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THE FLUORESCENCE OF TYROSINE IN ALKALINE SOLUTION

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SUMMARY

1. Contrary to previous reports in the literature, tyrosine in strongly alkaline solution (0.12 N NaOH in our experiments) was found to have a measurable fluorescence, and determinations of the relative quantum yield of this species are presented.

2. It was found that tyrosine and two proteins exhibiting tyrosine fluorescence, insulin and RNAase, show a shift in the peak of their fluorescence spectra from 315 mμ in neutral H_2O to 345 mμ in 0.12 N NaOH.

3. Under neutral conditions the quantum yield of tyrosine incorporated in a protein is diminished when compared with that of free tyrosine in solution. This is not the case in 0.12 N NaOH, suggesting the presence under neutral conditions of a "structural quenching effect" not present under alkaline conditions.

INTRODUCTION

Previous reports in the literature have usually described alkaline tyrosine as a non-fluorescent species[1,2]. One published fluorescence spectrum of alkaline tyrosine indicated a peak near 400 mμ (see ref. 3). Observations which we have made indicate that tyrosine in alkaline solution, though far less fluorescent than in neutral solution, has an easily measurable fluorescence occurring in the near ultraviolet region, at approx. 345 mμ. In order to characterize this fluorescence, we have recorded the fluorescence spectrum and calculated the quantum yield of tyrosine under strongly alkaline conditions (0.12 N NaOH). We have also recorded the fluorescence spectra and made preliminary measurements under similar conditions of pH of the quantum yields of two proteins exhibiting tyrosine fluorescence, insulin and RNAase.

MATERIALS AND METHODS

We have used commercially available DL-tyrosine (Fisher Scientific Co.), as received and also after two recrystallizations by precipitation from an alkaline solution titrated

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to neutrality with dilute HCl. Salts were carefully removed by washing the precipitate with 4 portions of double-distilled water of 10–20 times the volume of the precipitate. RNAase crystallized from ethanol was purchased from Worthington Biochemical Co. The insulin used included a highly purified zinc-free preparation kindly provided by Dr. M. SIMPSON and also a commercially available crystalline zinc insulin supplied as Iletin by Eli Lilly Co.

For fluorescence measurements we have used an Aminco–Bowman spectrophotofluorometer with a xenon arc (Aminco Cat. No. 4-8360) and an RCA 1P28 photomultiplier tube with its amplified output coupled to the Y-axis of an X–Y recorder. For our purposes, we have assumed that the fluorescence detecting and recording systems respond linearly to fluorescence intensity at any given wavelength. Thus, we have used the recorded curves of fluorescence spectra as the basis for our computations of quantum yields.

We have used the method for obtaining relative quantum yields described by TEALE AND WEBER[4], and our results are relative to the quantum yield of 0.21 which they have reported for tyrosine in neutral water solution. According to this method the ratio of the areas under the curves of $\lambda F(\lambda)$ *vs.* λ of two fluorescent substances is equal to the ratio of their quantum yields if the conditions of excitation are identical. $F(\lambda)$ is defined as the response of the detection system corrected for the change of response with wavelength of the photomultiplier tube and the loss of light energy in the grating of the fluorescence monochromator. For tyrosine, where there is considerable overlap of absorption and emission spectra, $F(\lambda)$ must be further corrected for reabsorption of fluorescence by the solution.

To obtain data for quantum-yield determinations we recorded the fluorescence spectra of tyrosine in double-distilled water (pH 5–7), in McIlvaine's buffer (pH 7.6), and in 0.12 N NaOH, of RNAase in double-distilled water, in McIlvaine's buffer (pH 7.6), and in 0.12 N NaOH, and of insulin in McIlvaine's buffer (pH 7.6), and in 0.12 N NaOH. Spectra of insulin in neutral water solution were not recorded owing to the fact that the absorption and emission characteristics of our preparations of insulin in double-distilled water are unstable. Light of identical wavelength was used for excitation of the samples of which the fluorescence was compared in quantum-yield determinations, and the solutions were of equal absorbancy (± 0.003) at the wavelength of excitation. Absorbancies were measured with a Beckman DU spectrophotometer. The normality of the NaOH solution was maintained at 0.12 ± 0.01 N by titration to a phenolphthalein endpoint with standard HCl. On standing for 24 h or longer the NaOH solutions were found to develop an increasing intrinsic fluorescence. For this reason all solutions were freshly prepared on the day of use. The normality of NaOH (0.12 N) was arbitrarily chosen in the pH range where virtually complete dissociation of the tyrosine hydroxyl can be expected. All solutions for fluorescence studies were handled in acid-washed glassware and cuvettes.

For the wavelength of excitation, we set the excitation monochromator of the Aminco–Bowman as close as possible to 277.3 mμ, which is an isosbestic point of the absorption curves of neutral and alkaline tyrosine as measured by our equipment. By this choice of wavelength, the neutral and alkaline tyrosine solutions used for relative quantum-yield determinations were of equal concentration as well as of equal absorbancy at the exciting wavelength, 277.3 mμ. The tyrosine solutions used ranged in concentration from 7.03–$8.59 \cdot 10^{-5}$ M ($A = 0.090$–0.110 at 277.3 mμ).

Fluorescence measurements were made using Pyrocell No. S18-120 1-cm path length quartz cuvettes.

To process the raw data obtained from the recorded curves of the fluorescence spectra of alkaline and neutral tyrosine, the ordinates of the recorded curves were measured in arbitrary units at intervals of 10 mμ. These readings were then corrected for variation in the sensitivity of the phototube with wavelength by use of the spectral-response curve supplied by the manufacturer for the RCA 1P28 phototube. We further corrected our data for reabsorption of fluorescence by the solution, using the formula.

$$F(\lambda)_{\text{corrected}} = \frac{F(\lambda)}{\alpha(\lambda)^{l}} \qquad (1)$$

where $\alpha(\lambda)$ is the transmission coefficient of the solution for fluorescent light of wavelength λ and l is the average thickness of solution traversed by the fluorescent light[4]. Because of the difficulty of calibration, we did not correct for attenuation of light by the grating of the fluorescence monochromator, and we did not correct for re-emission caused by the reabsorption of fluorescence, since in extremely dilute solutions of both neutral and alkaline tyrosine this factor is negligibly small compared to other sources of error. The data corrected as described were used as $F(\lambda)$ in plotting $\lambda F(\lambda)$ *vs.* λ for alkaline and neutral tyrosine.

Measurement of the fluorescence spectrum of tyrosine in the experimental concentrations in 0.12 N NaOH required the use of the maximum sensitivity settings of the Aminco–Bowman instrument, and at these sensitivities an appreciable though much less intense fluorescence could be recorded from the solvents and cuvettes alone. For this reason, we recorded in each experiment control spectra of the solvents used, and for quantum-yield determinations, subtracted the area of $\lambda F(\lambda)$ *vs.* λ of the blank (water, 0.12 N NaOH, or buffer), from the area of $\lambda F(\lambda)$ *vs.* λ of the substance being studied.

RESULTS

In addition to the quenching of fluorescence at alkaline pH (see refs. 1, 5, 6), we have observed that tyrosine and two proteins exhibiting tyrosine fluorescence, insulin and RNAase, show a shift in the peaks of their fluorescence spectra from 315 mμ in near neutral solutions (water or buffer) to 345 mμ in 0.12 N NaOH[7]. This shift begins above pH 11 and is complete in the range of pH 12–13. At intermediate pH's separate spectral peaks can be resolved at 315 and 345 mμ. Upon neutralization of the alkaline tyrosine solutions a single peak at 315 mμ is again observed.

Figures for the quantum yields of neutral and alkaline tyrosine, insulin, and RNAase are presented in Table II. These have been computed according to the method described above. Examples of the fluorescence excitation and emission spectra recorded for these substances are presented in Figs. 1–3*, and a summary of the con-

* The conditions under which the recording were made are presented in Table I. The ordinates for all figures are adjusted for absorbancy differences (in no case greater than 5%) so as indicate true relative intensities, but are otherwise uncorrected, except for Fig. 2b, (.....). The curves are presented to show the position of spectral peaks and relative fluorescence intensities, and are not in all cases the spectra on which quantum-yield determinations were made. They are therefore only approximately proportional to quantum yield.

TABLE I

DATA ON SPECTRAL CURVES*

Substance and solvent	Type of spectrum**	Wavelength of excitation (mμ)	Wavelength at which fluorescence monitored (mμ)	Absorbancy at 277.3 mμ	Concentration (where known)
DL-Tyrosine in H_2O	F	277 ± 3			
	E		315 ± 3	0.104	$8.17 \cdot 10^{-5}$ M
DL-Tyrosine in buffer pH 7.6	F	277 ± 3			
	E		315 ± 3	0.109	$8.56 \cdot 10^{-5}$ M
DL-Tyrosine in 0.12 N NaOH	F	277 ± 3			
	E		345 ± 3	0.105	$8.25 \cdot 10^{-5}$ M
RNAase in H_2O	F	277 ± 3			
	E		315 ± 3	0.100	123 mg/ml
RNAase in buffer pH 7.6	F	277 ± 3			
	E		315 ± 3	0.107	
RNAase in 0.12 N NaOH	F	277 ± 3			
	E		345 ± 3	0.106	
Insulin (Iletin) in buffer pH 7.6	F	277 ± 3			
	E		315 ± 3	0.098	
Insulin (Iletin) in 0.12 N NaOH	F	277 ± 3			
	E		345 ± 3	0.107	

* These curves are intended to show the position of spectral peaks and curves and are not in all cases the spectra on which quantum-yield determinations are based.

** Fluorescence spectrum (F) or excitation spectrum (E).

TABLE II

QUANTUM-YIELD DETERMINATIONS

Substance	Individual determination	Mean
DL-Tyrosine in H_2O (reference)		(0.21)*
DL-Tyrosine in buffer pH 7.6	0.103	0.088 ± 0.023
	0.070	
DL-Tyrosine in 0.12 N NaOH	0.011	0.009 ± 0.0013
	0.010	
	0.010	
	0.009	
	0.008	
	0.009	
	0.009	
	0.009	
	0.010	
	0.009	
	0.006	
Insulin in buffer pH 7.6	0.064	0.050 ± 0.020
	0.036	
Insulin in 0.12 N NaOH	0.008	0.008 ± 0.0017
	0.007	
	0.010	
RNAase in H_2O	0.025	0.024 ± 0.0014
	0.023	
RNAase in buffer pH 7.6	0.017	0.016 ± 0.0014
	0.015	
RNAase in 0.12 N NaOH	0.008	0.008 ± 0.0010
	0.007	
	0.009	

* The absolute value of 0.21 obtained by TEALE AND WEBER for the quantum yield of tyrosine in water was used as the reference value to which all other values listed are relative.

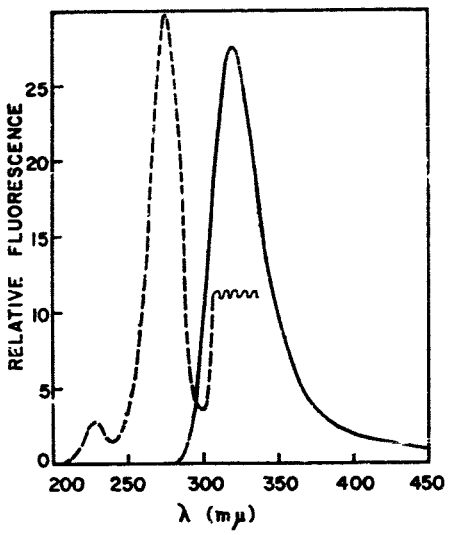

Fig. 1a.

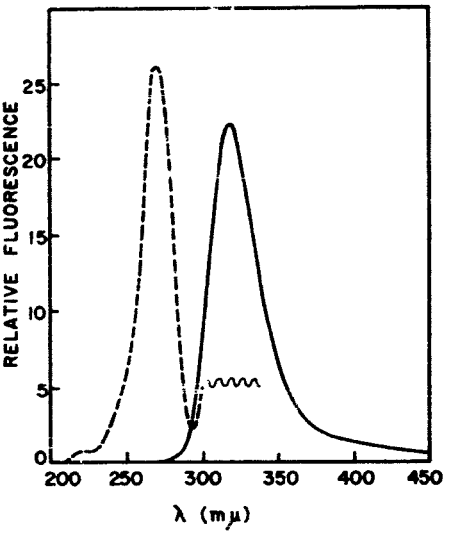

Fig. 1 b.

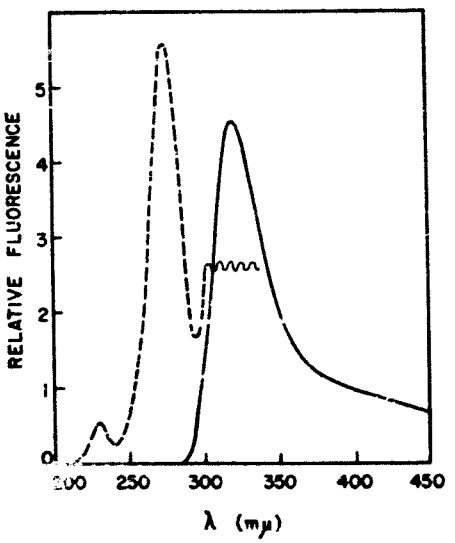

Fig. 1c.

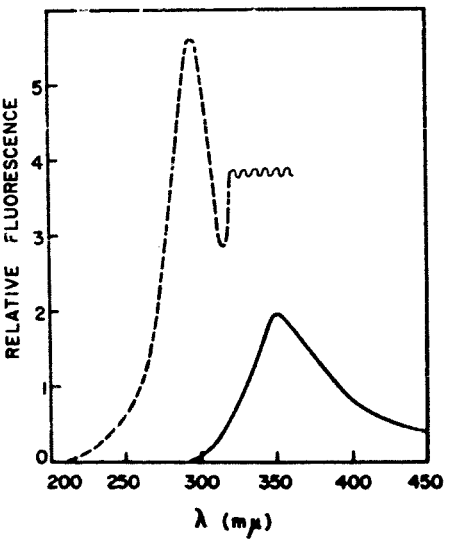

Fig. 1d.

ditions under which the recordings were made is given in Table I. A typical emission spectrum corrected for the spectral response of the phototube and the reabsorption of fluorescence is given in Fig. 2b. It can be seen that this correction yields higher values for the emission in the longer-wavelength portion of the emission band but does not significantly alter the position of the spectral peak.

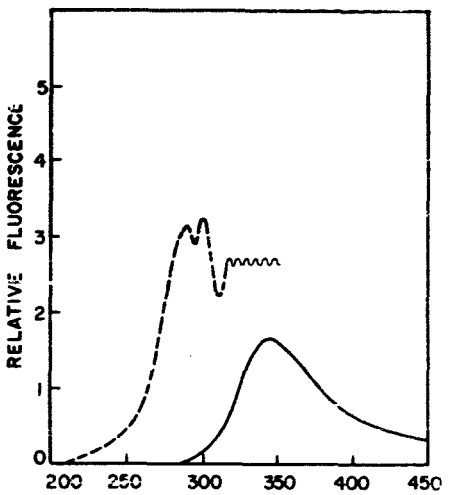

Fig. 1e.

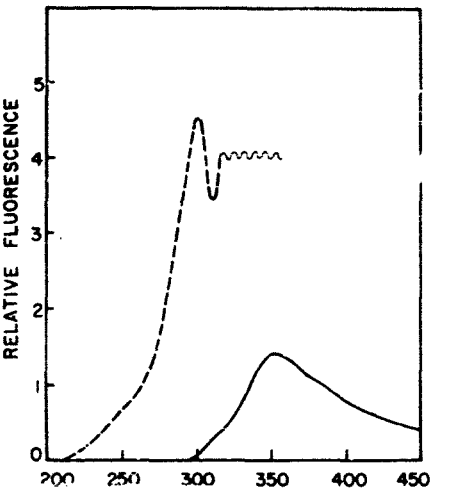

Fig. 1f.

Fig. 1. Uncorrected excitation (– – – –) and emission (———) spectra for tyrosine, insulin and RNAase. Spectra of neutral solutions (McIlvaine's buffer, pH 7.6) are shown for a, tyrosine; b, insulin; c, RNAase. Spectra in 0.12 N NaOH are shown for d, tyrosine; e, insulin; and f, RNAase. Scattered exciting light is indicated in the excitation spectra by (~~~~).

DISCUSSION

All our work was carried out at pH's where more than 99 % of the tyrosine hydroxyls would be expected to have lost a proton. We should like to point out, however, that we have no data, experimental or theoretical, which would allow an accurate estimate of the degree of dissociation of the sodium salt of the tyrosine phenolate group. We are therefore unable to state to what extent the observed fluorescence of alkaline tyrosine is due to the phenolate ion on the one hand, or to the corresponding sodium salt on the other. It is not unlikely that the sodium salt plays a role, especially in view of the relatively high concentration of sodium ion and the low concentrations of tyrosine employed. In order not to falsely imply a knowledge of this factor we have refrained from using the term "phenolate ion", using the less specific "alkaline tyrosine" instead.

The shift of the fluorescence spectrum of tyrosine in alkaline solution towards longer wavelengths corresponds to a similar shift in the absorption spectrum. Since even distilled water prepared by ordinary means may have ultraviolet fluorescence detectable by the Aminco–Bowman instrument, it seems clear that the terms "fluorescent" and "non-fluorescent" used in a qualitative sense must be interpreted with regard to the threshold of sensitivity of the detection equipment. Our attempts to determine the quantum yield of alkaline tyrosine are intended to illustrate that this species does have a florescence which is measurable with appropriate measuring devices, and which differs both qualitatively and quantitatively from that of neutral tyrosine.

It is interesting that the pH at which the shift in the fluorescence spectrum of tyrosine can first be seen is considerably above the tyrosine-hydroxyl-group p*K*

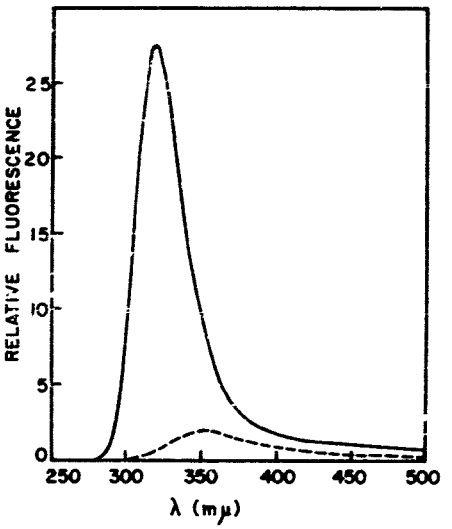

Fig. 2a.

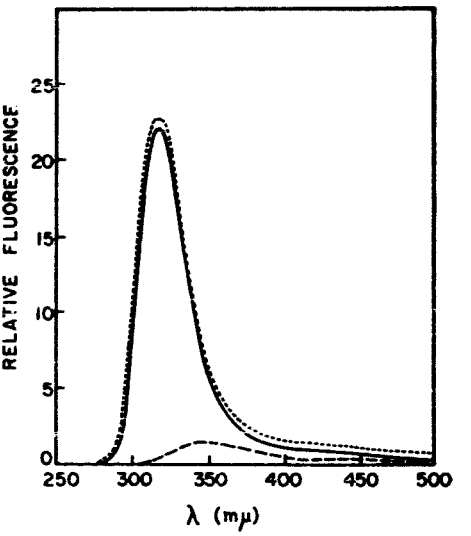

Fig. 2b.

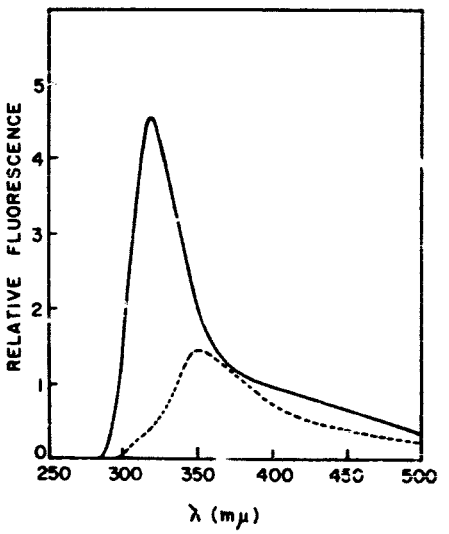

Fig. 2c.

Fig. 2. Illustrating the diminution in intensity of fluorescence and shift in wavelength under highly alkaline conditions (0.12 N NaOH) (– – –) compared to neutral solutions (McIlvaine's buffer, pH 7.6) (———), for a, tyrosine; b, insulin; c, RNAase. In addition, in b, (.....) depicts the magnitude of the correction factor when the curve for neutral insulin (———) is corrected for variation in spectral response of phototube and for re-absorption of fluorescence according to Eqn. 1.

of 10.1. Because the quantum yield of the phenolate form is much less than that of the unionized form, it is apparently necessary for a considerable excess of the former to be present before its characteristic fluorescence spectrum can be observed. We have found that this spectrum could best be observed in freshly prepared NaOH of pH approx. 13 and that a fall in pH allowed the appearance of the peak at 315 mμ associated with the unionized phenol group of tyrosine in addition to the peak at 345 mμ characteristic of alkaline tyrosine. Increasing the normality of the NaOH from 0.12 N to 0.2–0.3 N caused slight quenching of the fluorescence but did not shift the spectral peak from 345 mμ.

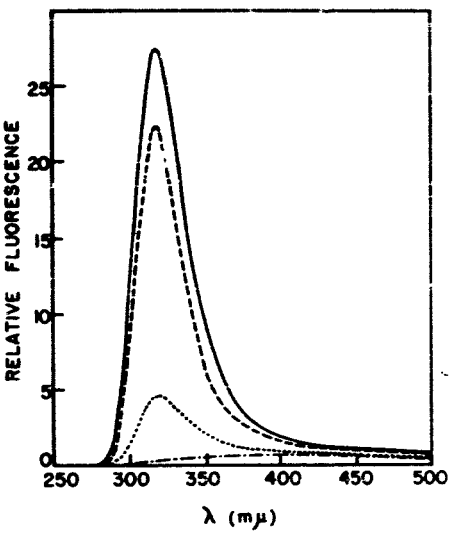

Fig. 3a.

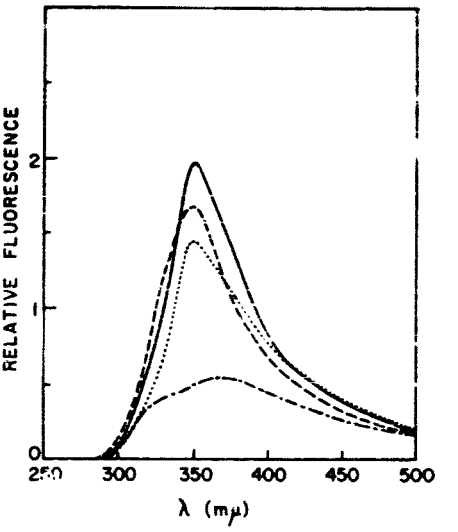

Fig. 3b.

Fig. 3. Illustrating the relative fluorescence intensities of a, neutral solutions (McIlvaine's buffer, pH 7.6) and b, alkaline solutions (0.12 N NaOH) of tyrosine (———), insulin (– – – –), and RNAase (.....). NaOH (0.12 N) blank (–·–·–).

The only previously published fluorescence spectrum which we have been able to find of tyrosine in alkaline solution (0.1 g/l = $5.53 \cdot 10^{-4}$ M; pH 10.77) shows a peak near 400 mμ with a shoulder at 350 mμ (ref. 7), thus differing significantly from the spectrum which we have recorded. However, we have obtained curves of similar form with the Aminco–Bowman instrument by the use of tyrosine solutions with concentrations greater than 3 mM in 0.12 N NaOH. When such solutions are diluted to the range of concentrations which we have used in measuring quantum yields, they give fluorescence spectra with a single peak at 345 mμ. Further dilution to the limits at which fluorescence measurements can be made causes no further shift in the peak of the fluorescence spectrum of tyrosine in 0.12 N NaOH. It is possible that the shape of the curves obtained with concentrated alkaline solutions may result in part from the high degree of re-absorption and re-emission of fluorescence together with changes in the geometrical distribution of fluorescence in the sample cuvette.

An important source of inaccuracy in our work is revealed by a comparison of the correction factors obtained by the use of the manufacturer's response curve for the 1P28 phototube[8] with those obtained by WHITE, HO, AND WEIMER[10] in a direct calibration of an Aminco–Bowman instrument, in which grating attenuation and other factors besides phototube sensitivity are taken into account. The discrepancies in these two sets of correction factors indicate that our data must be regarded as only partially corrected.

Reference to Table II will show that there is variation in the results obtained in different experiments. Part of this variation may be due to a probable error of at least ±3 mμ in setting the coarsely graduated dials of the monochromators of the Aminco–Bowman instrument. For this reason, it was impossible to duplicate exactly the wavelength of excitation on successive days. On any one day the excitation-wave-

length dial was set at the beginning and not touched until completion of the day's experiments. Because of the inaccuracies of setting, reading and recording wavelengths with the Aminco–Bowman instrument we believe that the peak at 345 mμ which we have observed in the fluorescence spectrum of alkaline tyrosine may be no more accurate than ±5 mμ.

The absorbancies which we have used at the excitation wavelength (A = 0.090–0.110) are slightly above those regarded as optimal by TEALE AND WEBER[11] for quantum-yield determinations, but were chosen because of the greater accuracy and convenience of absorbancy measurements in this range. Furthermore, pilot experiments with solutions of A = 0.050 gave no significant difference in quantum-yield results.

The figures for the quantum yields of RNAase and insulin are based on only two or three experimental determinations and thus must be regarded as preliminary. The figure for insulin especially is open to question since it is based on the use of a commercial preparation, Iletin, which, according to the manufacturer's description, contains 0.2% phenol and 1.6% glycerin. The spectral shift to 345 mμ in 0.12 N NaOH was also observed with the highly purified insulin preparation described above. In view of the potential sources of inaccuracy in our work, there seems to be quite good agreement between our figure of 2.4% for the quantum yield of RNAase in water and the figure 1.7% previously reported by TEALE[12].

It is of interest to note (Table II) that while there are considerable differences between the quantum yields of neutral solutions of tyrosine, insulin, and RNAase, the values for the respective alkaline solutions are to a first approximation the same. It would thus appear that when the amino acid tyrosine is incorporated in a protein its fluorescence is "structurally" quenched to a variable degree under neutral conditions but not under alkaline conditions. It seems reasonable that this "alkaline quench release" may be related to pH-dependent structural changes in the protein molecule. No data bearing on this possibility are available at the present time, however; and further speculation now would be premature.

All of our measurements are undoubtedly susceptible of greater accuracy through the use of more precise equipment and elaborate technique. Our data are presented at this time to demonstrate that tyrosine in strongly alkaline solution, either alone or as a tyrosine-containing protein, exhibits detectable fluorescence in the near ultraviolet region, and that, compared with neutral tyrosine, the quantum yield is the order of twenty-fold lower with concomitant shift of the emission peak from 315 mμ to 345 mμ.

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* The author remarks that the value of 303 mμ reported by TEALE AND WEBER[4] for the peak of the fluorescence spectrum of neutral tyrosine is lower than that usually recorded by commercial fluorescence spectrophotometers.