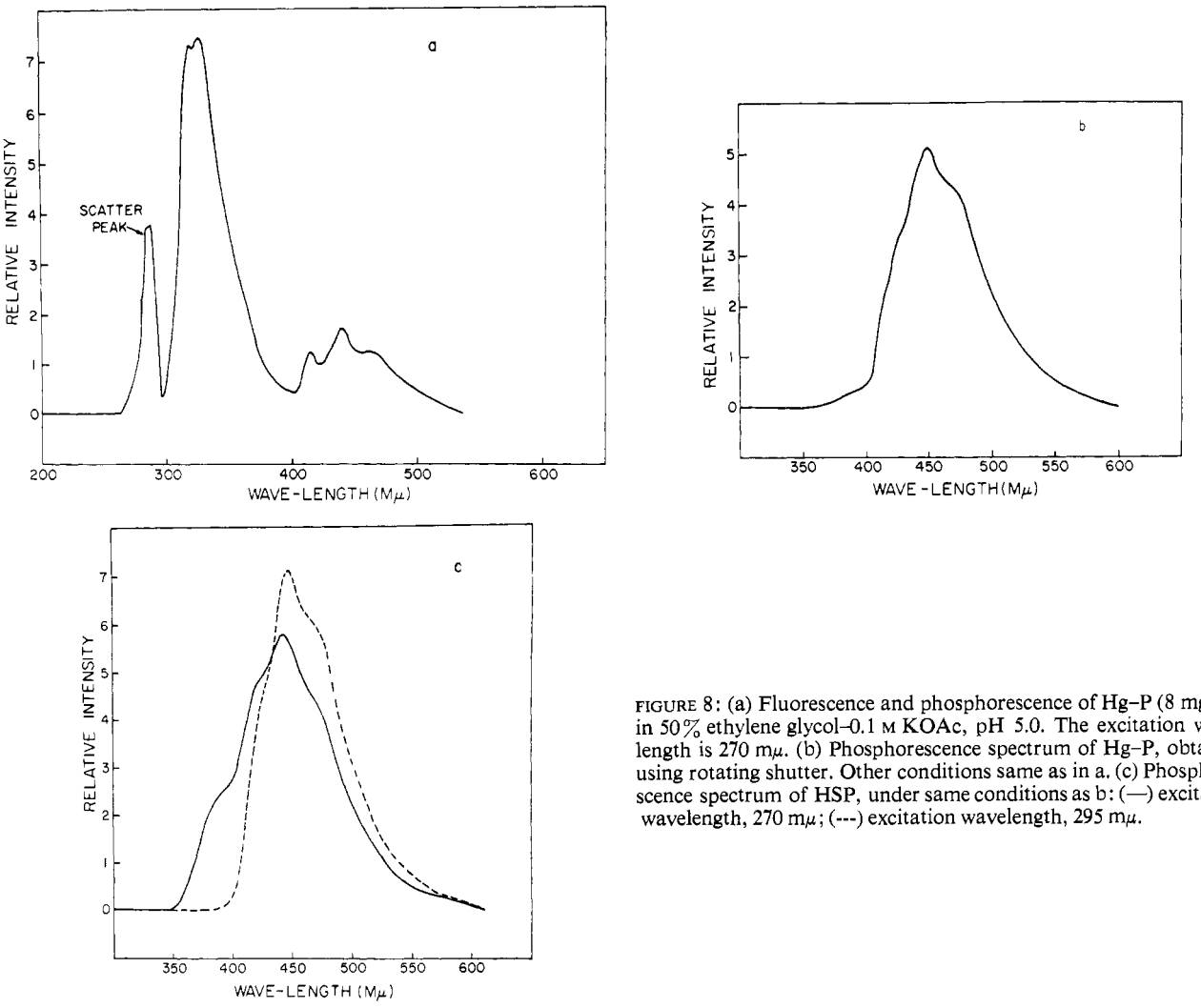

FIGURE 8: (a) Fluorescence and phosphorescence of Hg–P (8 mg/ml) in 50% ethylene glycol–0.1 M KOAc, pH 5.0. The excitation wavelength is 270 mμ. (b) Phosphorescence spectrum of Hg–P, obtained using rotating shutter. Other conditions same as in a. (c) Phosphorescence spectrum of HSP, under same conditions as b: (—) excitation wavelength, 270 mμ; (---) excitation wavelength, 295 mμ.

their microenvironments are so different that it would be surprising if they had similar emission properties.

For these reasons, which are admittedly inconclusive, we shall discuss the emission properties of papain in terms of a single dominant tryptophan, although the above reservations should be kept in mind.

The properties of this dominant tryptophan, or possibly tryptophans, are decidedly unusual. Its quantum yield and excited lifetime are remarkably high. From the fractional drop in energy yield occurring upon forming the Hg derivative of HSP or upon the acid quenching of CM-P or HSP, it can be estimated that 50–60% of the total fluorescence yield of papain may be accounted for by this residue (Table I). If the tryptophan-based yield for excitation at 280 mμ is taken as about 0.28 for CM-P in the unquenched state (Table I) (assuming a yield for tryptophan of 0.14) then the yield for the dominant tryptophan of CM-P would be >0.5. If two tryptophans are involved, the yield would be >0.25. This may actually be a lower limit, since quenching of the dominant tryptophan may not be quantitative. The apparent excited lifetime is about 6 nsec when the dominant tryptophan is unquenched. Since this represents a weighted average, the lifetime of the dominant tryptophan itself may be higher.

The spectral distribution of the fluorescence of the dominant tryptophan is similar to that for tryptophan itself, or its oligopeptide derivatives, in water, suggesting that its microenvironment is highly polar. Since it can be oxidized by NBS, it must be to some extent accessible to reagents.

Nevertheless, the dominant tryptophan is not sensitive to the action of quenchers, or neutral solvent perturbants. This is not necessarily inconsistent with its oxidation by NBS, since even a limited accessibility could permit attack by a chemical reagent, which requires only a single transient contact with the critical portion of the residue.

The dominant tryptophan is quenched by an Hg atom linked to the sulfur of the cysteine residue which is a component of the catalytic site, as well as by the free sulfhydryl group itself. The quenching by Hg is presumably an instance of the well-known "heavy-atom" effect, which induces quenching by enhancing the rate of intersystem crossing. Sulfhydryl compounds are also known to quench tryptophan and other indole derivatives (Steiner and Kirby, 1969). Since both effects are expected to be relatively short range, there is a definite implication that the dominant tryptophan is in proximity to the cysteine of active papain. It must also be close to a second potential quenching group with a pK close to 5.5 in the case of CM-P. This could be either a carboxyl group or an imidazole group of histidine, both of which have pK's in the expected range (Steiner and Kirby, 1969; Shinitzky and Goldman, 1967). The X-ray crystallographic studies of Drenth *et al.* (1968) provide a basis for selecting the most likely possibility for the dominant tryptophan. It must, of course, be recognized

that the conformation of papain in solution may not be identical with that of the enzyme in the crystalline state. Subject to this reservation, we can make the following speculative argument.

It is logical to expect the dominant tryptophan to be near the single sulfhydryl group. On this basis, Trp 26 and Trp 177 are likely possibilities, since both are placed close to Cys 25. Trp 7 and Trp 181 are less likely, being somewhat more removed from Cys 25. Trp 69 is also somewhat unlikely, being largely shielded from Cys 25 by other residues.

If, on this speculative basis, one accepts Trp 26 and Trp 177 as the leading candidates, one finds that each has some, but not all, of the characteristics expected for the dominant tryptophan. Trp 26 is the only tryptophan residue which is almost entirely buried and shielded from solvent. This is consistent with the inaccessibility of the dominant tryptophan to quenchers and neutral perturbants, and may not be inconsistent with its susceptibility to NBS oxidation, since a minor conformational change in solution might permit transient contact with reagent. The solvent-shielded character of Trp 26 would be expected to favor an increased quantum yield.

The strongest argument against identifying the dominant tryptophan with Trp 26 is the spectral distribution of fluorescence of the former, which is typical of a tryptophan exposed to aqueous solvent (Konev, 1967; Weber and Teale, 1965). Indeed, the quenching of the dominant tryptophan produces a shift in λ_{max} to lower values, as if the remaining tryptophans were less, rather than more, exposed to solvent.

Trp 177 (and Trp 69) are the only papain tryptophans with a high degree of exposure to solvent. This is consistent with the spectral distribution of the dominant tryptophan, but not with its lack of susceptibility to external quenchers or perturbants.

Both Trp 26 and Trp 177 are in proximity to a potential quenching group with a pK in the appropriate acid range. Trp 26 is near a carboxyl group and Trp 177 is close to a histidine, as is Trp 181.

In summary, if Trp 26 is the dominant tryptophan, then some unexplained aspect of its microenvironment must endow it with a spectral distribution of fluorescence characteristic of a polar medium. If Trp 177 is the dominant tryptophan, then it must somehow be shielded from external perturbation, possibly as a result of a structural alteration from the crystalline form, which blocks the approach of quencher or perturbant, but leaves the tryptophan in a polar solvent medium.

If Trp 26 proves to be the dominant tryptophan, a speculative possibility for the origin of the displacement of its fluorescence band to relatively long wavelengths might be the proximity of a charged group and the existence of an ion–dipole interaction involving the first excited singlet state.

There remains the problem of accounting for the unusually high quantum yield and lifetime of the dominant tryptophan. If the tentative assignment made earlier is correct, there is no very obvious explanation in terms of the known microenvironment, nor is any clue provided by the existing studies upon tryptophan-containing model compounds. It is clear that our understanding of the factors governing the luminescence properties of tryptophan residues in proteins is still far from complete. In all probability, studies with much more sophisticated model compounds will be required.

The remaining tryptophan residues have collectively an emission spectrum suggestive of a relatively nonpolar microenvironment. Although the relative magnitudes of their contributions to the quantum yield are unknown, it is possible that both the buried residue 26 and the unburied, or incompletely buried, residues contribute to the observed spectrum. The observation of quenching by KI indicates that at least some of this class of tryptophan residues are accessible to solvent. It should be mentioned that even the two unburied residues 69 and 177 have their indole rings parallel to, and partially shielded from solvent by, the enzyme surface in the crystalline state (J. Drenth, 1969, personal communication). Hence one, or both, of these could have emission characteristics which are not typical of a wholly polar microenvironment.

The two or three residues which are resistant to NBS oxidation are included in the above group and show, as expected, emission properties suggestive of a nonpolar environment.

The results reported here provide further evidence for the occurrence of significant energy transfer from tyrosine to tryptophan at the singlet level. This is less important for the dominant tryptophan than for the remaining tryptophans, possibly as a consequence of quenching of its vicinal tyrosines by other mechanisms.

It is not as yet possible to do more than speculate as to the origin of the significant splitting of the tryptophan fluorescence band at low temperatures. Possibly, it arises from the differing microenvironments of the exposed and buried tryptophans.

The suppression of tyrosine emission at 25° is not entirely due to radiationless transfer to tryptophan, at least in the case of HSP, in view of the appearance of a prominent tyrosine phosphorescence band at liquid nitrogen temperatures. Part of the quenching of tyrosine must arise from other competitive radiationless deactivation processes, which are temperature dependent. Since tyrosine emission is largely quenched for Hg–P, it appears that the tyrosines affected by the latter lie within the quenching radius of the Hg atom.

References

Barel, A. O., and Glazer, A. N. (1969), *J. Biol. Chem. 244*, 268.

Chen, R. F., Vurek, G. G., and Alexander, N. (1967), *Science 156*, 949.

Drenth, J., Jansonius, J. N., Koekoek, R., Swen, H. M., and Wolthers, G. B. (1968), *Nature (London) 218*, 929.

Konev, S. V. (1967), Fluorescence and Phosphorescence of Proteins and Nucleic Acids, New York, N. Y., Plenum.

Longworth, J. W. (1968), *Photochem. Photobiol. 7*, 587.

Purkey, O., and Galley, W. C. (1970), *Biochemistry 9*, 3569.

Shinitzky, N., and Goldman, R. (1967), *Eur. J. Biochem. 3*, 139.

Sluyterman, L. A. A. E., and De Graaf, M. J. M. (1970), *Biochim. Biophys. Acta 200*, 593.

Sluyterman, L. A. A. E., and Wijdenes, J. (1970), *Biochim. Biophys. Acta 200*, 595.

Steiner, R. F., and Kirby, E. P. (1969), *J. Phys. Chem. 73*, 4130.

Teale, F. W. J., and Badley, R. A. (1969), *J. Mol. Biol. 44*, 71.

Weber, G. and Teale, R. W. J. (1965), *Proteins 3*, 445.

Weinryb, I., and Steiner, R. F. (1970), *Biochemistry 9*, 135.

Weinryb, I., and Steiner, R. F. (1968), *Biochemistry 7*, 2488.

Witkop, B. (1961), *Advan. Protein Chem. 16*, 221.