Near-Ultraviolet Absorption Bands of Tryptophan. Studies Using Horseradish Peroxidase Isoenzymes, Bovine and Horse Heart Cytochrome *c*, and *N*-Stearyl-L-tryptophan *n*-Hexyl Ester*

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ABSTRACT: Cooling to 77°K permitted observing the long-wavelength tryptophanyl absorption bands of several proteins containing a single tryptophan residue. The 0–0 ^1L_a and 0–0 ^1L_b tryptophanyl bands were resolved in the 77°K absorption spectra of horseradish peroxidase A1, peroxidase C, and horse and bovine heart ferri- and ferrocytochrome *c*. In addition, a 0 + 850 cm^{-1} ^1L_a tryptophanyl band was resolved in both peroxidase isoenzymes and in horse ferrocytochrome *c*. The 0–0 ^1L_b tryptophanyl absorption band was located between 288 and 290 nm in these proteins. In contrast, the position of the 0–0 ^1L_a tryptophanyl band ranged from 302 nm (apoperoxidase C) to 292 nm (bovine ferricytochrome *c*). The interactions affecting the wavelength position of the ^1L_a bands were deduced from the following reference spectra: *N*-stearyl-L-tryptophan *n*-hexyl ester dissolved in methylcyclohexane, L-tryptophan in water–glycerol (1:1, v/v), and horse ferricytochrome *c*, whose three-dimensional structure is known from X-ray analysis (Dickerson *et al.* (1971), *J. Biol. Chem. 246*, 1511). The 0–0 ^1L_a band was not resolved when the indolyl ring was fully exposed to the water–glycerol solvent used for protein spectra at 77°K. When the ring was in a hydrocarbon environment, the 0–0 ^1L_a band occurred at about 289 nm. In horse ferricytochrome *c* the 0–0 ^1L_a tryptophanyl band was red shifted to 293 nm, evidently because its indolyl ring is hydrogen bonded within an otherwise nonpolar region. After ferricytochrome *c* was reduced to the ferro form, the ^1L_a bands underwent an additional 1.5-nm red shift. For peroxidase A1 and C, the 0–0 ^1L_a band was red shifted about 12 nm; apparently their indolyl rings occur in partially polar regions, but are not extensively exposed to the solvent. The fine structure absorption bands of phenylalanine and tyrosine residues were also prominent in the 77°K absorption spectra of peroxidase A1 and C. Control experiments showed that the heme moiety has only relatively broad absorption bands in the near-ultraviolet region even at 77°K.

Many characteristics of the near-ultraviolet absorption bands of tryptophan have been clarified by recent studies using 3-methylindole as a model (Strickland *et al.*, 1970a). When this compound is dissolved in perfluorinated hexane, extensive vibrational fine structure can be observed in the two overlapping electronic transitions (^1L_a and ^1L_b bands). The major ^1L_b bands are 0–0, 0 + 730, and 0 + 980 cm^{-1} transitions. The latter two transitions have appeared as a single unresolved band (0 + 850 cm^{-1}) when 3-methylindole or tryptophan is dissolved in other solvents (Strickland *et al.*, 1969, 1970a). The studies of 3-methylindole in perfluorinated hexane solvents have also permitted identifying the 0–0 ^1L_a transition and have placed certain restrictions on the possible spacings of the remaining ^1L_a vibrational bands. For 3-methylindole dissolved in hydrocarbon solvents, both the 0–0 ^1L_a and the 0–0 ^1L_b bands occur at 290 nm. When 3-methylindole or tryptophan is dissolved in alcoholic solvents, the 0–0 ^1L_a band is shifted to the long-wavelength side of the 0–0 ^1L_b band and is blurred. In these solvents the 0–0 ^1L_a band can only be resolved after cooling to 77°K. The remaining ^1L_a bands are not clearly resolved in polar solvents either. In all these solvents the ^1L_b bands are much sharper than the ^1L_a bands (Strickland *et al.*, 1970a; Bernardin, 1970).

Recently we have observed that the two lowest energy ^1L_a tryptophanyl absorption bands are well resolved at 77°K in several proteins which contain only a single tryptophan residue. The present communication describes the near-ultraviolet absorption spectra of horseradish peroxidase A1, horseradish peroxidase C, and ferri- and ferrocytochrome *c* from both horse and bovine. In all these proteins, cooling to 77°K brings out a number of fine structure absorption bands which arise from the aromatic amino acid residues. These spectra reveal the long-wavelength tryptophanyl absorption bands of both the ^1L_a and ^1L_b electronic transitions. The position of the 0–0 ^1L_a tryptophanyl band varies greatly among these proteins, reflecting differences in the environments of their indolyl rings. In addition, we describe the absorption spectra of *N*-stearyl-L-tryptophan *n*-hexyl ester in methylcyclohexane (297°K) and of L-tryptophan in water–glycerol (297 and 77°K). These results give additional insights concerning the factors which determine the wavelength position of the 0–0 ^1L_a band of tryptophan residues in proteins. Control experiments using the apoperoxidases, hematin, and the heme undecapeptide from cytochrome *c* confirmed that the heme moiety does not have fine structure bands in the near-ultraviolet region even at 77°K.

Materials and Methods

L-Tryptophan n-Hexyl Ester Hydrochloride. L-Tryptophan

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(4.08 g) was added to a chilled solution of 5 ml of thionyl chloride in 50 ml of 1-hexanol. The mixture was stirred at room temperature for 2 hr and then heated under reflux until the solid had dissolved. The reaction mixture was permitted to stand for another 3 hr at 25° and 250 ml of ether was added. The precipitate was filtered, washed with ether, and recrystallized from isopropyl alcohol: yield, 5.1 g (78%); mp 222°. *Anal.* Calcd for $C_{17}H_{25}ClN_2O_2$: C, 62.96; H, 7.71; N, 8.64. Found: C, 62.81; H, 7.58; N, 8.72.

N-Stearyl-L-tryptophan n-Hexyl Ester. Triethylamine (1.4 ml) was added to a solution of L-tryptophan hexyl ester hydrochloride (3.2 g) in 30 ml of chloroform. This was mixed with a solution of stearic acid (2.84 g) in 25 ml of chloroform at room temperature. *N,N'*-Dicyclohexylcarbodiimide (2.1 g) was added and the solution was stirred overnight at room temperature. *N,N'*-Dicyclohexylurea was removed by filtration and the filtrate was washed successively with 0.5 N hydrochloric acid, water, and 5% sodium bicarbonate solution, and finally dried over sodium sulfate. The solvent was evaporated *in vacuo* to dryness. The compound was recrystallized from petroleum ether (bp 60–70°). The compound was chromatographically pure on thin-layer chromatography: yield, 4.3 g (80%); mp 67–69°. *Anal.* Calcd for $C_{35}H_{58}N_2O_3$: C, 75.81; H, 10.47; N, 5.05. Found: C, 75.90; H, 10.28; N, 4.94.

Peroxidase A1 and C. The isolation of horseradish peroxidase A1 and C and the preparation of their apoenzymes have been described elsewhere (Shannon *et al.*, 1966; Strickland *et al.*, 1970b). The concentrations of the peroxidase solutions were calculated from the absorbance of their Soret bands at 297°K (Shannon *et al.*, 1966).

Cytochrome c. Horse heart ferricytochrome *c* (type III, lot 59B-7070 and type VI, lot 65B-7300) and bovine heart ferricytochrome *c* (type V, lot 119B-7190) were obtained from Sigma Chemical Co. (St. Louis). Examination of the visible absorption spectra (500–600 nm) confirmed that the material was predominantly in the oxidized form (about 5% ferrocytochrome *c*). The reduced forms were prepared by adding a minimal amount of sodium dithionite (Baker Chemical Co.) to the ferricytochrome *c*. The formation of ferrocytochrome *c* was verified by recording the visible absorption spectrum. The concentration of the cytochrome *c* solutions were calculated using ϵ (27,700) at 550 nm for the reduced and ϵ (11,200) at 528 nm for the oxidized form (Margoliash and Frohwirt, 1959).

The heme undecapeptide of horse heart ferricytochrome *c* was prepared essentially according to the procedure of Harbury and Loach (1960). An amino acid analysis, following digestions of the peptide in 6 N HCl at 100° for 20 hr, agreed with the results obtained by Harbury and Loach (1960). No aromatic amino acid residues were present in the heme undecapeptide.

Low-Temperature Technique. The 77°K absorption spectra were recorded on a Cary Model 15 spectrophotometer using a spectroscopic dewar described previously (Horwitz *et al.*, 1969). The proteins were dissolved in water–glycerol (1:1, v/v) containing either 50 or 100 mM sodium phosphate. After cooling these solutions to 77°K in short path-length cuvets (0.07–0.2 mm), there was no significant increase in light scattering (Strickland *et al.*, 1969). Since multiple reflections did not occur in our experimental arrangement, cooling from 297 to 77°K did not produce any increase in the total area under the absorption spectrum. These characteristics distinguish our glasses from the devitrified water–glycerol samples used to record the visible absorption bands of heme proteins at 77°K (Estabrook, 1961).

Near-ultraviolet circular dichroism spectra were recorded to verify that the presence of glycerol did not alter the conformations of the proteins used in our study. At 297°K the circular dichroism spectra (330–255 nm) of horse heart ferricytochrome *c* and of the peroxidase isoenzymes were unaffected by the presence of 50% glycerol in the buffer (1-cm path length).

In view of the high protein concentrations used to record spectra at 77°K, the possibility exists that the proteins may have been aggregated. Neither the circular dichroism nor the absorption spectra (310–270 nm), however, gave any indication of aggregation having occurred. (A) The circular dichroism spectra observed at high concentrations were the same as those previously reported for dilute solutions of the peroxidase isoenzymes (Strickland *et al.*, 1968, 1970b) and of horse and bovine cytochrome *c* (Vinogradov and Zand, 1968). (B) The ratio of circular dichroism to absorption intensity at 297°K was constant over a 100-fold concentration range for the peroxidase isoenzymes and for horse heart ferricytochrome *c*. (C) Cooling these proteins did not cause any changes in either the band shapes or areas that could not be accounted for by the anticipated band sharpening. If aggregation did occur with any of these proteins, it did not affect the near-ultraviolet circular dichroism or absorption bands arising from the aromatic amino acid residues. Furthermore, the circular dichroism spectra suggest that cooling to 77°K cannot have much effect upon the conformations of the aromatic amino acid side chains in these proteins.

A DuPont 310 curve resolver was used to determine the approximate widths of tryptophanyl absorption bands in certain 77°K spectra. These curves were fit using Gaussian bands.

Results

Hematin. The spectrum of hematin was examined under several conditions to search for any heme fine structure absorption bands which might be observable. The initial studies were carried out with hematin dissolved in dimethyl sulfoxide at 297°K. No fine structure absorption bands were found between 320 and 260 nm. Measurements at 77°K were carried out using hematin dissolved in butanol–glycerol (9:1, v/v) saturated with imidazole. Again only broad absorption bands were observed, as cooling did not produce any detectable fine structure bands between 320 and 240 nm.

Heme Undecapeptide of Cytochrome c. A further examination of the near-ultraviolet heme absorption bands was made on the heme undecapeptide from horse cytochrome *c*. Urry (1967) previously reported that neither the ferri- nor the ferroheme undecapeptide of cytochrome *c* possesses any vibrational fine structure absorption bands (320–240 nm) when dissolved in an aqueous borate buffer at room temperature. We recorded absorption spectra under the following conditions: ferriheme undecapeptide dissolved in dimethyl sulfoxide–water (1:1, v/v) at 297°K, and ferri- and ferroheme undecapeptide in water–glycerol (1:1, v/v) containing 25 mM $Na_2B_4O_7$ and 250 mM imidazole (pH 7) at 297 and 77°K. In all cases, the near-ultraviolet absorption bands were relatively broad and not sharpened much by cooling to 77°K.

N-Stearyl-L-tryptophan n-Hexyl Ester. The two alkyl moieties of this compound greatly enhanced its solubility in hydrocarbon solvents. Thus the absorption spectrum

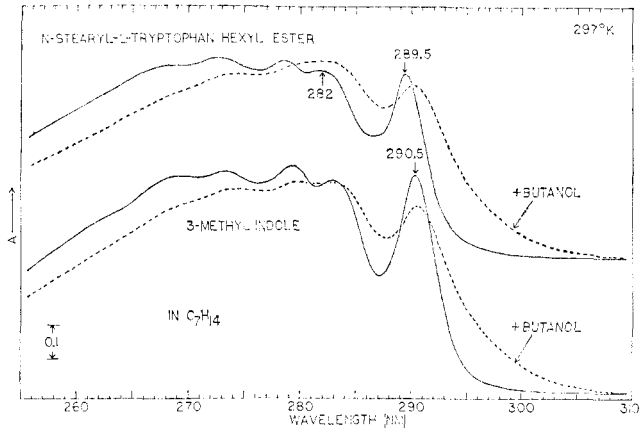

FIGURE 1: (——) Comparison of absorption spectra of *N*-stearyl-L-tryptophan *n*-hexyl ester (top) and 3-methylindole (bottom) dissolved in methylcyclohexane. (---) Effect of adding 0.1 ml of 1-butanol to 2.0 ml of these compounds dissolved in methylcyclohexane. 1.0-cm path length, 297°K. The absorption spectra of *N*-stearyl-L-tryptophan *n*-hexyl ester in methylcyclohexane followed Beer's law over a 100-fold concentration range, varying from 1/50th to twice the concentration shown in this figure.

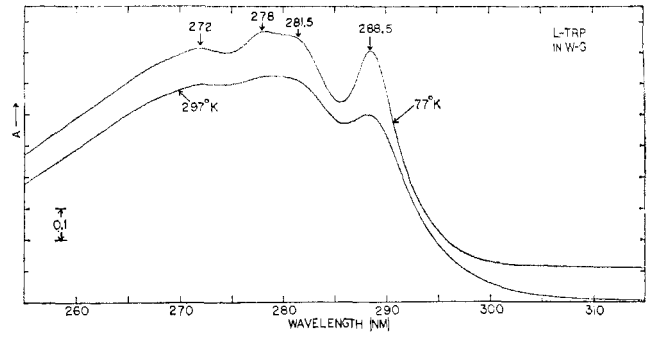

FIGURE 2: Absorption spectra of 6.5 mM L-tryptophan dissolved in water–glycerol (1:1, v/v) adjusted to pH 1.0 with phosphoric acid. 0.2-mm path length. Base line for the 77°K spectrum was offset 0.1 unit to separate the spectra. Both solvent base lines were flat. *N*-Ac-L-TrpNH$_2$ in water–glycerol (pH 7) gave a 77°K absorption spectrum similar to that of L-tryptophan except that the bands were red shifted by 0.5 nm.

of this tryptophan derivative can be compared with that of 3-methylindole in a solvent giving relatively well-resolved bands. In methylcyclohexane both *N*-stearyl-L-tryptophan *n*-hexyl ester and 3-methylindole have essentially identical absorption spectra (Figure 1). Each spectrum has five bands resolved, and the spacings between these bands are the same. In *N*-stearyl-L-tryptophan *n*-hexyl ester these bands are shifted toward shorter wavelengths by 1 nm from the corresponding bands in 3-methylindole. When a small concentration of 1-butanol was added to these solutions, the red edge of the longest wavelength absorption band was distorted in the same manner for both *N*-stearyl-L-tryptophan *n*-hexyl ester and 3-methylindole (Figure 1).

These findings confirm that the electronic transitions identified for 3-methylindole can also be applied to tryptophan derivatives (Strickland *et al.*, 1970a). Thus for *N*-stearyl-L-tryptophan *n*-hexyl ester in methylcyclohexane, the 0–0 ^1L_a band overlaps the 0–0 ^1L_b band at 289.5 nm. When butanol is allowed to interact with the indolyl ring, the ^1L_a bands are red shifted and also broadened greatly; in contrast, the ^1L_b bands are not much affected. Presumably the red shift of the ^1L_a band arises mainly from the formation of a hydrogen bond between the indolyl >NH group and the butanol oxygen atom (Konev, 1967). Interestingly, even small amounts of butanol in the methylcyclohexane blurred the spectrum as much as completely polar solvents did (compare Figures 1 and 2).

The absorption spectrum of *N*-stearyl-L-tryptophan *n*-hexyl ester in methylcyclohexane could not be obtained at low temperatures, because aggregation occurred upon cooling below 253°K. Furthermore, the circular dichroism spectrum of this compound was too weak to be useful in identifying the ^1L_a and ^1L_b bands.

L-Tryptophan Dissolved in Water–Glycerol. These absorption spectra are presented in Figure 2. At 297°K the bands are poorly resolved. After cooling to 77°K, the band at 288.5 nm is sharpened greatly and the short-wavelength bands (272, 278, and 281.5 nm) are somewhat better resolved. No bands are resolved in the region above 290 nm. The 0–0 ^1L_b and the 0 + 850 cm^{-1} ^1L_b bands occur at 288.5 and 281.5 nm, respectively (Strickland *et al.*, 1969). The 0–0 ^1L_a band is not resolved.

Horseradish Peroxidase A1. This glycoprotein was selected because it contains only one tryptophan and two tyrosine residues (Strickland *et al.*, 1968). The only other moieties having significant absorption in the near-ultraviolet region are the 16 phenylalanine residues and a single heme group (Shannon *et al.*, 1966; Shih *et al.*, 1971). The near-ultraviolet absorption of the peroxidase heme was approximated by subtracting the absorption of apoperoxidase A1 from that of peroxidase A1 at 297°K (dashed line in Figure 3). The absorption of the heme moiety causes the aromatic absorption bands to be superimposed upon a sloping background. Examination of this spectrum using a curve resolver indicated that the apparent positions of the fine structure bands are shifted to slightly shorter wavelengths (about 0.3 nm) by the heme absorption.

The absorption spectrum of peroxidase A1 at 297°K has a prominent shoulder at 292 nm and several phenylalanyl bands located below 270 nm (Figure 3). Cooling to 77°K brings out a remarkable number of fine structure bands in the region from 300 to 272 nm and greatly sharpens the phenylalanyl fine structure (268.2–252.2 nm). The positions of the phenylalanyl absorption bands of peroxidase A1 correspond to the bands observed previously for phenylalanine derivatives dissolved in nonaqueous solvents (Horwitz *et al.*, 1969). The prominence of the 261.7-nm absorption band suggests that the phenyl rings of most of these residues are relatively unexposed to the water–glycerol solvent (unpublished experiments).

The 300- and 293-nm bands of peroxidase A1 must arise from the tryptophan residue, since no other amino acid residues have fine structure in this region (Strickland *et al.*, 1969). A distinction between the ^1L_a and ^1L_b tryptophanyl transitions may be made on the basis of the wavelength positions, the relative widths of each band, and the vibronic spacings (Table I). Previous studies have shown that the 0–0 ^1L_b band may be located anywhere between 287 and 293 nm and that the 0–0 ^1L_a band may be shifted to even longer wavelengths (Strickland *et al.*, 1969, 1970a). Evidently the peroxidase A1 band at 300 nm is the 0–0 ^1L_a band. The broadness of this band also supports the ^1L_a assignment. Previous studies of 3-methylindole revealed that the 0–0 ^1L_a band is broader than the 0–0 ^1L_b band, even in solvents

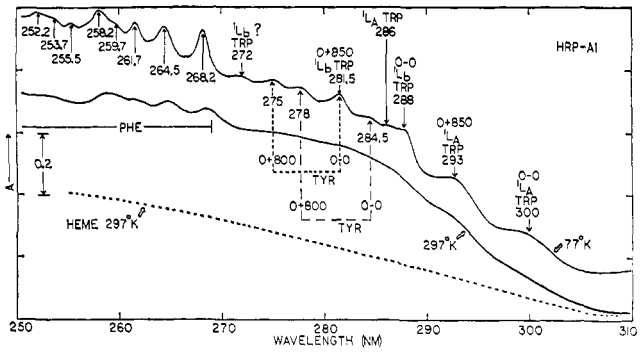

FIGURE 3: Absorption spectra of 1.9 mM peroxidase A1 at 297 and 77°K (——). The 77°K curve has been offset about 0.14 unit to separate the two records. Approximate absorption of heme moiety in peroxidase A1 at 297°K is given by dashed line. The approximate heme absorption at 77°K can be obtained by displacing the dashed line upward by 0.14 unit, *i.e.*, no tryptophan absorption above 307 nm in peroxidase cooled to 77°K. Peroxidase A1 was dissolved in water–glycerol (1:1, v/v) containing 50 mM sodium phosphate (pH 7). 0.2-mm path length. The 0–0 ^1L_a tryptophan band at 300 nm has ϵ of 2000 at 77°K after subtracting the heme absorption. The widths of the tryptophanyl absorption bands at half-intensity, assuming Gaussian bands and correcting for heme absorption, are: 0–0 ^1L_a, 5 nm; 0 + 850 ^1L_a, 5 nm; 0–0 ^1L_b, 4 nm. These spectra did not have fine structure between 310 and 330 nm.

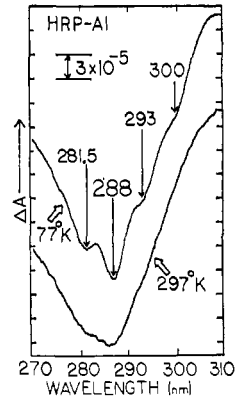

FIGURE 4: (——) Instrument traces of circular dichroism spectra of 1.9 mM peroxidase A1 at 297 and 77°K. The solvent was water–glycerol (1:1, v/v) containing 50 mM sodium phosphate (pH 7). The 77°K trace was offset to facilitate viewing both records. For 77°K the trace was the average of 40 scans; for 297°K, 20 scans (0.3-nm/sec scanning speed, 1-sec time constant, 0.12-mm path length, and 1.8-nm spectral half-intensity bandwidth). The peak-to-peak noise in the 77°K record was 3×10^{-6} ΔA or less. The wavelength positions of the fine structure at 77°K were verified in a separate experiment using a 0.3-sec time constant, which precluded any tracking error.

giving a minimal broadening of bands (Strickland *et al.*, 1970a). The 293-nm tryptophanyl band of peroxidase A1 is more difficult to identify since either ^1L_a or ^1L_b bands may occur at this wavelength. The 293-nm band, however, is also relatively broad (Figure 3), which suggests that it is a ^1L_a band. The broadness of this band is more obvious in the spectrum of peroxidase C (see below).

The next band at 288 nm must also be a tryptophanyl band, since it is too intense to have arisen from a tyrosine residue (Horwitz *et al.*, 1970). Its wavelength is appropriate for the 0–0 ^1L_b band. The width of the 288-nm band is not easily recognized in the peroxidase A1 absorption spectrum, because additional bands are located close by on the short wavelength side. Nevertheless, the relatively steep slope of the long wavelength edge of the 288-nm band indicates that it is sharper than either the 300- or the 293-nm band (Figure 3).

These tryptophanyl bands also give rise to fine structure in the circular dichroism spectrum of peroxidase A1 (Figure 4). Here again, the 288-nm band is sharper than either the 293- or the 300-nm band. Furthermore, the 288-nm circular dichroism band is accompanied by a second sharp circular dichroism band at 281.5 nm, which is the position expected for the 0 + 850 cm^{-1} ^1L_b tryptophanyl band (Strickland *et al.*, 1969, 1970c). Thus the circular dichroism spectra strongly support our assignments of the 288-nm band (0–0 ^1L_b), the 293-nm band (0 + 850 cm^{-1} ^1L_a), and the 300-nm band (0–0 ^1L_a).

Interpretation of the remaining absorption bands of peroxidase A1 is somewhat uncertain, because many tyrosyl bands overlap tryptophanyl bands in the region from 270 to 288 nm. An additional difficulty is that the short-wavelength tryptophanyl bands are broad and poorly characterized (Strickland *et al.*, 1970a). The tyrosyl bands of peroxidase A1, however, seem to be unusually narrow, permitting their detection even against the large background absorption from the tryptophan residue. Interestingly, most of the remaining fine structure bands of peroxidase A1 fit the wavelength pattern observed previously for the tyrosyl absorption bands of ribonuclease A (Horwitz *et al.*, 1970). This finding suggests that each of the two tyrosyl side chains of peroxidase A1 has a spectroscopically distinct site. Apparently the 0–0 band at 284.5 nm belongs to a buried residue, whereas the 0–0 band at 281.5 nm probably results from a phenolic ring having the hydroxyl group exposed to the solvent or perhaps to the carbohydrate moiety (Figure 3).

To examine further a possible influence of the heme absorption bands upon the near-ultraviolet fine structure bands, the 77°K absorption spectrum of peroxidase A1 (1.5 mM) was also recorded after treatment with 5 mM H_2O_2. The visible absorption bands revealed that the enzyme had been converted into a mixture of compounds I and II (George, 1956; Chance, 1963). The H_2O_2 treatment, however, did not alter the positions of the fine structure bands in the region from 300 to 250 nm.

The 77°K absorption spectrum of apoperoxidase A1 was also recorded. After removal of the heme, the 0–0 and 0 + 850 ^1L_a tryptophanyl bands were resolved at 299 and

TABLE I: Characteristics of the Major Tryptophanyl Absorption Bands of Proteins.[a]

	^1L_a		^1L_b	
Characteristics	0–0	0 + 850 cm^{-1}	0–0	0 + 850 cm^{-1}
Wavelength position (nm)	*302–289*	*295–282*	*293–287*	*286–280*
Band shape at 297°K	Diffuse	Diffuse	Broad	Broad
Band shape at 77°K	Broad[b]	Broad[b]	Sharp	Sharp

[a] Except for the characteristics of the 0 + 850 cm^{-1} ^1L_a band, these data are based upon previous findings (Strickland *et al.*, 1969, 1970a). [b] Bands not resolved if the indolyl ring is fully exposed to the water–glycerol solvent.

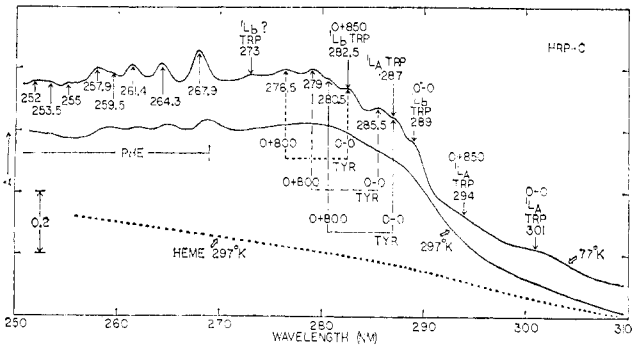

FIGURE 5: Absorption spectra of 1.7 mM peroxidase C at 297 and 77°K (——). The 77°K curve has been offset 0.1 unit to separate the records. Approximate absorption of the peroxidase C heme (---) was obtained by subtracting the absorption of apoperoxidase C from that of peroxidase C at 297°K. Peroxidase C was dissolved in water–glycerol (1:1, v/v) containing 50 mM sodium phosphate (pH 7). 0.2-mm path length. The 0–0 ^1L_a tryptophanyl band at 301 nm has ϵ of 1700 at 77°K after subtracting the heme absorption. No fine structure was observed between 310 and 330 nm.

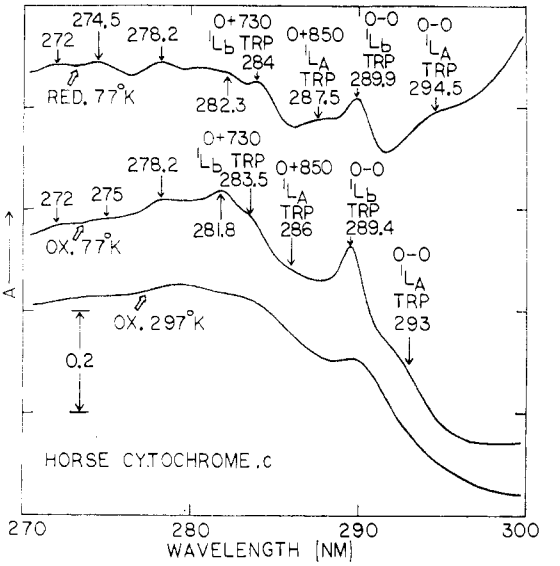

FIGURE 6: Absorption spectra of 3.4 mM horse heart ferri- and ferrocytochrome *c* dissolved in water–glycerol (1:1, v/v) containing 100 mM sodium phosphate (pH 7) (0.12-mm path length). The curves have been offset to permit viewing each record. The spectrum of ferrocytochrome *c* is distorted somewhat by absorption of sodium dithionite, which was used for reduction. Control experiments showed that dithionite does not have any fine structure absorption bands when dissolved in water–glycerol at 77°K. Curve fitting using Gaussian bands revealed that in ferricytochrome *c* at 77°K the widths of the bands at half-intensity are: 0–0 ^1L_a, 4–5 nm; 0–0 ^1L_b, 2–3 nm. The spectra shown in this figure were obtained from type VI cytochrome *c*. Identical results were obtained from type III cytochrome *c* at pH 7 (3 and 10 mM) and from type VI cytochrome *c* at pH 6 (3.4 mM). No fine structure occurred between 300 and 320 nm.

292 nm. These bands were somewhat broader than the corresponding bands in peroxidase A1. The bands between 270 and 290 nm were smeared into a pair of broad bands centered at 277 and 283 nm. Apparently removal of the heme from peroxidase A1 somewhat alters the environments of the two tyrosine residues.

Horseradish Peroxidase C. This glycoprotein contains 5 tyrosine residues, 1 tryptophan residue, 20 phenylalanine residues, and 1 heme group (Shannon *et al.*, 1966; Strickland *et al.*, 1968; Shih *et al.*, 1971). The absorption bands observed in peroxidase C at 77°K (Figure 5) are generally similar to those of peroxidase A1 except for a 1-nm red shift in all bands above 270 nm. The 0–0 ^1L_a tryptophanyl band is located at 301 nm and the 0 + 850 cm^{-1} ^1L_a band is at 294 nm. The latter band is not as well resolved in peroxidase C as in peroxidase A1. The 0–0 ^1L_b tryptophanyl band of peroxidase C occurs at 289 nm. Our suggested identifications of the remaining bands are summarized in Figure 5. The tyrosyl fine structure absorption bands are relatively more intense in peroxidase C as a result of its greater tyrosine content. The intensities of the bands suggest that two tyrosyl side chains have their 0–0 bands at 282.5 nm (hydroxyl group probably exposed to the solvent or to the carbohydrate moiety), that two tyrosyl side chains (buried) have their 0–0 bands at 285.5 nm, and that the final tyrosyl side chain (buried) has its 0–0 band at 287 nm. The existence of three different tyrosyl sites was observed previously in ribonuclease A (Horwitz *et al.*, 1970).

The 77°K absorption spectrum of apoperoxidase C is similar to that observed for apoperoxidase A1. The 0–0 ^1L_a and 0 + 850 cm^{-1} ^1L_a tryptophanyl bands occur at 302 and 295 nm, respectively, and a pair of relatively broad bands exist at 277.5 and 284 nm.

Horse Heart Cytochrome c. This heme protein contains one tryptophan and four tyrosine residues (Dickerson *et al.*, 1971). The extent of band sharpening induced by cooling is illustrated using ferricytochrome *c*. At 297°K this protein has a prominent shoulder at 289.5 nm, a maximum at 279 nm, and weak shoulders at 283 and 274 nm (Figure 6). Upon cooling ferricytochrome *c* to 77°K, the fine structure bands arising from the aromatic amino acid residues are greatly sharpened. Absorption bands are clearly resolved at 289.4, 281.8, and 278.2 nm (Figure 6). Prominent shoulders occur at 293, 283.5, and 272 nm.

The remarkable sharpness of the intense band at 289.4 nm in ferricytochrome *c* shows that it is the 0–0 ^1L_b tryptophanyl band. The broad shoulder at about 293 nm has the characteristics expected for the 0–0 ^1L_a tryptophan band. The sharpness of the 0–0 ^1L_b tryptophanyl band of cytochrome *c* suggests that the usual 0 + 850 cm^{-1} ^1L_b tryptophanyl band may actually be resolved into its component transitions (0 + 730 and 0 + 980 cm^{-1}), as previously observed in a high-resolution spectrum of 3-methylindole (Strickland *et al.*, 1970a). The 283.5-nm band of ferricytochrome *c* has the correct spacing to be the 0 + 730 cm^{-1} ^1L_b tryptophanyl band. The 0 + 980 cm^{-1} ^1L_b tryptophanyl band may contribute partially to the band at 281.8 nm, although a major part of the 281.8-nm band must arise from other sources (see below). In ferricytochrome *c* the tyrosyl absorption bands are not sufficiently prominent to be identified on the basis of their characteristic pair of fine structure bands (Horwitz *et al.*, 1970).

The tryptophanyl absorption bands were also examined at 77°K after the reduction of cytochrome *c* with sodium dithionite. In the spectrum of ferrocytochrome *c* shown in Figure 6, the long-wavelength end is skewed upward by a broad band at 312 nm, which arises partly from the excess dithionite and partly from the reduced heme moiety (Margoliash and Frohwirt, 1959). Minor changes in the heme absorption intensity occur throughout the region from 300 to 270 nm. The positions of the tryptophanyl bands are red shifted slightly. Both the 0–0 ^1L_b and the 0 + 730 cm^{-1} ^1L_b

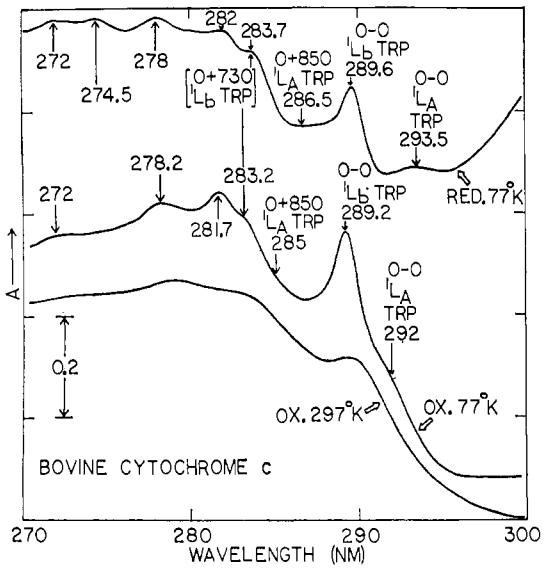

FIGURE 7: Absorption spectra of 6.3 mM bovine heart ferri- and ferrocytochrome *c* at 0.07-mm path length (equivalent to 3.7 mM at 0.12-mm path length). Cytochrome *c* was dissolved in water–glycerol (1:1, v/v) containing 100 mM sodium phosphate (pH 7). These spectra did not possess any fine structure between 300 and 320 nm.

band are moved 0.5 nm, and the 0–0 ^1L_a band is moved about 1.5 nm (Figure 6). In contrast, only part of the absorption at 281.8 nm in ferricytochrome *c* is shifted by reduction, implying that the 281.8-nm band cannot arise entirely from tryptophan. The ferrocytochrome *c* spectrum reveals a weak, broad band located in the trough at 287.5 nm. This band has the characteristics anticipated for the 0 + 850 cm^{-1} ^1L_a tryptophanyl band. Apparently in ferricytochrome *c* this ^1L_a band occurs at 286 nm and is thereby obscured by the onset of the next major absorption band (Figure 6).

To what extent are the apparent positions of the tryptophanyl bands red shifted by the presence of the broad absorption band at 312 nm in the reduced spectrum? This question was examined by using a curve resolver to add a 312-nm band to the ferricytochrome *c* spectrum. Even when the intensity of this broad band was made excessively large, the 0–0 ^1L_a tryptophanyl band was not red shifted beyond 293.5 nm, and the sharp 0–0 ^1L_b tryptophanyl band was shifted only about 0.2 nm. Evidently reduction of ferricytochrome *c* does cause an intrinsic red shift in the tryptophanyl bands (at least 1 nm for the ^1L_a bands and probably about 0.2–0.4 nm for the ^1L_b bands).

Bovine Heart Cytochrome c. This protein has the same aromatic amino acid residues as does horse cytochrome *c* (Dickerson *et al.*, 1971). Figure 7 summarizes the 77°K absorption spectra of bovine cytochrome *c* under conditions equivalent to those used for the spectra presented in Figure 6 for horse cytochrome *c*. Although the spectra of bovine ferri- and ferrocytochrome *c* are similar to those of horse, the exact wavelength positions and intensities of the tryptophanyl bands differ somewhat. The 0–0 ^1L_b tryptophanyl absorption band is even sharper in bovine cytochrome *c* than in horse cytochrome *c*. The 0–0 ^1L_a band of bovine ferricytochrome *c* (292 nm) is not shifted as far to the red, which may partially account for the band appearing less prominent than is the case for horse ferricytochrome *c*. In bovine cytochrome *c* both ^1L_b tryptophanyl bands are located about 0.2 nm toward shorter wavelengths, and the ^1L_a bands are about 1 nm more toward the short wavelength end than is the case for horse cytochrome *c*. As in horse cytochrome *c*, a red shift of the tryptophanyl absorption bands occurs when the bovine ferricytochrome is reduced.

Discussion

The heme moieties of cytochrome *c* and the peroxidase isoenzymes do not contribute any fine structure absorption bands in the region from 320 to 250 nm even at 77°K. First of all, neither hematin nor the heme undecapeptide of cytochrome *c* possessed any fine structure absorption bands in this region. These experiments, however, do not preclude the possibility that the heme moiety has ultraviolet fine structure at 77°K when buried in a nonpolar region of proteins. This possibility was investigated by changing the oxidation state of the heme, which causes major shifts in the ultraviolet absorption of the heme undecapeptide of cytochrome *c* (Urry, 1967). The fine structure observed for ferrocytochrome *c* at 77°K is identical with that of ferricytochrome *c* (Figures 6 and 7) except for small shifts that are consistent with the fine structure bands known for tryptophan. In the case of peroxidase A1, oxidation of the ferric protoporphyrin moiety with H_2O_2 (George, 1956) did not alter the positions of the fine structure bands. Evidently the heme moiety does not have any near-ultraviolet fine structure bands comparable to the bands arising from the aromatic amino acid residues of proteins.

Cooling the peroxidase isoenzymes and the cytochrome *c*'s to 77°K brought out many fine structure absorption bands belonging to their aromatic amino acid residues. The resolution of bands was especially impressive in the peroxidase isoenzymes. The major vibrational bands of the phenylalanine, tyrosine, and tryptophan residues of the peroxidases seem to be individually resolved (Figures 3 and 5). This exceptional resolution depends partly on the low aromatic amino acid content and partly upon the type of site each residue occupies in the peroxidases. For example even though horse cytochrome *c* has only four tyrosine residues, their characteristic vibronic bands (Horwitz *et al.*, 1970) could not be identified with any confidence.

In the region where tyrosine residues do not absorb (290–300 nm), the tryptophanyl bands were well resolved in the 77°K spectra of the peroxidases and the cytochrome *c*'s proteins having only a single tryptophan residue. We have identified many of the ^1L_a and ^1L_b tryptophanyl bands after considering their wavelength positions, relative band widths, and vibronic spacings (see results). The position of the 0–0 ^1L_a tryptophanyl band ranged from 302 nm (apoperoxidase C) to 292 nm (beef ferricytochrome *c*). The 0–0 ^1L_b tryptophanyl band was located at shorter wavelengths than the 0–0 ^1L_a band in all the proteins examined.

A band located +850 cm^{-1} from 0–0 ^1L_a tryptophanyl band was clearly resolved in horse ferrocytochrome *c* (Figure 6) and in both peroxidase isoenzymes at 77°K (Figures 3 and 5). The consistency of this spacing in these 3 proteins indicates that this band is a 0 + 850 cm^{-1} ^1L_a band. Its existence is consistent with the spectra of *N*-stearyl-L-tryptophan *n*-hexyl ester and of 3-methylindole in nonpolar solvents (Strickland *et al.*, 1970a). For example, the 282-nm band of *N*-stearyl-L-tryptophan *n*-hexyl ester in methylcyclohexane (Figure 1) can arise from both the 0 + 850 cm^{-1} ^1L_a and the 0 + 850 cm^{-1} ^1L_b bands. Poor resolution of the 0 + 850 cm^{-1} ^1L_a band in both ferricytochrome *c*'s and in

bovine ferrocytochrome *c* can also be accounted for by other overlapping bands (Figures 6 and 7). In view of the wavelength positions where the 0 + 850 cm^{-1} ^1L_a band may occur (Table I), it frequently may be obscured by other bands in proteins even at 77°K.

What causes the wavelength positions of the tryptophanyl bands to differ among the various proteins? Model studies using tryptophan and 3-methylindole dissolved in various solvents do not necessarily give a complete catalogue of the positions of the 0–0 ^1L_a and the 0–0 ^1L_b bands. The tryptophan residues in proteins may exist in a wide variety of environments, which cannot be duplicated by solvents. For example, part of the indolyl ring may be exposed to the aqueous solvent and the remainder may be in contact with a variety of polar or nonpolar amino acid side chains. In addition, the indolyl ring may be buried within many different protein environments, and its >NH group may or may not be hydrogen bonded. Each of these environments may produce different wavelength positions for the 0–0 ^1L_a and the 0–0 ^1L_b band.

On the other hand, the spectra of model compounds probably do represent accurately the spectra of tryptophan residues which are fully exposed to the solvent or which are buried within a completely nonpolar environment. The spectrum of L-tryptophan in water–glycerol at 77°K suggests that for a fully exposed indolyl ring the 0–0 ^1L_a band cannot be resolved and the 0–0 ^1L_b band occurs at about 289 nm (Figure 2). The spectrum of *N*-stearyl-L-tryptophan *n*-hexyl ester in methylcyclohexane (Figure 1) indicates that an indolyl ring buried in a completely nonpolar environment should have its 0–0 ^1L_a and 0–0 ^1L_b bands overlapping at about 289 nm (see Results). Judging from the effect of butanol upon the spectrum of *N*-stearyl-L-tryptophan *n*-hexyl ester in methylcyclohexane (Figure 1), the 0–0 ^1L_a band should be red shifted by several nm when the >NH moiety of the ring is hydrogen bonded. A 6- or 7-nm red shift of the 0–0 ^1L_a band occurs for tryptophan derivatives dissolved in alcoholic solvents at 77°K (Strickland *et al.*, 1970a, 1969). In contrast, the 0–0 ^1L_b band undergoes much less red shift when the indolyl ring is exposed to polar molecules (Figure 1 and Strickland *et al.*, 1969).

In the case of horse ferricytochrome *c*, X-ray analysis has revealed that the indolyl ring of tryptophan-59 is buried in a completely nonpolar region except for a carboxyl group which is hydrogen bonded to the indolyl >NH group (Dickerson *et al.*, 1971). The 0–0 ^1L_b tryptophanyl band of this residue occurs at the same wavelength as that of *N*-stearyl-L-tryptophan *n*-hexyl ester dissolved in methylcyclohexane (with or without butanol). The position of the 0–0 ^1L_a band in horse ferricytochrome *c* at 77°K (Figure 6) is consistent with the red shift observed when butanol was added to *N*-stearyl-L-tryptophan *n*-hexyl ester in methylcyclohexane (Figure 1). These results suggest that hydrogen bonding of the indolyl >NH group causes about a 4-nm red shift of the ^1L_a band. Some variation in the size of the red shift may be anticipated to result from changes in the strength of the hydrogen bond (Pimentel, 1957).

For the peroxidase isoenzymes the sharpness and positions of the tryptophanyl absorption bands show that their indolyl rings cannot be fully exposed to the water–glycerol solvent (compare Figures 2 and 3). The 0–0 ^1L_a tryptophanyl band of the peroxidases (300–301 nm) is red shifted much more than is the case with cytochrome *c*, even though the 0–0 ^1L_b band remains at about 289 nm. The 12-nm red shift of the ^1L_a band in the peroxidases seems much too large to be explained solely by a hydrogen bond. Thermodynamic considerations, of course, suggest that the indolyl >NH group probably is hydrogen bonded. Evidently, the large red shift in the peroxidases results because at least one additional polar group strongly perturbs the indolyl ring; *i.e.*, part of the ring is located near polar groups. These might be polar amino acid side chains, the carbohydrate moiety, or perhaps even well-ordered solvent molecules.

Suzuki (1967) has described the mechanism by which a wavelength shift results from the interaction between a polar chromophore and a polar environment. Apparently the ^1L_a excited state of the indolyl ring is more polar than the ground state.[1] A polar group suitably oriented near the indolyl ring of peroxidase could interact more strongly with the ^1L_a excited state than with the ground state, thereby causing a large red shift.

For other proteins having indolyl rings in a partially polar region, the 0–0 ^1L_a bands may occur at different wavelengths (about 290–302 nm). The position of the 0–0 ^1L_a band should vary from one tryptophan site to another, because the magnitude and sometimes even the direction of the wavelength shift depend on the orientation and number of polar groups (Suzuki, 1967). Furthermore, a polar group would not be expected to cause the same shift in both the ^1L_a and the ^1L_b electronic bands (Bernardin, 1970; Mataga *et al.*, 1964).

The wavelength positions and shapes of the tryptophanyl absorption bands at 77°K may provide a method to detect small differences between proteins. The 1-nm shift of the 0–0 ^1L_a tryptophanyl band of bovine cytochrome *c* (Figure 7) relative to horse cytochrome *c* (Figure 6) may indicate an intrinsic difference in these proteins. If so, then the effect on Trp-59 may result from the substitution of a glycine (bovine) for a lysine residue (horse) at position 60, even though these side chains are not in contact with each other (Dickerson *et al.*, 1971). On the other hand, it is difficult to exclude the possibility that the absorption spectrum of either horse or bovine cytochrome *c* may be altered due to changes which might have occurred during purification (Margoliash and Schejter, 1966; Myer, 1970). Interestingly, though, both type III and type VI horse heart cytochrome *c* (Sigma) gave the same absorption fine structure. (Type III is prepared using trichloroacetic acid, whereas type VI is prepared without.)

The reduction of ferricytochrome *c* shifts the 0–0 ^1L_a tryptophanyl bands by about 1 nm to the red (Figures 6 and 7). This shift may result from a rearrangement of a polar group(s) after the reduction of ferricytochrome *c*. One possibility is a small movement of the carboxyl group that is hydrogen bonded to the indolyl ring. However, even polar groups located some distance away may cause small shifts in the position of the ^1L_a bands. Since the region close by the indolyl ring is predominantly nonpolar in ferricytochrome *c* (Dickerson *et al.*, 1971) and thus has a low dielectric constant, the electric fields from more distant polar groups can be effective in spite of a larger separation. In any case, the smallness of the observed red shift suggests that reduction of ferricytochrome *c* does not bring any additional polar groups close by the indolyl ring.

As our final point, we consider whether the tryptophanyl absorption bands may be resolvable in other proteins cooled

[1] This conclusion is based upon the observation that in nonpolar solvents the largest red shift of the 0–0 ^1L_a band of 3-methylindole occurs with the solvent having the greatest polarizability (see Suzuki, 1967). The reasons for the red shift of the ^1L_a band have been described in more detail by Bernardin (1970) and Mataga *et al.* (1964).

to 77°K. When a protein contains two or more tryptophan residues, the numerous absorption bands will tend to overlap so extensively that only a few bands may be resolved. Sometimes even for proteins containing a single tryptophan residue, only the 0–0 ^1L_a tryptophanyl band may be well resolved; *e.g.*, the 0 + 850 cm^{-1} ^1L_a band may accidentally overlap the 0–0 ^1L_b tryptophanyl band. Experiments at 77°K have shown that in a number of proteins containing several tryptophan residues the longest wavelength ^1L_a bands give rise to shoulders located above 295 nm (Fretto and Strickland, 1971; Strickland *et al.*, 1969; E. H. Strickland, J. Horwitz, and C. Billups, 1970, unpublished experiments; Beaven, 1961). These long-wavelength tryptophanyl bands have also been detected in proteins using other techniques: by fluorescence (Weber, 1960; Anderson *et al.*, 1970), by phosphorescence (Purkey and Galley, 1970), by difference absorption spectra (Ananthanarayanan and Bigelow, 1969), and by circular dichroism (Strickland *et al.*, 1969). In many cases, the individual bands seem to be resolved best in either the circular dichroism or the absorption spectra of proteins cooled to 77°K.

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References

Ananthanarayanan, V. S., and Bigelow, C. C. (1969), *Biochemistry 8*, 3723.

Anderson, S. R., Brunori, M., and Weber, G. (1970), *Biochemistry 9*, 4723.

Beaven, G. H. (1961), *Advan. Spectrosc. II*, 331.

Bernardin, J. E. (1970), Ph.D. Thesis, University of Oregon.

Chance, B. (1963), *in* Techniques of Organic Chemistry, Friess, S. L., Lewis, E. S., and Weissberger, A., Ed., Vol. 8, Part II, 2nd ed, New York, N. Y., Interscience, p 1314.

Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A., and Margoliash, E. (1971), *J. Biol. Chem. 246*, 1511.

Estabrook, R. W. (1961), *in* Haematin Enzymes, IUB Symposium Ser. Vol. 19, Part 2, Falk, J. E., Lemberg, R., and Morton, R. K., Ed., Oxford, Pergamon, p 436.

Fretto, L., and Strickland, E. H. (1971), *Biochim. Biophys. Acta 235* (in press).

George, P. (1956), *in* Currents in Biochemical Research 1956, Green, D. E., Ed., New York, N. Y., Interscience, p 338.

Harbury, H. A., and Loach, P. A. (1960), *J. Biol. Chem. 235*, 3640.

Horwitz, J., Strickland, E. H., and Billups, C. (1969), *J. Amer. Chem. Soc. 91*, 184.

Horwitz, J., Strickland, E. H., and Billups, C. (1970), *J. Amer. Chem. Soc. 92*, 2119.

Konev, S. V. (1967), *in* Fluorescence and Phosphorescence of Proteins and Nucleic Acids, Udenfriend, S., Ed., New York, N. Y., Plenum, p 9.

Margoliash, E., and Frohwirt, N. (1959), *Biochem. J. 71*, 570.

Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 113.

Mataga, N., Torihashi, Y., and Ezumi, K. (1964), *Theor. Chim. Acta 2*, 158.

Myer, Y. P. (1970), *Biochim. Biophys. Acta 214*, 94.

Pimentel, G. C. (1957), *J. Amer. Chem. Soc. 79*, 3323.

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

Shannon, L. M., Kay, E., and Lew, J. Y. (1966), *J. Biol. Chem. 241*, 2166.

Shih, J. H. C., Shannon, L. M., Kay, E., and Lew, J. Y. (1971), *J. Biol. Chem.* (in press).

Strickland, E. H., Horwitz, J., and Billups, C. (1969), *Biochemistry 8*, 3205.

Strickland, E. H., Horwitz, J., and Billups, C. (1970a), *Biochemistry 9*, 4914.

Strickland, E. H., Kay, E., and Shannon, L. M. (1970b), *J. Biol. Chem. 245*, 1233.

Strickland, E. H., Kay, E., Shannon, L. M., and Horwitz, J. (1968), *J. Biol. Chem. 243*, 3560.

Strickland, E. H., Wilchek, M., Horwitz, J., and Billups, C. (1970c), *J. Biol. Chem. 245*, 4168.

Suzuki, H. (1967), Electronic Absorption Spectra and Geometry of Organic Molecules, New York, N. Y., Academic, p 97.

Urry, D. W. (1967), *J. Biol. Chem. 242*, 4441.

Vinogradov, S., and Zand, R. (1968), *Arch. Biochem. Biophys. 125*, 902.

Weber, G. (1960), *Biochem. J. 75*, 345.