Studies of the Triplet State of Proteins by Electron Spin Resonance Spectroscopy*

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ABSTRACT: We have examined some features of the triplet states of proteins and model compounds in part to explore the relationship of this property to the structures of metalloenzymes. Self-quenching of the L-tryptophan triplet state was observed above 10^{-4} M. The triplet yield of poly-L-tryptophan was *ca.* half of that of glycyl-L-tryptophan. The lifetimes of the triplet states in different proteins (ovalbumin, β-lactoglobulin, bovine serum albumin, at 77°K in 50% glycerol glass) were not the same, but all approached that of tryptophan upon urea denaturation. The presence of Cu(II) completely quenched the triplet states of glycyl-L-tryptophan and poly-L-tryptophan, but not that of bovine serum albumin. Apohemocyanin and anaerobic hemocyanin gave similar triplet yields, but upon oxygenation of hemocyanin, the triplet state was quenched, suggesting dipolar interaction between the copper–oxygen system and tryptophan in the protein.

The excited states of various proteins and aromatic amino acids have been extensively studied by methods involving fluorescence. The properties of the excited singlet state in proteins have been particularly well worked out by fluorescent measurements and related to the structure and interactions of proteins (Weber and Teale, 1965). However, the phosphorescing or triplet state of proteins is still poorly understood.

The triplet states of aromatic amino acids (Ptak and Douzou, 1963; Shiga and Piette, 1964; Maling *et al.*, 1965) and of proteins (Shiga and Piette, 1964) have been observed by electron spin resonance spectroscopy. It has been shown that the esr[1] signal of phosphorescing proteins at 1450 gauss (X-band spectrometer) originates from the triplet state of tryptophan residues in the protein (Shiga and Piette, 1964) and corresponds to the $\Delta m = \pm 2$ transition. In this study, we have examined some features of the triplet states of proteins and model compounds to provide more information upon which to base structural interpretations, and to explore the relationship of the triplet state to the ligand system and the oxidation state of metals in metalloproteins. We have examined self- (concentration) quenching, changes in triplet state lifetimes with conformational changes, and the effect of paramagnetic species (Cu^{2+}) upon the triplet state yield in the following systems: L-tryptophan, glycyl-L-tryptophan, poly-L-tryptophan, ovalbumin, β-lactoglobulin, bovine serum albumin, and native, apo- and oxyhemocyanins (*Cancer magister* and *Limulus*).

Materials and Methods

Materials. L-tryptophan and glycyl-L-tryptophan were obtained from Nutritional Biochemicals Corp.; poly-L-tryptophan, from Mann Research Laboratory. $Cu(NO_3)_2 \cdot 3H_2O$ and phosphate salts were obtained from Matheson Coleman and Bell Co.; urea, from J. T. Baker Chemical Co.; and methanol and glycerol, from Allied Chemical, General Chemical Division. All these chemicals were reagent grade and not further purified. Crystalline ovalbumin was obtained from Pentex, Inc.; β-lactoglobulin, from the Bureau of Agriculture and Industrial Chemistry, U. S. Department of Agriculture; and crystalline bovine serum albumin, from Calbiochem Co. *C. magister* hemocyanin was prepared essentially as already described (Thomson *et al.*, 1959), with omission of the dialysis step and the substitution of Tris buffer, 0.01 M in calcium ion, for phosphate buffer. Apohemocyanin was prepared by dialysis of an aliquot of freshly prepared concentrate against cyanide (Ghiretti, 1956). All native and apohemocyanin solutions were passed through a column of Sephadex G-75 before use in the esr experiments to minimize an effect of any contaminating paramagnetic species. *Limulus* hemocyanin was prepared by the same method. The hemocyanins were prepared in 0.1 mM stock solutions.

Apparatus. A Varian V-4500 epr spectrometer with Fieldial magnetic field control unit and with 100-kc

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[1] Abbreviations used: esr, electron spin resonance.

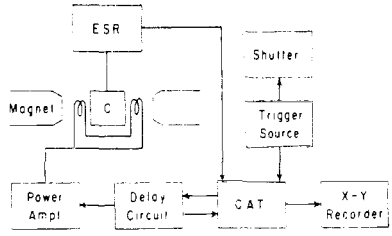

FIGURE 1: A diagram of esr–CAT operation (see text). C is esr cavity. The power amplifier was a Harrison Laboratory Model 855C power supply. The delay time between each sweep was set to 30 msec. The trigger switch was manually operated; shutter was operated by a solenoid.

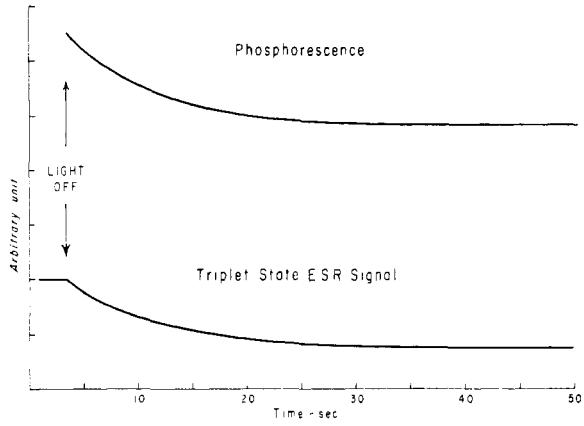

FIGURE 2: Decay curve of esr triplet signal and of phosphorescence of tryptophan. Excitation: 290 mμ (half band width, 12.5 mμ), 3×10^{-4} cal sec^{-1} cm^{-2}. L-Tryptophan concentration, 10^{-3} M in 1:1 glycerol–phosphate buffer (0.1 M, pH 7.0) containing dissolved air, at 77°K.

field modulation unit was used. The apparatus included a microwave circulator,[2] by means of which the power of the incident microwave radiation at the cavity could be systematically varied and accurately measured. The light source was a Bausch and Lomb monochromater No. 33-86-26 with an Osram HBO 200-w high-pressure mercury lamp. Fluorescence was measured by an Aminco spectrofluorometer. The recording systems used for esr measurements were a Varian F-80 XY-recorder, a Sanborn model 320 dual-channel recorder, and a Varian C-1024 time-averaging computer (CAT). A simultaneous measurement of the phosphorescent and of the esr triplet signal was carried out, using a Varian V-4534 optical transmission cavity, a modified Bausch and Lomb Spectronic 20 photometer, and a Tektronix type 564 storage oscilloscope with a dual trace plug-in unit. The absorption spectra of the materials were checked with a Cary Model 14 spectrophotometer. The excitation light intensity was measured by a calibrated Eppley thermopile.

Methods. A quartz tube (i.d., 2.7 mm) containing the sample solution was put into a liquid nitrogen filled Varian V-4546 quartz dewar. The dewar was inserted into the Varian V-4531 multipurpose cavity. The samples were irradiated through the window of the cavity. (a) In most cases, the esr signal was recorded ordinally by an XY-recorder. For the decay time measurement, the magnetic field was fixed at the maximal deflection point of the first derivative curves of the esr signal, and the decay of the signal amplitude, after shutting off the excitation light, was recorded by the Sanborn recorder or by the CAT. (b) In the case of very weak signals, a method developed by Varian Associates was employed in connection with the CAT. As shown in Figure 1, external modulation coils (ϕ 4.5–6.0 cm) were attached to the cavity. At a fixed magnetic field, an amplified sawtooth sweep from the CAT time base was applied to the external modulation coils. The esr signals, obtained during each sawtooth sweep, were stored in the CAT memory. After 1–600 sweeps, the stored memory was plotted by the XY-recorder. Usually the sweep rate was 110 gauss/250 msec, and the time constant of the esr spectrometer was 3–30 msec. The lifetime measurements were also repeated and fed consecutively into the CAT, when the esr signal was very weak. (c) Simultaneous observations of phosphorescent and esr signals were carried out, using an optical transmission cavity. The sample was irradiated from the front window, and a Corning 5433 glass filter was placed at the rear window, in front of a phototube used to observe phosphorescence. The signals from the epr spectrometer and photometer were recorded by an oscilloscope.

Solvent. The choice of the solvent was a most critical part of the experiments. Since we were dealing with the biological material, the organic solvents which are commonly used for this kind of work (Smith *et al.*, 1962) could not be used. It is preferable that the solvent cools to a transparent rigid glass at low temperature for the accuracy of the triplet state concentration measurement and for a high yield of triplet state molecules. Aqueous buffer solutions change to opaque polycrystalline solids at liquid nitrogen temperature. Using this opaque solid sample, only the decay rate measurements were possible, but no quantitative work could be done, due to a considerable light scattering. A mixture of 50% glycerol–water (v/v) gave a good glass at 77°K, so that a 1:1 mixture of glycerol and buffer was adopted for most of the experiments. Yasumatsu *et al.* (1965) report that high concentrations of polyhydric alcohols repress denaturation of enzymes, and hemocyanin was observed to oxygenate satisfactorily in the mixture. However, our results must be referred to the specific solvent used, because of the possibility that conformational changes may have been induced and because glycerol binds Cu^{2+}, and may compete for this ion in some cases. Either pure methanol or a 1:1 methanol–glycerol mixture was used as solvent

[2] We thank Mr. Roy Hansen and Dr. H. Beinert for the design of the circulator.

for poly-L-tryptophan. In general, a 1:1 glycerol–phosphate buffer (0.1 M, pH 7.0) mixture was used in the quantitative measurements. For decay time measurements, phosphate buffer only was used as a solvent.

Molecular oxygen dissolved in the solvent is known to affect the triplet state (Porter and Windsor, 1958). However, we had difficulty in obtaining proven oxygen-free protein solutions, especially with very concentrated proteins. Therefore, all experiments were carried out in the presence of dissolved oxygen, except for those in which the effect of O_2 binding upon the hemocyanin triplet state was examined. In this case, the glycerol was deoxygenated with a stream of pure nitrogen passed through a syringe needle into a flask capped with a rubber serum-bottle closure. The exhaust gas was vented through a second needle. After 48 hr of bubbling with nitrogen, both needles were withdrawn, leaving a rubber-capped flask of anaerobic glycerol from which the solvent could be readily removed by a nitrogen-flushed syringe and needle. Hemocyanin solutions were deoxygenated in a similar manner, except that the flask was swirled in an ice bath and the nitrogen was passed without bubbling through the protein solution. When the hemocyanin had become completely anaerobic, as indicated by complete loss of blue color due to oxyhemocyanin (2–3 hr), the needles were withdrawn. This system remained anaerobic for several hours. In order to prepare an anaerobic solution in an esr tube, the tube was capped with a small serum-bottle stopper, through which two syringe needles had been passed, and nitrogen was allowed to flow into (and out of) the tube for 1 hr. The needles were then withdrawn, and anaerobic hemocyanin and glycerol were transferred from their anaerobic flasks by means of nitrogen-flushed gas-tight syringes to the bottom of the esr tube. The hemocyanin remained colorless during this procedure and the subsequent spectrometric manipulations.

Concentration Measurement. Since the observed esr signal is a forbidden transition, there is no way of measuring the concentration of the triplet state at the moment. The "triplet yield" is, therefore, expressed simply by the peak-to-peak height of the observed first derivative curves, divided by the measured excitation light intensity at that wavelength and slit settings, to correct for large differences in lamp output over the range of irradiating wavelength. The solvent used gives a good glass at 77°K, but sometimes makes small crevices. The error due to the light scattering from these small crevices was not taken into account. There was also no correction made for the absorption of the excitation light by the liquid nitrogen dewar and sample tube in the action spectrum measurements.

Microwave power saturation was observed above 2 mw of incident power at the entrance of the cavity. However, as shown later, the patterns of the saturation curves were almost the same for all esr signals, so that 25 mw of incident power was used throughout the experiments.

In view of the complexity of the irradiation and detection system, a test of the relationship between the concentration of protein and triplet yield was made, using bovine serum albumin in 50% glycerol buffer (pH 7.0) glasses. At 25 mw of incident microwave power, and using illumination at 285 mμ, it was found that the triplet yield could be repeated within 20% at the higher concentrations (0.5–1.0 mM) and larger signal heights, but that errors became substantial (100%) in the low-yield range. The relationship was linear, however. The contents of a single esr tube, repeatedly frozen in liquid N_2 and thawed, gave high reproducibility of signal height, showing that variations in the number and size of the cracks in the glycerol–buffer glass, and denaturation of the protein under these conditions, did not play a detectable role.

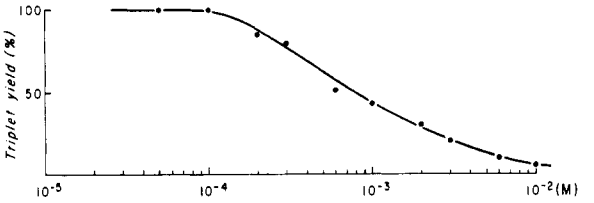

FIGURE 3: Self-quenching of the L-tryptophan triplet state in 1:1 glycerol–phosphate buffer (0.1 M, pH 7.0) containing dissolved air, measured at 77°K. Excitation: 290 mμ (half band width 12.5 mμ), 3×10^{-4} cal sec^{-1} cm^{-2}. Esr-CAT recording: microwave power, 25 mw at the entrance of the cavity; field modulation, 100 kc, 3.8 gauss; time constant, 0.1 msec; magnetic field scanning speed, 110 gauss/50 msec; repetition, 100–600 times. The ratio of triplet yield (η/η_0) is in per cent, and η_0 is a calculated value assuming no self-quenching, and η is an observed value.

Results

L-Tryptophan. The esr signal of the L-tryptophan triplet state, in 1:1 glycerol–phosphate buffer (0.1 M, pH 7.0) at 77°K, was similar to that reported previously (Shiga and Piette, 1964). The half-life was 4.4 sec. The decay curve of the esr signal, after the excitation light was shut off, was parallel to that of the phosphorescence (Figure 2). A quenching of the triplet yield occurred at high concentrations of L-tryptophan (Figure 3). This self-quenching (or concentration quenching) began above 10^{-4} M. The lifetime of the L-tryptophan triplet state seemed to be independent of the L-tryptophan concentration within experimental error. It was uncertain that the light intensity was strong enough to irradiate totally all of the sample at high concentrations of L-tryptophan. A rough judgment could be made by observing the phosphorescence. At a relatively low concentration, the phosphorescence could be observed all over, from the front, facing the excitation light, to the back of the sample tube. However, at higher concentrations ($>2 \times 10^{-3}$ M), the rear of the sample was not phosphorescent, *i.e.*, all the excitation light was absorbed before it reached the rear of the sample. An

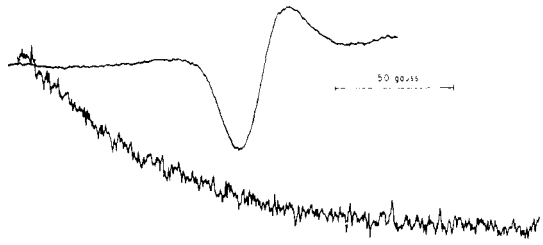

FIGURE 4: Esr signal and decay curve of the glycyl-L-tryptophan triplet state. Concentration 10^{-3} M, excitation same as that of Figure 2, at 77°K. Esr recording: microwave power, 25 mw; modulation, 100 kc, 3.8 gauss; time constant, 0.3 sec; magnetic field scanning speed, 250 gauss/2.5 min. Decay time recording: time constant, 30 msec, fed into CAT once and plotted by XY-recorder. The total trace of the decay curve corresponds to 25 sec.

addition of urea to L-tryptophan led to a decrease of triplet yield (*ca.* 15%) in 1:1 glycerol–phosphate buffer, but no change in lifetime.

Glycyl-L-tryptophan. The esr signal of the glycyl-L-tryptophan triplet state was the same as that of L-tryptophan (Figure 4) and the half-life was 4.5 sec in 1:1 glycerol–phosphate buffer (0.1 M, pH 7.0) at 77°K. However, the triplet yield was increased *ca.* 25%, compared with that of L-tryptophan, in 1:1 glycerol–methanol, per unit optical density.

Poly-L-tryptophan. The esr signal was the same as that of the L-tryptophan triplet state. The half-life of the poly-L-tryptophan triplet state was 4.2 sec in 1:1 glycerol–phosphate buffer (0.1 M, pH 7.0) at 77°K. The triplet yield in a 1:1 glycerol–methanol was approximately half that observed in glycyl-L-tryptophan, per optical density unit.

Proteins. The resonant magnetic field and the shape of the triplet state signals of proteins (ovalbumin, β-lactoglobulin, bovine serum albumin, and hemocyanins) were almost the same and were similar to that of L-tryptophan. However, the half-lives of the triplet state of the proteins were not the same when they were measured in a polycrystalline solid of phosphate buffer (0.1 M, pH 7.0) at 77°K. Upon urea treatment (protein dissolved in the phosphate buffer containing 8 M urea and kept at room temperature for 3 hr), the half-lives were prolonged. The half-lives were also prolonged in 1:1 glycerol–phosphate buffer (0.1 M, pH 7.0), either in the presence or absence of 8 M urea. The measured half-lives are summarized in Tables I and II.

The action spectra were taken in 1:1 glycerol–phosphate buffer (0.1 M, pH 7.0) at 77°K (Figures 5 and 6). Urea treatment (protein dissolved in the phosphate buffer containing 8 M urea, kept at room temperature 3 hr, and then mixed with a same amount of glycerol) altered the action spectra slightly.

The effect of oxygen was studied in the case of native hemocyanin because the oxidation state of copper in oxyhemocyanin is an interesting problem. We found that the copper-free apohemocyanin and anaerobic hemocyanin gave triplet signals of the same intensity, whereas the yield from oxyhemocyanin under the same conditions was much lower (Figure 5 and Table III).

TABLE I: The Half-Lives of the Tryptophan Triplet State in Proteins at 77°K (sec).

Solvent	Ovalbumin	β-Lactoglobulin	Bovine Serum Albumin
Buffer[a]	3.3	3.9	3.7
Buffer + 8 M urea[b]	4.3	4.7	4.0
1:1 Glycerol–buffer[c]	4.1	4.0	4.6
1:1 Glycerol–buffer + urea[d]	4.4	4.8	4.7

[a] 100 mg/ml (dry weight) of protein was dissolved in phosphate buffer (0.1 M, pH 7.0). [b] 100 mg/ml (dry weight) of protein was dissolved in phosphate buffer (0.1 M, pH 7.0) containing 8 M urea, and incubated for 3 hr at room temperature. [c] 60 mg/ml (dry weight) of protein was first dissolved in phosphate buffer (0.1 M, pH 7.0), then a same amount of glycerol was added. [d] 60 mg/ml (dry weight) of protein was dissolved in phosphate buffer (0.1 M, pH 7.0) containing 8 M urea, incubated at room temperature for 3 hr, then a same amount of glycerol was added.

TABLE II: The Half-Lives of the Triplet State of Tryptophan in Hemocyanin at 77°K (sec).

Solvent	Native Hemocyanin (0.05 mM; 1.2 mM Cu) Oxy-	Native Hemocyanin (0.05 mM; 1.2 mM Cu) Deoxy-	Apohemocyanin (0.05 mM; 0.04 mM Cu) Deoxy-
(a) Tris buffer [0.05 M, pH 6.8, containing 0.01 M Ca(II)]	1.7	4.8	4.8
(b) 1:1 glycerol–Tris buffer and same as above	3.4 3.2	4.4 3.9	3.8 4.0

Effect of Cupric Ion. The cupric ion quenched the triplet yield of glycyl-L-tryptophan and of poly-L-tryptophan, but not of L-tryptophan. The quantitative data are summarized in Figure 7. The half-quenching

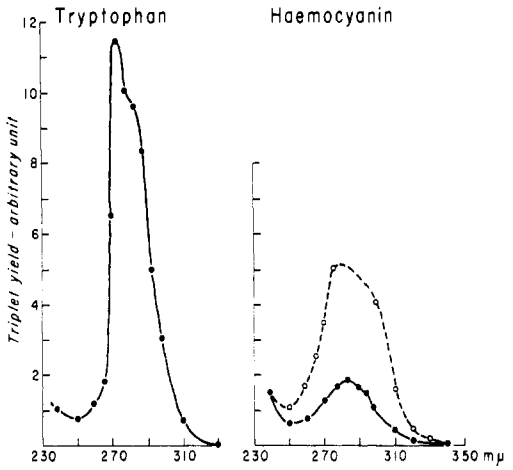

FIGURE 5: Action spectra of the triplet state of L-tryptophan and of hemocyanin. Tryptophan concentration 3×10^{-3} M in 1:1 glycerol–phosphate buffer (0.1 M, pH 7.0) at 77°K. Half band width of the excitation light was 7.4 mμ. Hemocyanin concentration: 0.05 mM in Tris buffer (Table II), at 77°K. Dotted line: apohemocyanin, 0.05 mM (containing dissolved air). Solid line: oxygenated native hemocyanin (0.05 mM).

concentration of Cu(II) was 2×10^{-4} M for 4.6×10^{-4} M of glycyl-L-tryptophan and 3×10^{-4} M for poly-L-tryptophan (equivalent to 1.25×10^{-3} M of L-tryptophan), in 1:1 glycerol–phosphate buffer (0.1 M, pH 7.0) and in methanol, respectively. The molar ratio of Cu(II) to L-tryptophan at half-quenching was 1:2 and 1:4.

TABLE III: The Effect of Oxygen on the Tryptophan Triplet Yield in Hemocyanin at 77°K (%).

C. magister Hemocyanin[a]	Native Hemocyanin Oxy-	Native Hemocyanin Deoxy-	Apohemocyanin Deoxy-
b	100	40	180
c	100	20	60
Limulus hemocyanin	100	40	100

[a] *C. magister* hemocyanin in glycerol–Tris buffer (same as Table II). *Limulus* hemocyanin, 1 mM containing 0.159% Cu, in glycerol–Tris buffer. *Limulus* apohemocyanin, 1 mM but containing 0.026% Cu. [b] See *a* of Table II. [c] See *b* of Table II.

The half-life, the signal shape, and the resonant magnetic field were not modified by an addition of Cu(II). In these experiments, the concentration of the glycyl-L-tryptophan and poly-L-tryptophan was somewhat higher than the concentration at which no self-quenching occurred, because at concentrations of 10^{-4} M or less, the poor signal:noise ratio led to an ambiguity in the measurement of the quenching effect of Cu(II).

Cu(II) was added to bovine serum albumin up to eight atoms of Cu(II) per molecule of protein, also to ovalbumin up to 10 atoms of Cu(II) per molecule of protein, but no appreciable change in triplet yield was observed at pH 7. The esr line shapes of Cu(II) protein and glycerol are very different. Using this basis, a small

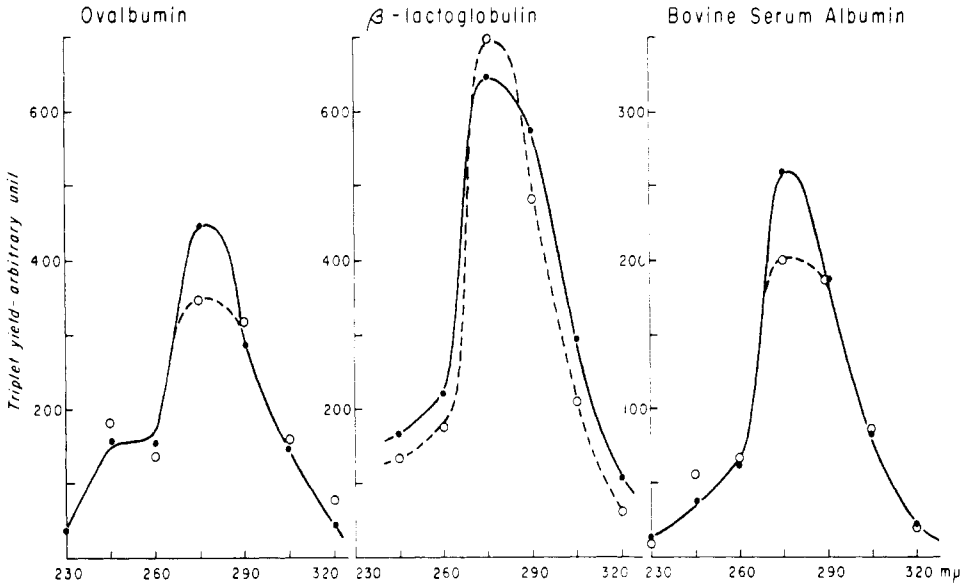

FIGURE 6: Action spectra of the triplet state of proteins. Concentration of protein 25 mg (dry weight)/ml in 1:1 glycerol–phosphate buffer (0.1 M, pH 7.0), at 77°K. (a) Ovalbumin, (b) β-lactoglobulin, and (c) bovine serum albumin. Half band width of the excitation light was 12.5 mμ. Esr-CAT recording: same as that of Figure 2. Preparation of the sample: same as that of Table I.

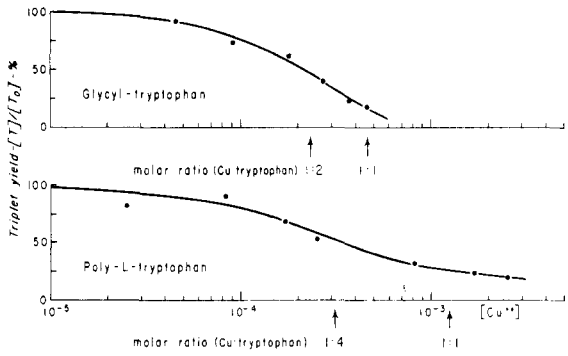

FIGURE 7: Quenching effect of Cu(II) on the triplet yield of glycyl-L-tryptophan and of poly-L-tryptophan. Concentration: glycyl-L-tryptophan 4.6×10^{-4} M in 1:1 glycerol–phosphate buffer (0.1 M, pH 7.0); poly-L-tryptophan, equivalent to 1.25×10^{-3} M of tryptophan, in methanol. Excitation at 290 mμ, half band width 12.5 mμ, 3×10^{-4} cal sec^{-1} cm^{-2}, at 77°K.

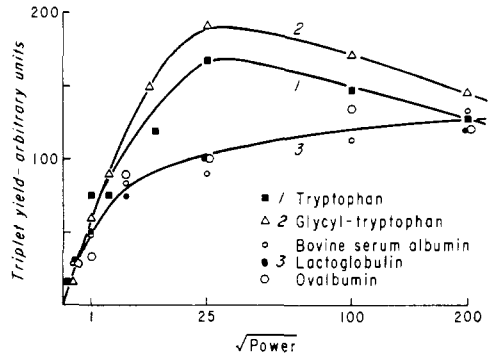

FIGURE 8: Microwave power saturation of the triplet signals. (Microwave power)$^{1/2}$ *vs.* (normalized signal amplitude in arbitrary units).

amount of Cu was found to be removed from the proteins by glycerol under the conditions of our experiments.

Light Intensity Saturation. The triplet yield was not proportional to the excitation light intensity, but saturated gradually as the light intensity was increased. This phenomenon was observed in all the triplet states studied (see Discussion).

Microwave Power Saturation. The power saturation of the esr signal was observed by changing the incident microwave power. Saturation of the signals began above 2 mw (measured at the entrance of the cavity), but the line width did not change. The saturation curves were similar in low power region for all the triplet states studied (Figure 8). An addition of Cu(II) did not affect the saturation behavior of the esr signal.

Discussion

Light Intensity Saturation and Self-Quenching. The phenomenon of light intensity saturation in phosphorescence was first mentioned in 1940 (Levshin and Tugarinov, 1940; Lewis *et al.*, 1941). As discussed in a previous paper (Shiga and Piette, 1965), this phenomenon can be treated in the following manner. Assuming the absence of electronic excited states higher than the first excited states, and the absence of a secondary photochemical reaction, *e.g.*, free radical formation of solute and/or solvent, then the photoinduced changes of the electronic states can be simplified as follows

$$S_0 + h\nu \longrightarrow S_1 \qquad (1)$$

$$S_1 \xrightarrow{k_2} S_0 + \text{fluorescence} \qquad (2)$$

$$S_1 \xrightarrow{k_2'} S_0 + \text{heat} \qquad (2')$$

$$S_1 \xrightarrow{k_3} T \qquad (3)$$

$$T \xrightarrow{k_4} S_0 + \text{phosphorescence} \qquad (4)$$

$$T \xrightarrow{k_4'} S_0 + \text{heat} \qquad (4')$$

Here, S_0, S_1, and T are the ground, the first excited singlet, and triplet states, respectively. For simplification, $k_2 + k_2' = k_s$ and $k_4 + k_4' = k_d$. The reverse reaction of reaction 3 can be neglected at low temperature. For the steady-state approximation, $d[S_1]/dt = 0$ and $d[T]/dt = 0$, the following equation can be derived

$$\frac{[A]}{[T]} = 1 + \frac{k_d}{k_3} + \frac{(k_s + k_3)k_d}{I\alpha k_3} \qquad (5)$$

where $[A] = [S_0] + [S_1] + [T]$ is the total concentration of solute, I is the light intensity, and α is a correction factor corresponding to a change in the oscillator strength.

Applying eq 5 to the experimental data of L-tryptophan triplet state, a number of straight lines were obtained from the [A]/[T] *vs.* $1/I$ plot (Figure 9). At concentrations below 10^{-4} M (no self-quenching), the lines overlapped, showing no change in rate constants or α. However, once self-quenching began (2×10^{-4} M), the slope increased. Since k_d and the intercept did not change, it was certain that the high concentration did not appreciably alter k_3 (a rate constant for intersystem crossing)[3] but caused a decrease in α (corresponding to a change in the excitation coefficient), and/or caused an increase in k_s (corresponding to a shortening of the excited singlet state lifetime). The reason for this may be dimer or excimer (Stevens and Hutton, 1960;

[3] Since $k_d \ll k_3$, the intercept will be 1, in this case; thus, a decrease in k_3 could occur without changing the intercept, although unlikely.

Stevens, 1961)[4] formation. At concentrations greater than 2×10^{-3} M, the intercept and the slope increased. This apparently strong quenching may be due to a shielding effect. That is, the light intensity was not enough to irradiate all of the sample, as was mentioned before.

These results showed that it is permissible to discuss certain properties of the tryptophan triplet state in proteins (*e.g.*, lifetime, signal shape, etc.) without knowing the degree of self-quenching occurring in a given protein.

Glycyl-L-tryptophan showed a somewhat greater triplet yield than L-tryptophan, in 1:1 glycerol–methanol. However, an accurate measurement was not possible, since the quantitation should be carried out at low concentration where no self-quenching occurs. Under these conditions, the poor signal:noise ratio is critical. Even the small crevices in the solvent glass may cause significant error. It is, however, noteworthy that the glycyl-L-tryptophan triplet yield was not less than that of L-tryptophan. This fact is in sharp contrast with fluorescence measurements (White, 1959; Cowgill, 1963a,b), which show a considerable decrease in quantum yield for glycyl-L-tryptophan. Since the fluorescence measurements were carried out in the aqueous phase at room temperature, but the triplet state experiments were made in 1:1 glycerol–methanol at 77°K, an obvious hypothesis for the difference in the quantum yield lies in a solvent effect and/or a temperature effect. The difference in pK values in the excited singlet state and pK^T (pK in the excited triplet state) of the peptide nitrogen may contribute to the difference in quantum yield between two excited states. In fact, the fluorescent yield depends upon the dissociation of the imino proton, and it has been shown that pK values change dramatically with change in electronic states (Förster, 1950; Weller, 1952). More detailed experiments are needed to clarify this point.

The poor triplet yield of poly-L-tryptophan may be related to a concentration quenching phenomenon, but a quantitative explanation is difficult, because it is necessary to consider the unknown distances and orientations of the indole rings, as well as the effect of peptide imino and carboxyl protons.

The Triplet State Lifetime and Protein Conformation. As can be seen in Tables I and II, the nature of the solvent has a variable effect upon the triplet state lifetimes of different proteins. Glycerol buffer and urea buffer increase the lifetime of the ovalbumin triplet state compared to buffer alone. In this case, it is clear that the conformation of the protein has been affected by the addition of glycerol but, in the cases of β-lactoglobulin, bovine serum albumin, and hemocyanin, the situation is not at all clear-cut. However, the glycerol–buffer–urea system gave rise to the longest lifetimes.

[4] The concept of excimer was originally introduced for collisional complexing between an excited molecule and an unexcited molecule. However, taking account of a change in electronic overlapping between the ground and excited states, such a concept may be extended to the frozen state.

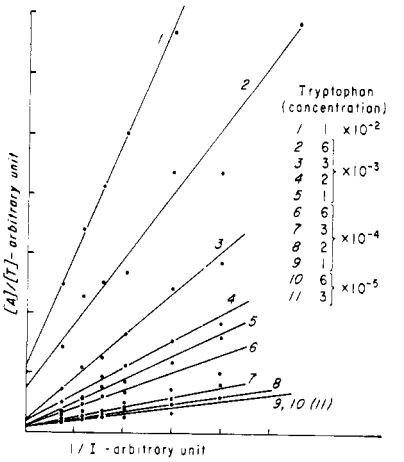

FIGURE 9: Light intensity saturation phenomena. [A]/[T] *vs.* $1/I$ plot (see text). Both axes are expressed in arbitrary units. Esr recording: same as that of Figure 2.

These facts suggest that the lifetime determining factors include the environment of the tryptophan residues in the individual proteins, especially around the indole ring. In the native proteins, the tryptophan residues may be surrounded by a more or less hydrophobic milieu, and their excited states may be capable of more or less probable resonance transfer with each other. Once the denaturation of the protein occurs, the electronic environments of the tryptophan residues become relatively independent of the conditions which exist in native conformations and more like that of tryptophan itself in the same solvent. In support of this view, Teale (1960) has already shown that the fluorescent band maxima of proteins varied from 328 to 342 mμ, but upon 8 M urea treatment, all the proteins showed a band at 350 mμ. An experiment intended to establish the relationship between the polarity of the solvent and the lifetime of the tryptophan triplet state did not give consistent results, although the lifetime changed from solvent to solvent.

The esr decay curves of the protein triplet state fit closely to a simple first-order plot, in spite of the presence of several tryptophan residues. If the tryptophan residues in a given protein were electronically independent of each other and had different environments, two or more overlapping decay curves would be expected, since it is improbable that all tryptophan residues would have identical environments. This result suggests that the resonance transfer of excitation energy between the different tryptophan residues in the same protein is quite efficient, and that the decay rate characterizes the over-all conformation and environment of the protein. However, rather poor signal:noise ratios, especially during the later stages of decay, made accurate analysis of the decay curves impossible, and the differences in lifetimes among the environments which were studied were small.

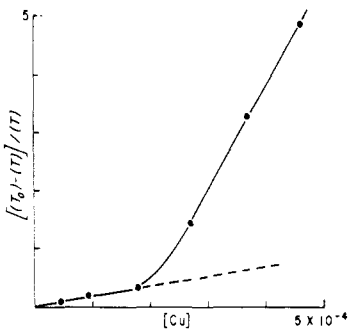

FIGURE 10: Quenching effect of Cu(II) on the triplet yield of glycyl-L-tryptophan. Stern–Volmer plot of the data of Figure 7 (see text).

Action Spectra and Quantum Yield. The action spectrum of the tryptophan triplet state (Figure 5) coincided with that of its fluorescence, corresponding to the absorption spectrum. The action spectra of proteins (Figures 5 and 6), however, were not exactly the same as that of tryptophan. In some cases (especially ovalbumin), there was a marked increase around 260 mμ. Also, the triplet yields of proteins varied from protein to protein. The differences of these properties must be related to (a) the variety of the distance and orientation between the tryptophan residues, (b) that between the tryptophan residues and the tyrosine and/or phenylalanine residues, (c) the variety of the degree of proton dissociation in the peptide chain related to tryptophan residues, and (d) the variety of the environment of the tryptophan residues. In addition, the action spectra were obtained in 1:1 glycerol–phosphate buffer, which may alter the configuration of the proteins, as discussed earlier.

Effect of Complex Formation with Cupric Ion. The cupric ion forms stable complexes with peptides (*e.g.*, Gould and Mason, 1966). The Cu(II) may be covalently bound to a peptide nitrogen atom, terminal amino nitrogen atom, and other ligands, depending upon the conditions of pH and concentration.

Cu(II) decreased the triplet yield of glycyl-L-tryptophan (Figure 7), but no other properties of the esr signal, such as its shape, resonant magnetic field, lifetime, light intensity saturation, or microwave power saturation, were found to alter. At a molar ratio of 1:1 between glycyl-L-tryptophan and Cu(II), nearly complete triplet quenching occurred. In this case, Stern–Volmer's equation (Stern and Volmer, 1919), which is ordinarily applied to fluorescent quenching, may be applicable to triplet state quenching. The data of Figure 7 were plotted according to eq 6 (Figure 10):

$$\frac{[T_0] - [T]}{[T]} = K[Cu] \qquad (6)$$

Here, $[T_0]$ and $[T]$ represent triplet yield in the absence and presence of Cu; [Cu] is concentration of Cu(II); and K is a quenching constant or an apparent binding constant.[5] In this case, the concentration of glycyl-L-tryptophan was higher than the concentration at which self-quenching began, and the concentration of Cu(II) was not low enough to satisfy the condition which led to the equation. These conditions were necessary in order to obtain good (less noisy) esr signals. However, in the low concentration range ($<2 \times 10^{-4}$ M) of Cu(II), assuming a complete quenching of triplet state by Cu(II) binding, the plot is a straight line, and K has a value of the order of 10^{+3} M.

The fluorescence of glycyl-L-tryptophan was quenched by Cu(II) in a manner parallel with that of the triplet state. The ultraviolet absorption spectrum of the Cu(II)–glycyl-L-tryptophan was similar to that of the glycyl-L-tryptophan at 270–300 mμ in methanol. Therefore, the first excited singlet state was also quenched. A concept of "paramagnetic quenching" may be applicable in this case (Porter and Wright, 1959). It is of interest that Cu(II) does not directly bind to the indole ring, which is responsible for the fluorescent and for the esr signal of the triplet state. Judging from the fluorescence emission of tryptophan and the absorption spectrum of the complex, a resonance energy transfer mechanism (Förster, 1948) from the excited (singlet) indole ring to Cu peptide is improbable. Attempts to observe an electron transfer mechanism, such as a photoinduced valency change of copper ion, were not successful.

Since the quenching by Cu(II) occurred indirectly, another problem is raised, *i.e.*, how far the quenching effect can spread over a polypeptide chain. It has been observed (Bersohn and Isenberg, 1964) that one Mn(II) can quench the phosphorescence of many purine bases in deoxyribonucleic acid (DNA). Thus, the presence of a delocalized exiton was suggested. In the case of poly-L-tryptophan, less Cu(II) was necessary to quench the triplet state than in the case of glycyl-L-tryptophan, *e.g.*, the ratio of Cu(II) to indole rings at half-quenching was 1:2 for glycyl-L-tryptophan and 1:4 for poly-L-tryptophan. The pattern of the Cu(II) quenching to poly-L-tryptophan (Figure 7) did not seem to complete quenching; instead, there may exist a certain limitation of the quenching. These facts suggest that a chelated Cu(II) atom could quench only two adjacent indole rings in poly-L-tryptophan. The limitation of the quenching may be contributed by unbound tryptophan residues, which will always exist when the binding occurs randomly or by competition of methanol for Cu(II). If the former explanation is correct, there is no possibility of the existence of exiton concerning indole rings. However, since the triplet yield of poly-L-tryptophan is originally quenched compared with glycyl-L-tryptophan, and, since the consecutive copper-binding constants are unlikely to be the same, a definitive conclusion cannot be drawn.

Hemocyanin. In comparisons between apohemocyanin and native deoxygenated hemocyanin in deoxygenated condition, the presence of Cu(I) did not quench the triplet state of tryptophan in the protein.

[5] In this semiquantitative consideration, a rigorous definition and a possible difference in K at the different electronic states were not considered.

No diamagnetic ion quenched the triplet state of organic molecules (Porter and Wright, 1959); in contrast, a diamagnetic substance quenched the phosphorescence of chlorophyll (Fujimori and Livingston, 1957).

The effect of oxygen upon native hemocyanin was remarkable; oxyhemocyanin showed smaller (30–40%) triplet yields than deoxyhemocyanin. A number of molecular structures have been proposed for oxyhemocyanin (Mason, 1964). These include the resonating system, Cu(I)· · ·O_2^-–Cu(II), Cu(II)–O_2^-· · ·Cu(I), Cu(I)· · ·O_2· · ·Cu(I), Cu(II)· · ·O_2^{2-}· · ·Cu(II), or the charge-transfer complex, $Cu(I)_2$· · ·O_2. In either case, the electron distribution involves molecular orbitals, and the specification of explicit oxidation states for the Cu and O_2 is impossible. Nevertheless, although the oxygenated complex is diamagnetic and gives no esr signal (Nakamura and Mason, 1960), it may have low-lying excited states in which the Cu is effectively in an oxidation state of two and paramagnetic. Charge-transfer interaction itself may lead to a quenching mechanism. The contact charge-transfer complexes between O_2 and organic molecules enhance singlet–triplet conversion (Tsubomura and Mulliken, 1960), and a similar mechanism may underlie the quenching of the tryptophan triplet state in oxyhemocyanin.

Microwave Power Saturation. Since the esr signal of the $\Delta m = \pm 2$ transition of randomly oriented molecules in the triplet state is a superposition (or envelope) of the different esr absorptions, microwave power saturation of an inhomogeneous type may be expected (Portis, 1953). As seen in Figure 8, the esr signals saturated rather inhomogeneously (Castner, 1959). Saturation of the signal began above 2 mw of the incident microwave power. The saturation factor is dependent on the lifetime (Sohma, 1962). There were small differences in the saturation curves between the protein triplet state and L-tryptophan glycyl-L-tryptophan, probably due to interactions within the protein.

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