*STUDIES ON THE EXCITED STATES OF PROTEINS**

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INTRODUCTION

If a bullock lens is illuminated by near UV, it emits a brilliant blue light, which is due to its proteins.[1] This is unexpected because two of the three aromatic amino acids—phenylalanine and tyrosine—have no visible fluorescence, and tryptophane has but a very weak one. A similar blue light is emitted if the lens is cooled in dry ice or liquid N_2. In neither case does the lens show an afterglow. If, however, the lens is illuminated by shorter UV, then a long-lasting afterglow can be observed. The monomolecular kinetics of the decay indicate that the light, in this case, was emitted from a metastable triplet state.[2] In order to arrive at a better understanding of these phenomena, we studied the optical properties of the aromatic amino acids and a number of proteins better defined than the proteins of the lens. Our results can be summed up by saying that the optical behavior of the lens and other proteins, under our conditions, is dominated by their tryptophane and that tryptophane, in a frozen watery solution, also shows two emissions—a short-lived one elicited by near UV and a long-lived one elicited by a shorter wave length. This indicates that the tryptophane within the protein is present in two different states which can be identified by their different excitability and light emission. The analogous two states can be detected also in frozen watery solutions of this amino acid.

EXPERIMENTAL

Absorption spectra were measured with the Beckman DK-1 automatic recording spectrophotometer. A variable-path quartz absorption cell was used in the absorption-spectra measurements of the concentrated protein preparations.

Room-temperature emission spectra of the proteins were determined with the Beckman DK-1 spectrophotometer, using the technique described by Gemmill.[3] For low-temperature emission studies (77° K.) this technique was modified by positioning an unsilvered Dewar flask containing liquid nitrogen in the DK-1 lamp housing compartment. The samples under study were placed in quartz test tubes and precooled in an alcohol dry-ice bath and then inserted into the liquid nitrogen. Exciting light was supplied by a Hanovia high-pressure xenon arc (Model 10-C-1). The wave-length region for excitation was isolated with a Leiss single-prism (fused-quartz) monochromator. It should be recognized that, with the exceptions noted below, none of the emission spectra for proteins and the detailed study of trypto-

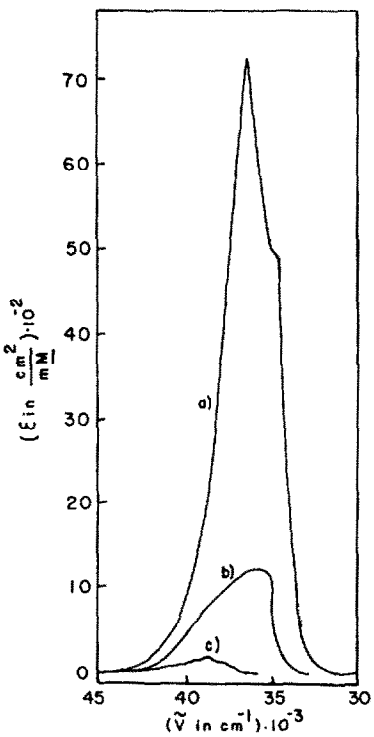

FIG. 1.—Molar extinction wave numbers for the aromatic amino acids: *a*, tryptophane; *b*, tyrosine; *c*, phenylalanine.

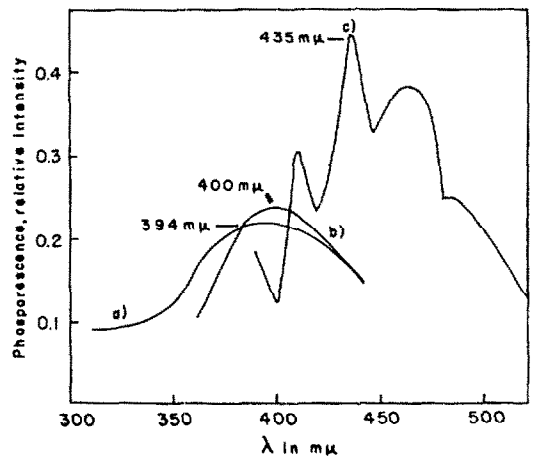

FIG. 2.—Phosphorescent spectra of (*a*) phenylalanine, (*b*) tyrosine, (*c*) tryptophane. Aqueous 0.001 *M* solutions containing 0.5 per cent glucose. $T = 77°$ K.

phane were corrected for the spectral-response sensitivity of the photomultiplier detector in the DK-1 spectrophotometer or for the prism efficiency of the monochromator of the DK-1. This uncorrected feature applies as well to the transmitted exciting light curves. It is to be noted, however, that the photomultiplier was an RCA 1P28 multiplier tube with S-5 response[4] with peak sensitivity at approximately 340 mμ. In practice, on the short-wave-length side of the 340-mμ maximum, the recorded curves (including the transmitted exciting light curves) are shifted approximately 5 mμ toward longer wave lengths, while on the long-wave-length side of 340 mμ the recorded curves are shifted approximately 5 mμ toward shorter wave lengths. These shifts are not important for any of the conclusions reported in this paper.

The fluorescent and phosphorescent spectra reported in Figure 2 were obtained

using Farrand grating monochromators to isolate the exciting light (xenon arc) and to analyze the emitted spectra. These curves were corrected for the spectral sensitivity of the RCA 1P28 photomultiplier tube.

RESULTS

Aromatic Amino Acids.—Figure 1 gives the absorption spectra of the three aromatic amino acids which are responsible for most of the 2300–3100 A absorption of protein. There is no apparent absorption at a longer wave length than 32,000 cm.$^{-1}$ (3120 A). The longest-wave-length absorption of tryptophane appears to be the unresolved peak masked under the shoulder at 34,000 cm.$^{-1}$ (2,900 A).

Our measurements of the fluorescent emission of these amino acids are in good agreement with those of Teale and Weber,[5] the maxima being at 284, 302.5, and 348 mμ for phenylalanine, tyrosine, and tryptophane, respectively. These emissions represent transitions from the lowest excited singlet state to the singlet ground state.[6]

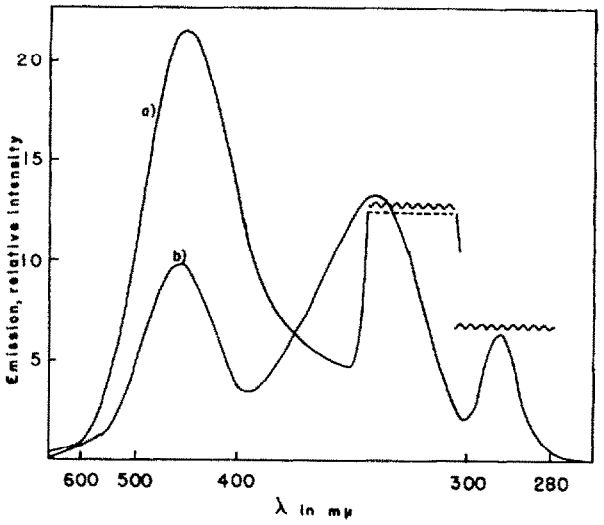

FIG. 3.—Emission spectrum of intact bullock lens, (*a*) excited in the 340-mμ region; (*b*) excited in the 280-mμ region. In all figures the wave line marks transmitted exciting light.

Figure 2 gives the phosphorescent spectra for the three amino acids. We have presented data[2] which led us to interpret the low-temperature emission for tyrosine and tryptophane as a transition from the lowest excited triplet state to the singlet ground state. Though the phenylalanine emission decay was too rapid for us to make kinetic studies with the equipment we had available, we have interpreted the long-wave-length shift with its close correspondence to the tyrosine emission maximum as evidence that this emission is also triplet-singlet in character.

PROTEINS

Bullock Lens.—At room temperature, excitation in the general region of the 280-mμ protein absorption band gave rise to an emission with a maximum at 340 mμ which we consider to be fluorescence originating from tryptophane residues in the protein. Shifting the exciting light to longer wave lengths elicited an intense emission with the same blue color as the phosphorescence we had observed from the same preparation at low temperature (77° K.).

Curve *b* in Fig. 3 gives the emission spectra of the bullock lens excited within the protein 280-mμ absorption band and by near UV at 77° K. (liquid N_2). The 330-mμ emission band had no delayed emission observable visually on our not too fast instruments. The 450 mμ band, in contrast, exhibited a considerable afterglow.

When we first observed the intense blue emission elicited by excitation in the 340-mμ region, we suspected that we might be inducing a direct singlet-triplet transi-

tion from the ground state or else that we were exciting the protein in a heretofore undetermined absorption band.

It might be reasoned that a singlet-triplet transition which would give rise to the intense emission we observed should manifest a reasonably strong absorption and that, consequently, a spectral examination of a concentrated protein preparation might reveal this fact. Such a study did, indeed, reveal a long-wave-length maximum in the absorption spectrum at 340 mμ. A long-wave-length shoulder was observed in this general region for all the proteins we have examined to date, but we have not felt justified in publishing the data, for we have not assured ourselves that scattering was not considerable in our experiments. Suffice it to point out that other workers, notably Goodwin and Morton,[7] have noted an "irrelevant absorption" in proteins in the 340–400-mμ region, where the aromatic amino acids do not absorb. When, however, the lens was excited in this absorption region at 77° K., the intense blue emission had no prolonged decay time. We may conclude that, if the low-temperature, long-wave-length emission elicited in the 340-mμ region was originating from a metastable triplet level, it was not the same level that is populated under the same conditions by exciting the protein in the 280-mμ absorption band.

Homogenized lens exhibited the same spectral behavior as did the intact material. The addition of glucose to the lens homogenate did not influence the lifetime of the 77° K. blue emission elicited by excitation in the long-wave-length 340-mμ region.

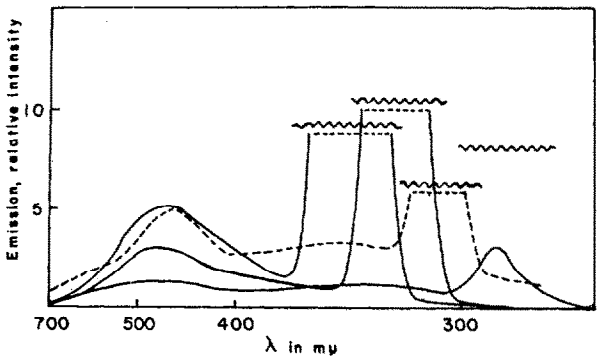

FIG. 4.—Emission spectrum of bovine serum albumin, excited by light of different wave lengths. 10 per cent aqueous solution, containing 1 per cent glucose. T = 77° K.

Albumin, Trypsin, Myosin.—Spectral data for bovine serum albumin, recrystallized trypsin, and myosin are represented in Figures 4–6. The emissions elicited by irradiation in the main protein absorption band around 280

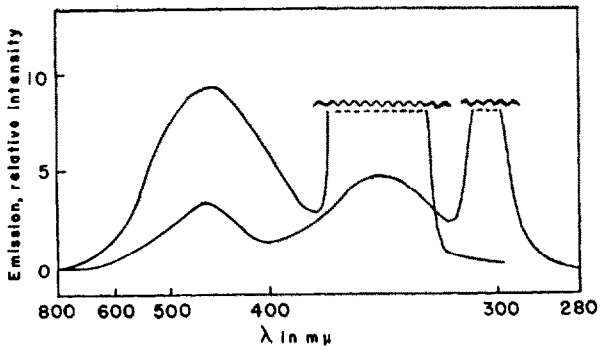

FIG. 5.—Emission spectra of recrystallized trypsin (0.245 gm. ml.$^{-1}$). T = 77° K.

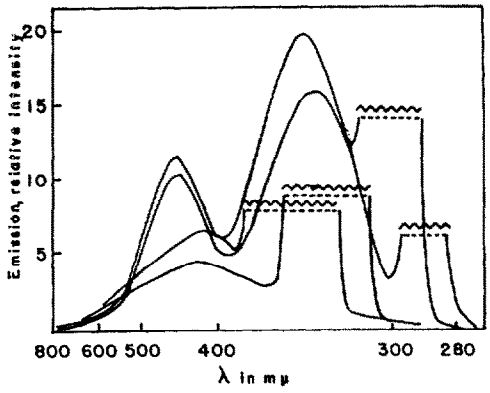

FIG. 6.—Emission spectra of myosin at different exciting wave lengths. 0.24 per cent aqueous solution. T = 77° K.

mμ were long-lived showing a considerable afterglow, while the emissions elicited by near UV around 340 mμ were short-lived. No afterglow could be seen visually or detected by our relatively slow instrumentation. The results are summed up in Table 1.

With the exception of the long-wave-length emission of myosin, elicited by exciting in the 340-mμ region, the emission spectra for the several proteins mirror the emission properties of tryptophane more than that of phenylalanine or tyrosine. Parenthetically, the close similarity between the low-temperature emission properties of the proteins and tryptophane, elicited by exciting in the absorption bands, supports the concept expressed by Teale and Weber[5] and ourselves,[8] that energy absorbed by phenylalanine or tyrosine is transferred to tryptophane, from which molecule emission occurs. The minimum in the emission spectra around 400 mμ, where phenylalanine and tyrosine phosphoresce (see Fig. 2), also bears out this idea. It might be considered that the high molar absorbancy of tryptophane relative to that of tyrosine or phenylalanine would result in tryptophane being the primary emitter. An examination of Table 2, where the molar ratios of the aromatic amino acid residues in the proteins are given, shows that, when allowance is made for the ratio of molar absorbancies (approximately 1:5:35 for phenylalanine, tyrosine, and tryptophane, respectively; see Fig. 1), phenylalanine and tyrosine should be almost as efficient emitters as tryptophane. Further, when the quantum yields are considered, tyrosine has approximately the same value as tryptophane, namely, 0.21 and 0.19, respectively (Teale and Weber[5]). The yield for phenylalanine is only 0.045, which may account for the nonobservance of an emission in the 300-mμ region, where phenylalanine and tyrosine fluoresce. Conversely, it may be

TABLE 1*

Protein	Long-Wave-Length Absorption in Aqueous Solution	Fluorescence 298° K.	Fluorescence 77° K.	Long-Lived Phosphorescence	Emission Elicited by Near UV (≅ 340 mμ) 298° K.	Emission Elicited by Near UV (≅ 340 mμ) 77° K.
Bullock lens	340	345	335	450	440	440
Bovine serum albumin	350	340	335	460	470	470
Trypsin	340	342	337	450	450	450
Myosin	340?	340	333	440	?	420

* Numbers stand for wave lengths of maxima in mμ.

TABLE 2*

Moles of Amino Acids in 10^5 Gm. Protein

Protein	Phenylalanine	Tyrosine	Tryptophane
Bullock lens	49	35	11
Bovine serum albumin	39	29	3
Trypsin	10	23	5
Myosin	28	18	4
Insulin	52	69	0

* compiled from date taken from R. J. Block and K. W. Weiss, *Amino Acid Handbook* (Springfield, Ill.: Charles C Thomas, 1956).

argued that the low quantum yield of fluorescence is evidence for an increased transition probability to the triplet state, where it may be more readily degraded as heat due to the increased lifetime. We, however, are inclined to the idea that the excitation energy is transferred by resonance to tryptophane and then emitted.[8]

Insulin and Tryptophane.—The close similarity in the emission properties of the

proteins and tryptophane led us to a comparative study of the emission behavior of tryptophane and of insulin, which contains no tryptophane.

Since we have previously noted[2] that the blue *delayed* emission of tryptophane (phosphorescence) was observable only after the addition of glucose to the system (in the absence of glucose what appeared to be the same blue emission was short-lived and decayed too rapidly for us to measure the decay kinetics), we examined the emission behavior with and without glucose. Though the kinetic behavior bore out our earlier observations, the emission spectra revealed interesting new details. These studies with watery solutions of tryptophane-no-glucose and tryptophane-glucose gave the following results:

1. Tryptophane-no-glucose excited in the absorption band at 77° K. displayed no fluorescent emission band, and the blue emission band with maximum at 435 mμ had no afterglow.

2. In the absence of glucose, 340-mμ excitation, at 77° K., elicited 435-mμ emission, just as found for proteins; this emission also had no afterglow.

3. Tryptophane *in the presence of glucose* at 77° K. exhibited a fluorescent band with a maximum at 325 mμ, and the blue band emitted at 440 mμ displayed a phosphorescent afterglow.

4. Long-wave-length excitation of tryptophane *in the presence of glucose* failed to elicit the 435-mμ short-lived emission. In these experiments aqueous 10^{-4} *M* tryptophane solutions were used.

The insulin was studied at 77° K., and the exciting light was varied from 260 to 450 mμ. When the protein was excited in the region of protein absorption (260–290 mμ), there was evidence of emission in the 290–340-mμ region, which was suggestive of fluorescence from the phenylalanine and tyrosine residues, but the intensity was too low to make positive assertion. It is of interest to note, however, that we observed no 440-mμ emission band, no matter what exciting wave-length region was selected. That is, phosphorescence, elicited by exciting in the region of protein absorption, was absent, as was the short-lived long-wave-length emission elicited by exciting the protein in the 340-mμ region. Insulin contains no tryptophane.

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[2] Steele, R. H., and Szent-Györgyi, A., these PROCEEDINGS, **43**, 477–91, 1957.

[3] Gemmill, Ch. L., *Anal. Chem.*, **28**, 1061–64, 1956.

[4] Radio Corporation of America, *Tube Handbook*.

[5] Teale, F. W. J., and Weber, G., *Biochem. J.*, **65**, 476–82, 1957.

[6] For all spectra reported in this paper (with the exception of that reported in Fig. 2) quartz monochromators were used to isolate both exciting and emitted spectral regions, and nondescript transmission was no problem.

[7] Goodwin, T. W., and Morton, R. A., *Biochem. J.*, **40**, 628–32, 1946.

[8] Karreman, G., Steele, R. H., and Szent-Györgyi, A., these PROCEEDINGS (in press).