Biophysical Chemistry 8 (1978) 87–104

PULSEFLUORIMETRY OF TYROSYL PEPTIDES

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Received 21 October 1977

The fluorescence quantum yield and the fluorescence decay of aqueous solutions of derivatives containing a single tyrosine residue have been measured at different pH. In these derivatives tyrosine was substituted on its amino end (series I) or/and, on its carboxyl end (series II), by acyl, amino or amino acyl groups. The fluorescence decays of series I derivatives are monoexponential regardless to the ionization state of their amino group. Upon deprotonation of the α-amino group, the quantum yields and the lifetimes increase in the case of dipeptides, and slightly decrease, for the tripeptides. The quantum yield and the lifetime increase with the side chain length of the aliphatic residue adjacent to the tyrosine residue, (the fluorescence of Val Tyr anion being identical to that of free Tyrosine). Quite different is the behavior of series II derivatives: their decays at pH 5.5 must be described by two exponential terms, one of them decaying with a short time constant (about 0.5 ns) and little side chain effect is observed. The fluorescence intensity increases upon deprotonation of the α-amino group (though to a lesser extent than for series I derivatives); a nearly monoexponential decay is observed at basic pH for dipeptides, but not for tyrosine amide, amide of dipeptides, or tripeptides. The following interpretation of our results is proposed: fluorescence quenching occurs in molecular conformations in which a peptide carbonyl can come in contact with the phenolic chromophore. This condition depends mainly on the value of the angle χ_1 which determines the conformation of the tyrosyl residue around its $C_\alpha - C_\beta$ bond. It appears that the rotamer in which quenching occurs are not the same for series I and series II derivatives, which can explain the different behavior of these two kinds of compounds. The interpretation of the fluorescence properties is developed taking into account on one side the relative population of the rotamers in the ground state, which is given by studies of crystals and of solutions, and on the other side the possibility of an exchange between these rotamers during the excited state time. In this scheme the protonated α-amino groups would act to reinforce the quenching efficiency of the carbonyl. At last it is found that the radiative lifetime of the phenolic chromophore is the same for all the compounds studies.

1. Introduction

White [1] was the first to report that the fluorescence quantum yield in aqueous solution of the tyrosyl residue in small peptide molecules was lower than that of the free aminoacid, and that its value depended on the pH of the solution. These studies were resumed and extended by a number of authors [2].

A quenching by the carbonyl group of the peptide bond was found to take place, especially when this bond was on the C-terminal end of the tyrosyl residue [3–6]. Cowgill [7] suggested that this quenching might be due to a direct intramolecular interaction between the carbonyl group and the aromatic ring; the quenching efficiency would depend on the spatial relationship between the two groups, on the polarizability of the carbonyl group, and on the possibility of the solvent to form a hydrogen bond with the latter [6].

The free α amino-group could also modify the fluorescence of the phenol chromophore. However its influence differed from one compound to another [7]. For example, the amino group deprotonation had no effect on the fluorescence of free tyrosine [1, 9] whereas it induced a raise of the fluorescence intensity in the derivatives of the type H_3^+N-Tyr-$(Gly)_n COO^-$ for $n > 1$; this effect remained for $n = 1$ in the series $H_3^+N(Gly)_n$-Tyr COO^-, but it disappeared for $n > 1$ [5]. On the other hand the deprotonated α-amino group seemed to have a quenching effect in the case of the peptide Gly Leu Tyr [8].

Also the presence of neighbouring side chains was found in certain cases to modify the fluorescence of the tyrosyl residue [2]. A few measurements suggested that the chromophore fluorescence was enhanced when

the glycyl residue of the dipeptide Gly Tyr was replaced by an alanyl or leucyl residue.

Most of the previous studies were made using a continuous excitation (fluorescence quantum yield measurements). Several workers reported the results of decay time measurements of free tyrosine or related compounds in aqueous solutions [9–12], but very few works concerned by tyrosine peptides [11].

The study of the transient fluorescence of these compounds is expected to bring additional information on the quenching of the fluorescence of the phenol chromophore, especially by the peptide and α-amino groups. Furthermore this kind of study can provide indications on the conformations of complex molecules in solution [12–15] and on their rates of exchange. This was illustrated in a recent study of the fluorescence of tryptophan diketopiperazines [15].

In the present work, fluorescence quantum yield and transient fluorescence measurements were carried out on aqueous solutions of several derivatives containing a single tyrosyl residue, substituted either on its amino end (series I) or on its carboxyl end (series II), by the acetyl, amino or aminoacyl groups. The pH of the solution was adjusted so that the carboxyl group, if it was free, was on its ionized state, and the dependence of the fluorescence parameters on the ionic state of the amino group was investigated. To carry out this study, it was necessary to resolve the technical problems raised by the rather low fluorescence quantum yield and lifetime of the tyrosyl derivatives, and by the short wavelength of the maximum of their fluorescence spectra. In addition to the present work, this permitted the study of more complex oligopeptides [16] and should allow the study of polypeptide and protein containing tyrosyl residues.

2. Experimental section

2.1. Materials

Samples of L-tyrosine obtained from two different sources (Merck and Fluka, puriss) gave the same results. Tyramine (Fluka) was purified by sublimation and *p*-cresol (Fluka) was recrystallized. AcTyrNHEt was a git of R. Mayer (C.B.M. Orléans). TyrGly and $TyrGlyNH_2$ was obtained from Bachem, AcTyr and the peptides TyrAla, AlaTyr, AlaAlaTyr from Sigma Chemical Co, LeuTyr from Fluka, and all other peptides from Cyclo Chemical Co. All the peptides except $TyrNH_2$ and $TyrGlyNH_2$ appeared to be homogeneous by thin layer chromatography using Merck's silicagel F 254 plates in three solvent systems (n-butyl alcohol/acetic acid/water (9:1:2.5), ethyl acetate/pyridine/water (30:15:16.5), methanol/chloroform/ammonium hydroxide/water (22:27:5:5) when they were revealed with ninhydrin, t-butyl hypochlorite and Pauly reagent. In the case of $TyrNH_2$ and $TyrGlyNH_2$ it appeared very difficult to eliminate traces of impurities; however the amount of these impurities was estimated to be less than one percent.

Imidazole (Fluka) was treated with charcoal and recrystallized from petroleum aether, and did not show any detectable fluorescence.

p-terphenyl was obtained from Koch-Light laboratories.

2.2. Methods

The samples were dissolved in a 2×10^{-3} M acetate buffer, or in solutions of imidazole 2×10^{-3} M, all prepared with twice distilled water. At these concentrations acetate and imidazole do not affect the fluorescence of tyrosine, as was checked preliminary.

The pH of imidazole solutions was adjusted with HCl or NaOH, and redetermined after dissolution of the peptides. In all cases, the value of the pH was determined before and after each fluorescence measurement. The Tacussel pH meter was standardized using standard buffer solutions obtained from Merck. The accuracy of the measured pH is estimated as ± 0.05 pH unit. Solutions with a concentration of 3×10^{-4} M were used, both for steady-state and transient fluorescence measurements.

Absorption spectra and optical densities were measured with a Beckman Acta III spectrophotometer. Steady-state fluorescence measurements were made using a Jobin Yvon spectrofluorometer modified in our laboratory. Thermostated 5 × 5 mm quartz cells were used.

Beside the phenol hydroxyle group, the molecules under study showed either a carboxyl group, an amino group, or both groups at the same time. Solutions of the anionic, cationic or zwitterionic forms of these molecules, respectively, were obtained by adjusting the pH to 5.5. This choice was supported by published pK

data [17] and fluorimetric titration results (ref. [5] and this work).

In the basic pH range, ionization of the phenol hydroxyl to give a non fluorescent species, introduces some uncertainty in the determination of fluorescence quantum yield. Hence the values given in the table of results were measured at pH 9, for which the phenolate concentration is low. Since for some compounds the ionization of the amino group is not completed at this pH, transient fluorescence measurements were carried out at slightly higher pH.

Fluorimetric titration curves were obtained by comparing the fluorescence intensity of the solutions at various pH to that of a solution at pH 5.5, the optical densities being identical. Excitation was at 278 nm.

Relative fluorescence quantum yields at pH 5.5 were measured using a solution of tyrosine at the same pH, as the reference.

Since all the samples except p-cresol have absorption and emission spectra very close to that of tyrosine, the fluorescence intensities of the samples (F) and of the reference (F_{Tyr}) measured at 305 nm, near the emission maximum were compared [18]. The respective optical densities at the excitation wavelength (270 nm) OD and OD_{Tyr} were chosen as close to each other as possible; the relative fluorescence quantum yield Q_r was given by:

$$Q_r = \frac{F}{F_{Tyr}} 10^{(OD-OD_{Tyr})/2} \frac{OD_{Tyr}}{OD} \qquad (1)$$

The inner filter correction included in this formula is based on the fact that the PM of the fluorimeter views a small area in the center of the sample cell.

In the case of p-cresol, the quantum yield was determined from the ratio of the areas under the corrected emission spectra curves measured by planimetry [18].

The quantum yields of solutions at pH 9 were determined by comparing their fluorescence intensities to that of solutions of the same samples at pH 5.5, as well as to that of a solution of tyrosine, pH 5.5. At pH 9, there exists a fraction f of molecules in the non fluorescent phenolate form. If we estimate the value of f from the value of the optical density at 298 nm, the measured quantum yield Q_r is related to the quantum yield Q'_r of the fluorescent species by the relation

$$Q_r = (1 - f) Q'_r . \qquad (2)$$

At pH 9, the difference between Q_r and Q'_r was less than 4%.

For the study of the variation of the quantum yield with temperature, the fluorescence intensity of the sample at a given temperature was compared to that of tyrosine at the same temperature. Temperature was determined with an accuracy of ± 0.2°C using a calibrated thermistance. For temperatures below 20°C, dry nitrogen was blown on the outer surfaces of the cell to avoid water condensation.

Transient fluorescence measurements at 20°C were performed in thermostated 1 × 1 cm quartz cells. Transient fluorescence curves were measured with the single photon counting method [19, 20], with an apparatus supplied with a peak stabilizer [21]. The exciting light was provided by a high pressure deuterium or deuterium-neon [22] flash lamp, with a frequency of 10 kHz. Its wavelength was selected at 270 nm through a 250 nm Bausch and Lomb monochromator, with the band-width set between 13.2 and 16.5 nm. The emission wavelength, selected through a 500 nm Bausch and Lomb monochromator, was chosen at 315 nm, to allow the use of p-terphenyl as a reference compound for the determination of the apparatus response function (see below). With the band-width used (4 to 6.4 nm) the amount of stray light was negligible (less than 0.5%). The intensity of the fluorescence beam collected on a Radiotechnique XP 2020 photomultiplier was, if necessary, reduced with a neutral MTO filter situated at the output of the excitation monochromator, so as to obtain counting rates ranging from 0.5% to 2% of the flash frequency. Data collection was stopped when the total count in the fluorescence curve was about one million, which was generally reached in less than two hours.

For the transient fluorescent measurements at other temperatures than 20°C, we used the same method than that described for steady-state fluorescence measurements.

Transient fluorescence analysis. Experimental transient fluorescence curve $i(t)$ is a convolution product of the type

$$i(t) = \int_0^t G(t)\, I(t - T)\, dT . \qquad (3)$$

which is conventionally written

$$i(t) = G(t) * I(t) . \qquad (3a)$$

$G(t)$ represents the apparatus response function to the

excitation light and $I(t)$ the decay corresponding to an infinitely short excitation function. $G(t)$ depends on the range of wavelength used for absorption and emission. This curve cannot be directly measured. However, it can be obtained from the transient fluorescence $i'(t)$ of a reference compound which has a monoexponential decay of known time constant τ' [21]. Furthermore, it can be shown that as long as the decays of the fluorescences under study are monoexponential or are described with a sum of exponential terms, it is not necessary to calculate $G(t)$ from $i'(t)$.

Thus for instance, if we assume that the decay is monoexponential, with a time constant τ, we have:

$$i(t) = \frac{M_0}{M_0'}\left[\frac{\tau - \tau'}{\tau^2}\, i'(t) * \exp(-t/\tau) + \frac{\tau'}{\tau}\, i'(t)\right], \qquad (4)$$

where M_0 and M_0' are the zero order moments of $i(t)$ and $i'(t)$ respectively.

More generally, $I(t)$ can be written as a sum of exponential functions of the form:

$$I(t) = \sum_{i=1}^{P} A_i \exp(-t/\tau_i) \qquad (5)$$

where τ_i are the decays times and A_i the amplitudes of the exponential terms. Eq. (4) then becomes:

$$i(t) = \frac{M_0}{M_0'}\frac{I}{\Sigma C_i \tau_i}\left\{\tau' i'(t) + \sum_{i=1}^{P} C_i \left(I - \frac{\tau'}{\tau_i}\right) i'(t) * \exp(-t/\tau_i)\right\}, \qquad (6)$$

where the C_i are the relative amplitudes given by

$$C_i = A_i/\Sigma A_i\,, \qquad \Sigma C_i = 1\,.$$

Contrary to the A_i, the C_i are independent from the arbitrary number of counts of $i(t)$, and will be employed in the following.

In the present work, p-terphenyl in deaerated cyclohexane was used as reference compound. The value of its time constant [21] is 0.96 ns at 20°C. $i'(t)$ was determined before and after each measurement of $i(t)$ in similar excitation and emission conditions.

It must be emphasized that the modulating function analysis of transient fluorescence [23] can easily be modified in order to use directly $i'(t)$ instead of the calculated $G(t)$. This is also true for the calculation of the amplitudes of the exponential terms by the cut-off moments methods [23].

Computer programs based on these methods were used to determine the values of C_i and τ_i which gave the best fit between the experimental curve $i(t)$ and the convolution function $i_c(t)$ calculated from eq. (6).

This fit can be appreciated from the value of the weighted mean residue R [24, 25] defined by

$$R = \frac{1}{n}\sum_{k=1}^{n} \frac{[i_c(k) - i(k)]^2}{i(k)}, \qquad (7)$$

where n is the total number of channels of the analyzer used for the curve record, $i(k)$ and $i_c(k)$ the number of counts in the kth channel of the experimental and calculated curves, respectively.

By drawing of the deviation function [26] defined as

$$\mathrm{DV}(k) = [i_c(k) - i(k)]/i(k)^{1/2} \qquad (8)$$

it is also possible to point out the time intervals, if any, where $i_c(t)$ diverge systematically from $i(t)$.

Photocounting obeys the Poisson statistics [24]. Then the best fit between $i_c(t)$ and $i(t)$ should be obtained when R becomes equal to one: in addition the deviation function should fluctuate randomly around zero, with an average amplitude equal to one. In fact, this ideal behaviour was never observed, for a number of reasons which have already been partly discussed in a preceding paper [27], and which we recall here in a somewhat different manner:

a) first it is to be noted that relations (7) and (8) do not take into account statistical errors in the determination of the apparatus response $G(t)$, or, in one particular case, of the transient fluorescence of the reference compound $i'(t)$. This error was found to be important especially when the time constants of the sample were smaller than the time constant of the reference, and when the half-height width of $G(t)$ was too great.

b) it has been observed that the minimal value of R increases with the duration of the experiment. This can be attributed to systematic errors such as:

- fluctuations of the shape of the exciting flash
- disturbance by electrical signals
- incomplete correction of the wavelength effect in the photomultiplication response, due to the shift between the fluorescence spectra of the sample and of the reference compound.

These considerations led us to introduce a new parameter P defined by the following expression:

$$P = [(R - 1)/M_0]^{1/2}, \quad (9)$$

which gives an estimation of the whole systematic error, independent on data collecting time. It must be noted that P is still dependent to some extent on the value of the time constants of the sample (see a).

Since it appears difficult to find an absolute criterion of the fit between $i_c(t)$ and $i(t)$, the following method was developed to reduce the uncertainties on the decay characteristics determination.

For all transient fluorescence curves $i(t)$, we searched the monoexponential function that gave the best value of P. For this purpose, a first estimation of the single time constant was achieved by using the modulation functions method and then the value obtained was varied systematically, the amplitude being calculated by the cut-off moments method.

For a number of substances this analysis gave relatively low values of P. Furthermore, the fluctuation of the deviation function approach very closely to a random one. Thus the monoexponentiality of these decays was quite probable. This indeed was expected for some of them, since they were decays of rather simple compounds. A reinforcement of this inference was obtained by analyzing the two transient fluorescence curves $i'(t)$ of the p-terphenyl, measured before and after each $i(t)$, with regard to one another, the time constant of 0.96 ns being assigned. The so-obtained values P' of the parameter P were very close to the best values provided by the monoexponential analysis of the $i(t)$ curves: so was it for the fluctuation of the deviation function.

For another class of decays, the values of P obtained from the monoexponential analysis were much higher. In those cases, the deviation functions diverged systematically from zero. Thus a biexponential analysis was tried, using a similar method than for the monoexponential analysis. Two dimensional tables of the values of P were set by varying the two time constants τ_1 and τ_2. In these tables, identical values of P are arranged on concentric elliptical-shaped curves, as is expected for a determination of parameters by residue minimization [25].

Theoretically, the true values of τ_1 and τ_2, and of the amplitudes of the exponential terms should correspond to the minimal value of P. Nevertheless, this value in a number of cases, was lower than the value of P' and than the value of P obtained from the analysis of a transient fluorescence having a monoexponential decay with a close time constant and measured under identical experimental conditions. Owing to the increased number of parameters, this is probably due to the erasing of the systematical divergences between $i(t)$ and $i'(t)$, which is allowed by small shifts in the values of the decay parameters.

Therefore we admitted among the possible solutions every couple of time constants which gave a value of P close to that obtained for the monoexponential decay. In the tables of results, the values of the decay parameters τ_1, τ_2, C_1 corresponding to the minimal value of P are given, together with the differences $\Delta\tau_1, \Delta\tau_2, \Delta C_1$ between the average of the acceptable values (which is in most cases distinct from the value corresponding to the minimum of P) and the utmost acceptable values. The lowest and highest acceptable values of the average time constant $\langle\tau\rangle$ defined as

$$\langle\tau\rangle = C_1\tau_1 + C_2\tau_2 \quad (10)$$

are also given, and provide another kind of estimate of the reliability of the analysis.

It must be emphasized that the possibility to resolve the exponential terms of a complex decay is greatly dependent on the relative values of their time constants and amplitudes. Especially, if the time constants of a biexponential decay become close to each other, the two exponential terms become very difficult to resolve, and the monoexponential analysis will give a satisfactory result. The same will hold if the amplitude of one of the exponential become very small. This has been mathematically discussed by Isenberg [28] in the case of the analysis by the method of moments.

3. Results

3.1. Fluorescence quantum yields

The values of the relative fluorescence quantum yields of the tyrosyl derivatives of series I and II at pH 5.5 and pH 9, are given in table 1, together with that of tyramine and p-cresol. Our values at pH 5.5 are in good agreement with that given in the literature [2], except for TyrAla which gave a value lower than the value cited by Cowgill [2].

Table 1
Fluorescence of tyrosine derivatives: relative quantum yields and monoexponential analysis of the transient fluorescence. A – Data for tyrosine, tyramine, p-cresol and tyrosine derivatives of series I. B – Data for tyrosine derivatives of series II, AcTyrNHEt and $GlyTyrNH_2$. (Excitation: 270 nm, emission: 315 nm.)

		Protonated amino group				Unprotonated amino group			
	Q_f(a)	pH	τ_{ns}(b)	$P(\times 10^{-3})$	τ/τ_T(c)	pH	τ ns	$P(\times 10^{-3})$	τ/τ_T(c)
Tyrosine	1	5.2	3.38	1.2	1	10.5	3.3	1.0	0.98
Tyramine	0.94	4.4–6.3	3.2	1.3	0.95				
p-cresol	1.05	5.77	3	1.2	0.89				
A									
N-acetyl-tyrosine	0.96 (–)	6.88	3.2		0.95				
GlyTyr	0.38 (0.69)	5.5	1.3	1.2	0.385	10.2	2.7	1.4	0.8
AlaTyr	0.46 (0.83)	5.5	1.51	1.5	0.45	10.6	3	1.4	0.89
LeuTyr	0.485 (–)	5.5	1.6	1.4	0.475				
ValTyr	0.63 (0.84)	5.5	2.16	1.2	0.64	10.5	3.37	1.4	1
GlyGlyTyr	0.56 (0.53)	5.5	1.9	1	0.56	10.1	1.7	1	0.5
AlaAlaTyr	0.75 (0.72)	5.5	2.56	1.2	0.76	10.1	2.37	1.1	0.7
B									
$TyrNH_2$	0.25 (0.3)	5.5	1.05	2.4	0.31	9.9	1.15	3.3	0.34
TyrGly	0.335 (0.63)	5.5	1.35	3.4	0.4	9.9	2.13	1.5	0.63
TyrAla	0.38 (–)	5.5	1.57	3	0.465				
TyrLeu	0.4 (–)	5.5	1.7	4.1	0.5				
TyrVal	0.33 (0.66)	5.5	1.64	4	0.485	9.9	2.26	1.5	0.67
$TyrGlyNH_2$	0.23 (0.33)	5.5	1.1	5	0.29	9.9	1.25	2.3	0.37
$TyrLeuNH_2$	0.25 (–)	5.5	1.37	6	0.4	10.7	1.3	2.6	0.385
TyrGlyGly	0.22 (0.315)	5.5	1.14	5	0.34	10.5	1.15	2.4	0.34
AcTyrNHEt	0.69 (–)	5.5	2.36	1.3	0.7				
$GlyTyrNH_2$	0.20 (0.3)	5.5	0.66	2.3	0.195				

(a) Estimated error ± 0.01; numbers in brackets refer to the values of Q_f measured at pH = 9. (b) Estimated error ± 0.05 ns. (c) τ_T is the fluorescence lifetime of tyrosine (3.38 ± 0.05 ns).

Our measurements of the variation of the quantum yields with pH, corroborate the results of Edelhoch and coworkers [5]; we have extended the study to a number of additional compounds, and examples of the titration curves obtained are given in fig. 1. In the case of GlyGlyTyr and AlaAlaTyr a slight decrease of the quantum yield is observed, beside the formation of non fluorescent tyrosinate. As will be shown, this decrease is not an artefact due to the imprecision of the measurement, since it affects the decay time constant itself. This behaviour is to be compared with that found for the peptide GlyLeuTyr [8].

Temperature dependence of the quantum yields of AcTyrNHEt, TyrAla, TyrLeu and TyrVal is depicted in fig. 2. It appears that the variation is faster in the case of the dipeptides.

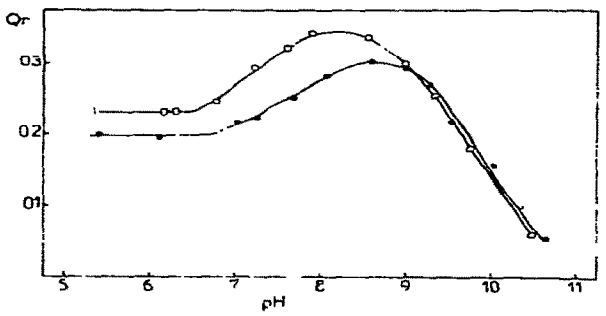

Fig. 1. Fluorimetric titration of $GlyTyrNH_2$ (•) and $TyrGlyNH_2$ (○), at 20°; in 2×10^{-3} M imidazole to stabilize pH. Excitation was at 278 nm, emission at 305 nm.

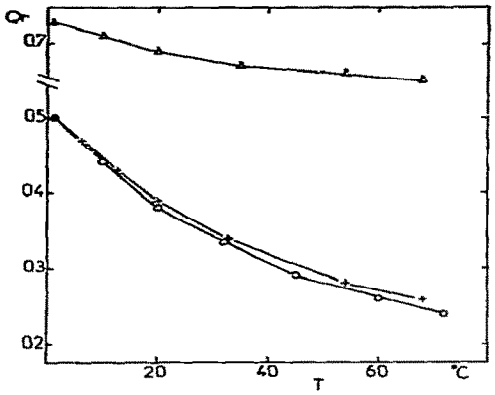

Fig. 2. Influence of temperature on the fluorescence quantum yield relative to tyrosine, of AcTyrNHEt (△), TyrAla (○) and TyrVal (+), measured in 2×10^{-3} M acetate buffer, pH 5.5. Excitation at 270 nm, emission at 305 nm.

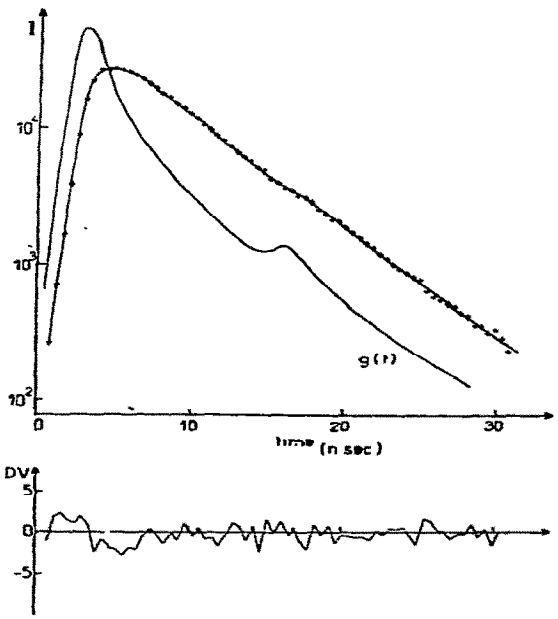

Fig. 3. Transient fluorescence of tyrosine at 20°, in 2×10^{-3} M acetate buffer pH 5.2. Reconstitution of the experimental curve (÷) with a convolution product assuming a monoexponential decay (τ = 3.38 ns) (——). $G(t)$: apparatus response function. The trace of the deviation function DV(t) is represented below the decay curves. Excitation at 270 nm, emission at 315 nm.

3.2. Transient fluorescences

Fluorescence decays of all compounds at pH 5.5 and for several of them at basic pH, were measured at 20°C. The results of the analysis of the transient fluorescence curves with a single exponential function are given in table 1; clearly, these fluorescences are distributed amongst two classes: those for which the monoexponential analysis gives a value of P between 10^{-3} and 1.5×10^{-3}, and those for which P is higher.

The lowest values of P are obtained not only for the fluorescences of tyrosine, tyramine and p-cresol, but also for the fluorescence of the derivatives of series I and of AcTyrNHEt. Furthermore, for all these compounds the deviation function fluctuates randomly around zero, with an amplitude lower than 4. This behaviour is illustrated for tyrosine in fig. 3 and for the dipeptide GlyTyr at pH 5.5 in fig. 4A.

Similar values of P, together with similar deviation functions are obtained for the analysis of two transient fluorescence curves of p-terphenyl with regard to one another (see sect. 2.2).

As we developed in the experimental section, the decay kinetics of these compounds can reasonably be described by a monoexponential function. The error on the values of the time constants is estimated to ± 0.05 ns.

The monoexponential analysis of the transient fluorescence of $GlyTyrNH_2$ at pH 5.5 gives a slightly different result: the deviation function still shows a random oscillation, but its amplitude and the value of P are relatively high. A similar behaviour has been observed when the time constant of NADPH was measured [27]. It does not mean that the decay is complex, but probably originates in the amplification of the counting statistical errors which occurs when the sample has a decay time smaller than the reference solution (see sect. 2).

On the contrary, the fluorescence decays of the compounds of series II can not be fitted to single exponential functions (table 1)[‡]: the values of P are high, and the deviation function diverges markedly from the time axis. This is illustrated in fig. 4B in the case of the transient fluorescence of TyrGly, pH 5.5 measured in the same conditions than that of GlyTyr (fig. 4A). The aspect of the deviation function suggests

[‡] Except for TyrGly and TyrVal at basic pH.

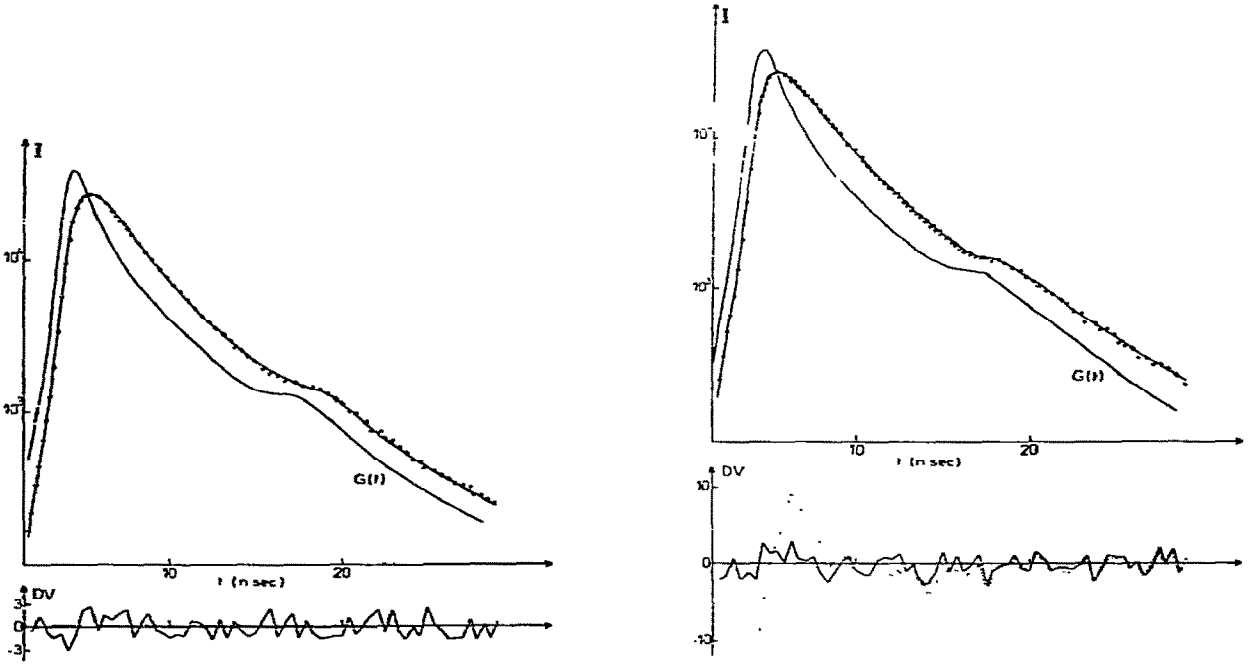

Fig. 4. A) Transient fluorescence of GlyTyr at 20° in 2×10^{-3} M acetate buffer pH 5.5. Reconstitution of the experimental curve (+) with a convolution product assuming a monoexponential decay ($\tau = 1.3$ ns) (—), and trace of the corresponding deviation function DV(t). Excitation at 270 nm, emission at 315 nm. B) Transient fluorescence of TyrGly, at 20°, in 2×10^{-3} M acetate buffer pH 5.5. Reconstitution of the experimental curve (+) with convolution products assuming: 1. a monoexponential decay (– – –), $\tau = 1.35$ ns; 2. a biexponential decay (—), $\tau_1 = 0.5$ ns, $\tau_2 = 1.6$ ns, $C_1 = 0.46$; and trace of the corresponding deviations functions. Excitation at 270 nm, emission at 315 nm.

that at least one additional exponential term with a shorter time constant is present in the decay.

We thus try to analyze these decays with sums of two exponentials. The new values of P, together with that of the time constants and relative amplitude of the exponential terms, are given in table 2.

The fit between the calculated curves and the experimental curves is good, and for a number of case, the values of P are even lower than those obtained for the monoexponential analysis of transient fluorescence with simple decay kinetics. This has been discussed in the experimental section, and can be connected to the finding that the biexponential analysis of simple decays also improves, but in a much smaller extent, the values of P and of the amplitudes of the deviation function. In addition, this latter result illustrates the fact that whenever a good fit is obtained with a single exponential function, it is also possible to obtain a good fit with a sum of two exponential terms or more. However, in these cases, we found that either the values of the two time constants are close to each other and to the value of the single time constant τ, or one of the exponential terms has a relative amplitude greater than 0.95 and a time constant with a well defined value close to that of τ (see also sect. 2). The latter case is illustrated in table 2 for GlyTyr pH 5.5.

The uncertainty on the values of τ_1, τ_2 and C_1 depicted by $\Delta\tau_1$, $\Delta\tau_2$, ΔC_1 in table 2, is of the same order for the decays for which the biexponential analysis led to a great improvement of the values of P.

It is generally greater for the short time constant ($\Delta\tau_1 = \pm 0.15$ to ± 0.2 ns) than for the long one ($\Delta\tau_2 = \pm 0.05$ to ± 0.1 ns), ΔC_1 being close to ± 0.05.

The parameters of the decays at basic pH of $TyrGlyNH_2$, $TyrLeuNH_2$ have much poorly defined values. This could have been forecasted, since the values of P and of the amplitude of the deviation function corresponding to the best single exponential curve (table 1) drew nearer to that obtained for a true monoexponential decay. It seems that this kind of behaviour

Table 2
Biexponential analysis of the transient fluorescence of tyrosine derivatives of series II, and of GlyTyr, pH 5.5. For the explanation of the various parameters, see text. (Excitation: 270 nm, emission: 315 nm).

	pH	τ_1 ns	$\Delta\tau_1$	τ_2 ns	$\Delta\tau_2$	C_1	ΔC_1	$P(\times 10^{-3})$	$P'(\times 10^{-3})$	$\langle\tau\rangle$ ns (a)	$\langle\tau\rangle/\tau_T$ (a)
GlyTyr	5.5	1.25	0.05	2.1	1.5	0.95	0.02	1.12	1.3	0.8 –0.9	0.24–0.27
$TyrNH_2$	5.5	0.4	0.175	1.2	0.05	0.41	0.075	1.4	1.5	0.9 –1.10	0.26–0.32
	9.9	0.4	0.2	1.4	0.075	0.44	0.075	1.6	1.5	1.0 –1.15	0.3 –0.34
TyrGly	5.5	0.50	0.15	1.6	0.1	0.46	0.055	1.25	1.3	1.0 –1.15	0.3 –0.34
TyrAla	5.5	0.6	0.2	1.8	0.05	0.38	0.06	1.4	1.3	1.3 –1.4	0.38–0.41
TyrLeu	5.5	0.5	0.15	2	0.05	0.45	0.05	1	1.1	1.25–1.40	0.37–0.42
TyrVal	5.5	0.6	0.15	2	0.05	0.49	0.04	0.9	1	1.10–1.3	0.33–0.39
$TyrGlyNH_2$	5.5	0.40	0.1	1.50	0.1	0.60	0.07	1	1.25	0.70–0.90	0.21–0.26
	9.9	0.65	0.35	1.5	0.15	0.42	0.22	0.9	1.2	1.05–1.20	0.31–0.36
$TyrLeuNH_2$	5.5	0.3	0.1	1.8	0.1	0.64	0.06	0.9	1.2	0.8 –1	0.24–0.29
	10.7	0.6	0.3	1.6	0.2	0.46	0.19	0.6	1.1	1.04–1.20	0.3 –0.36
TyrGlyGly	5.5	0.34	0.1	1.4	0.05	0.55	0.05	1.3	1.1	0.77–0.87	0.23–0.26
	10.5	0.7	0.25	1.4	0.20	0.50	0.23	1.4	1.2	1 –1.1	0.29–0.32

(a) Utmost values.

Table 3
Temperature dependence of the transient fluorescence of tyrosine, AcTyrNHEt, TyrAla and TyrVal, pH 5.5. Data of the monoexponential and biexponential analysis. (Excitation: 270 nm, emission: 315 nm.)

$T°C$		Monoexponential analysis		Biexponential analysis									
		τ_1 ns	$P(\times 10^{-3})$	τ_1 ns	$\Delta\tau_1$	τ_2 ns	$\Delta\tau_2$	C_1	ΔC_1	$P(\times 10^{-3})$	$\langle\tau\rangle$ (a)	$\langle\tau\rangle/\tau_T$ (a)	Q_r
1	Tyrosine	3.75	1.1			Δ monoexponential							
	AcTyrNHEt	2.8	1.3			monoexponential						0.75	0.73
	TyrAla	2.4	4.6	0.7	0.15	2.7	0.05	0.4	0.04	0.9	1.81–2.00	0.48–0.55	0.49
	TyrVal	2.45	5	1	0.35	2.9	0.15	0.47	0.05	1	1.8 –2.2	0.48–0.58	0.5
10	Tyrosine	3.55	1			monoexponential							
	AcTyrNHEt	2.55	1.4			monoexponential						0.72	0.71
	TyrAla	2.06	4.7	0.6	0.2	2.4	0.05	0.44	0.035	0.95	1.4 –1.7	0.39–0.48	0.44
	TyrVal	2.12	5	0.6	0.2	2.5	0.1	0.46	0.05	0.7	1.5 –1.7	0.43–0.48	0.44
35	Tyrosine	2.95	1.2			monoexponential							
	AcTyrNHEt	2.05	1.2			monoexponential						0.69	0.67
	TyrAla	1.22	2.9	0.4	0.2	1.4	0.05	0.40	0.07	1	0.90–1.1	0.31–0.37	0.32
	TyrVal	1.2	3.5	0.4	0.2	1.5	0.05	0.43	0.06	1.2	0.90–1.1	0.3 –0.35	0.33
54	Tyrosine	2.55	1.4			monoexponential							
	AcTyrNHEt	1.7	1.3			monoexponential						0.67	0.66
	TyrAla	0.77	4	0.5	0.15	1.05	0.15	0.62	0.18	2	0.65–0.75	0.25–0.29	0.27
	TyrVal	0.84	3.1	0.4	0.2	1.05	0.25	0.58	0.29	1.5	0.71–0.80	0.28–0.31	0.28
68	Tyrosine	2.24	1			monoexponential							
	AcTyrNHEt	1.45	1			monoexponential						0.65	0.65
	TyrVal	0.62	2.8	0.47	0.1	0.90	0.15	0.80	0.15	2.3	0.55–0.6	0.25–0.27	0.26

(a) Utmost values.

is relevant to the decrease of the relative amplitude of one of the exponential terms.

The transient fluorescences of tyrosine, AcTyrNHEt, TyrAla and TyrVal were measured at various temperature. The result of their analysis is given in table 3.

The fluorescence decays of tyrosine and AcTyrNHEt remain monoexponential in the whole range of temperatures studied. The variation of the fluorescence lifetime of tyrosine is consistent with the variation of the quantum yield measured by Turoverov [29].

The examination of the values of P given in table 3 shows that the decays of TyrAla and TyrVal can be fitted to sums of two exponential at all temperatures studied. As the temperature is raised from 1° to 35°, the time constants τ_1 and τ_2 decrease, whereas the amplitude of the exponential terms remain constant. Further increase of the temperature induces an increase of amplitude C_1, while the corresponding time constant τ_1 remains unchanged. Though the uncertainty on these values increases, the effect is clearcut, since C_1 reaches 0.80 for TyrVal at 68° (instead of 0.43 at 35°). It must be noted that the value of P given by the monoexponential analysis of the latter decay is only slightly higher than that obtained for $GlyTyrNH_2$ (see table 1). However the deviation function shows a systematic divergence from the time axis in the short times range, that vanishes with the insertion of the second exponential term of low amplitude.

3.3. Relation between the quantum yield and the decay

In this section, we will compare the results of steady state and transient fluorescence measurements, and draw out the various characteristics of the fluorescent behavior of the compounds under study.

In the general expression of a fluorescence decay (relation 5) the amplitude A_i is related to the absorption intensity and to the radiative lifetime τ_{0_i} of the ith chromophore. Assuming that all chromophores have identical extinction coefficient and radiative lifetime τ_0, the decay can be written

$$I(t) = (N_0/\tau_0) \sum C_i \exp(-t/\tau_i), \qquad (11)$$

where N_0 represents the total concentration of excited chromophores at $t = 0$. The absolute quantum yield Q is related to $I(t)$ by expression (12):

$$Q = \left(\int_0^\infty I(t)\right) \Big/ N_0 . \qquad (12)$$

Substituting $I(t)$ by expression (11) yields:

$$Q = \left(\sum C_i \tau_i\right) \Big/ \tau_0 . \qquad (13)$$

We have found, in agreement with a number of authors that tyrosine and its derivatives have similar absorption and similar fluorescence spectra. Since the value of the radiative lifetime τ_0 is directly related to these spectra [30], it must be nearly identical for all these compounds. In this work, the relative quantum yield we measured, Q_r, is the ratio of the absolute quantum yield Q of the sample to that of tyrosine taken as a standard, Q_T

$$Q_r = Q/Q_T . \qquad (14)$$

Let us call τ_T the lifetime of tyrosine; since τ_0 is the same for tyrosine and its derivatives, eq. (13) yields

$$Q_T = \tau_T/\tau_0$$

and eq. (14) becomes

$$Q_r = \left(\sum C_i \tau_i\right) \Big/ \tau_T . \qquad (15)$$

In the case of monoexponential decays relation (15) yields:

$$Q_r = \tau/\tau_T \qquad (16)$$

The values of τ/τ_T are given in table 1, and can be compared with the experimental value of Q_r.

It appears that relation (16) is verified for most derivatives the decay of which can be fitted to a monoexponential function: indeed, for these decays, the difference between τ/τ_T and Q_r never exceeds the estimated error on Q_r. This is the case for the derivatives in which the tyrosyl residue is substituted on its α amino group (series I). Thus N-acetyl-L-tyrosine has a quantum yield and a lifetime very close to those of tyrosine, suggesting that an N-terminal peptide bond has little effect on the fluorescence of the phenol chromophore. Addition of a protonated amino group on the methyl group of N-acetyl-L-tyrosine to yield the zwitterion of GlyTyr causes a simultaneous decrease of the quantum yield and of the lifetime. This drop is less important when the α carbon of the glycyl residue is substituted by aliphatic groups, as can be seen for the

zwitterions of AlaTyr, LeuTyr, ValTyr. Furthermore it appears that the more hydrophobic is the side chain, the less important is the quenching.

Though the decay of these dipeptides is monoexponential at basic pH, the values of their quantum yield and of their lifetime given in table 1 do not verify eq. (16). However it must be reminded that they were recorded at two different pHs. The values of the pK available in the literature [17] (8.45 for GlyTyr, 8.36 for LeuTyr) suggest that at pH 9, at which the quantum yields are measured, the amino group is not completely deprotonated. On the contrary the transient fluorescences are measured at higher pH, so that all the molecules have their amino group in the unprotonated form. At this pH, the phenol hydroxyl group of a certain ratio of molecules is dissociated. Since the phenolate ion is not fluorescent, the measured lifetime (which we call τ^{NH_2}) is characteristic of the peptide molecules with their amino and phenol groups both in the unionized forms. Let us call $Q_f^{NH_2}$ the quantum yield of these molecules, and $Q_f^{NH_3^+}$ and $\tau^{NH_3^+}$ the quantum yield and lifetime of the peptide in its zwitterionic form (namely the values measured at pH 5.5). Assuming that the deprotonation of the amino group do not appreciably affect the value of the radiative lifetime τ_0 one can write

$$Q_f^{NH_2} = \tau^{NH_2}(Q_f^{NH_3^+}/\tau^{NH_3^+}). \qquad (17)$$

Thus $Q_f^{NH_2}$ can be calculated from experimental results. The quantum yield of the peptide measured at pH 9, must be written:

$$Q_f = \alpha Q_f^{NH_3^+} + (1-\alpha)\, Q_f^{NH_2}, \qquad (18)$$

where α is the molar fraction of the peptide in the zwitterionic form. The value of α calculated from eq. (18) yields a value of pK of 8.51 in the case of GlyTyr, which is in good agreement with the above cited value. For AlaTyr and ValTyr, we find neighbouring values (8.2 and 8.3 respectively) which seem reasonable.

For GlyTyr, AlaTyr and ValTyr, the values of $Q_f^{NH_2}$ calculated from eq. (17) are given in table 1. These values, as well as the experimental values of τ^{NH_2} are close to that of tyrosine and N-acetyl-L-tyrosine; the deprotonation of the amino group greatly diminishes the quenching of the fluorescence. However some dependence of the quantum yield on the nature of the side chain is still observed.

At pH 5.5 the quantum yield and lifetime of GlyGlyTyr and AlaAlaTyr are increased with regard to that of the corresponding dipeptides. An important feature is that a slight decrease of the fluorescence is observed when the α-amino group is deprotonated. A similar result has already been obtained by Russel et al. [8] in the case of the GlyLeuTyr. Quenching of the phenol fluorescence by unionized amino groups has also been observed in copolymers of lysine and tyrosine [31] and in oligopeptides containing one tyrosyl residue surrounded by alanyl and lysyl residues [16].

This dynamic quenching could be due to an interaction in the excited state between the amino groups and the phenol hydroxyl [3]. Observation of molecular models of tripeptides indeed show that folding of the peptide backbone can bring the amino group in the immediate neighbouring of the hydroxyl.

Replacing the free carboxyl group of N-acetyl tyrosine by an amide group to yield AcTyrNHEt and $GlyTyrNH_2$ lowers the time constant and the quantum yield. The radiative lifetime must remain identical since relation (17) is still verified; furthermore table 3 shows that this is true at all temperature studied, for AcTyrNHEt.

For TyrGly and TyrVal at basic pH, the value of τ/τ_T is in good agreement with that of Q_f. This is not surprising, since the pK of the amino group is about one pH unit lower in these compounds, than in series I dipeptides [17]. This tend to confirm that the decay is truly monoexponential: hence the behavior of TyrGly and TyrVal at basic pH is close to that of AcTyrNHEt.

It must be noted that the discrepancy between the values of Q_f and τ in the case of *p*-cresol is consistent with the fact that the absorption and fluorescence spectra of this compound are shifted with regard to that of L-tyrosine. This can be related to the different inductive effects of the methyl and substituted ethyl groups on the π electron shell of the aromatic ring.

For all the compounds we have just surveyed (except *p*-cresol), the monoexponential behaviour of the decay is confirmed by the constancy of the ratio of the time constant to the relative quantum yield. On the contrary (see table 1) when the decay cannot be fitted to a monoexponential function, the difference between τ/τ_T and Q_f clearly exceeds the estimated error on Q_f. This is also illustrated in fig. 5: for TyrGly

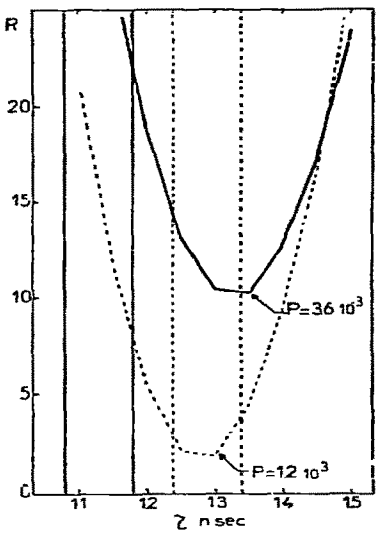

Fig. 5. Variation of the weighted mean residue R with the value of the time constant τ, in the monoexponential analysis of the transient fluorescence of TyrGly, pH 5.5 (——) and GlyTyr, pH 5.5 (– – –). Since the two experimental transient fluorescence curves have similar M_0, a given value of R yields the same value of P for both of them. Estimating the experimental error on Q_r and τ_T (3.38 ns) to ± 0.01 and ± 0.05 n.s respectively, eq. (16) (see text) is verified when τ takes a value of 1.13 ± 0.05, for TyrGly (area between the solid vertical lines) and of 1.29 ± 0.05 for GlyTyr (area between the dotted vertical lines).

In contrast with GlyTyr, the plot of the weighted mean residue R (see relation (7)) as a function of the single time constant τ, takes its minimum clearly outside the area defined by the utmost tolerable values of τ calculated from relation (16) taking into account experimental errors on the values of Q_r and τ_T (± 0.01 and ± 0.05 respectively).

For a biexponential decay, relation (15) becomes

$$Q_r = (C_1\tau_1 + C_2\tau_2)/\tau_T = \langle\tau\rangle/\tau_T . \qquad (19)$$

Relation (19) is verified, within experimental error, for $TyrNH_2$ and the dipeptides of series II at pH 5.5 and basic pH, $T = 20°C$, and at other temperatures, for TyrAla and TyrVal, as can be seen in tables 2 and 3; this is also illustrated in fig. 6, for the zwitterion of TyrGly: in the set of the values of R (related to P by eq. (9)) as a function of τ_1 and τ_2, each value of R corresponds to a given value of $\langle\tau\rangle$; taking into account experimental error on Q_r and τ_T, it is possible to point out the values of R for which $\langle\tau\rangle$ verifies eq. (19) (shadowed area): it appears that R (and thus P) takes its minimum amongst these values.

In the case of $TyrGlyNH_2$ and $TyrLeuNH_2$ at pH 5.5 and basic pH, as well as for TyrGlyGly at basic pH, the values of $\langle\tau\rangle/\tau_T$ is still in a rather good agreement with the values of Q_r.

For TyrGlyGly at pH 5.5, the lowest acceptable value of $\langle\tau\rangle/\tau_T$ is slightly higher than the value of Q_r. However the difference between these values is close to the estimated error on Q_r (0.01, for $Q_r = 0.22$), and the question remains, whether the discrepancy can be attributed to experimental error, or not. In the latter case, since the decay can be fitted to a sum of only two exponential terms, this would suggest that a small ratio of the tripeptide molecules are non-fluorescent.

4. Discussion

The fluorescence decay kinetics of the phenol chromophore of the tyrosine derivatives containing one or two amide or peptide groups is different according as the tyrosyl residue is substituted on its α-amino group (series I) or on its α-carboxyl group (series II). In the latter case, the fluorescence decay deviates markedly from the monoexponential function, which is characteristic of the decay of the derivatives of series I, and can be fitted to a sum of two exponential terms. For derivatives of close chemical structures, such a different behaviour is rather surprising. This cannot be due to impurities in samples of series II, since most of the compounds studied were chromatographically pure (see sect. 2.1), and since biexponential decay was a general feature for compounds of series II. The possibility of a mixture of ionic species may be discarded, because transient fluorescences were always measured in pH ranges where only a single fluorescent ionic species was present: this was verified by steady-state fluorimetric titrations, and from the pK values available in the literature. Occurence of intermolecular associations cannot be definitely eliminated. However it seems most unlikely at the concentration used (3×10^{-4} M). Furthermore we verified that the quantum yield of TyrGly is the same for peptide concentrations ranging from 3×10^{-4} M to 6×10^{-6} M.

It is expected that the fluorescence emission kinetics

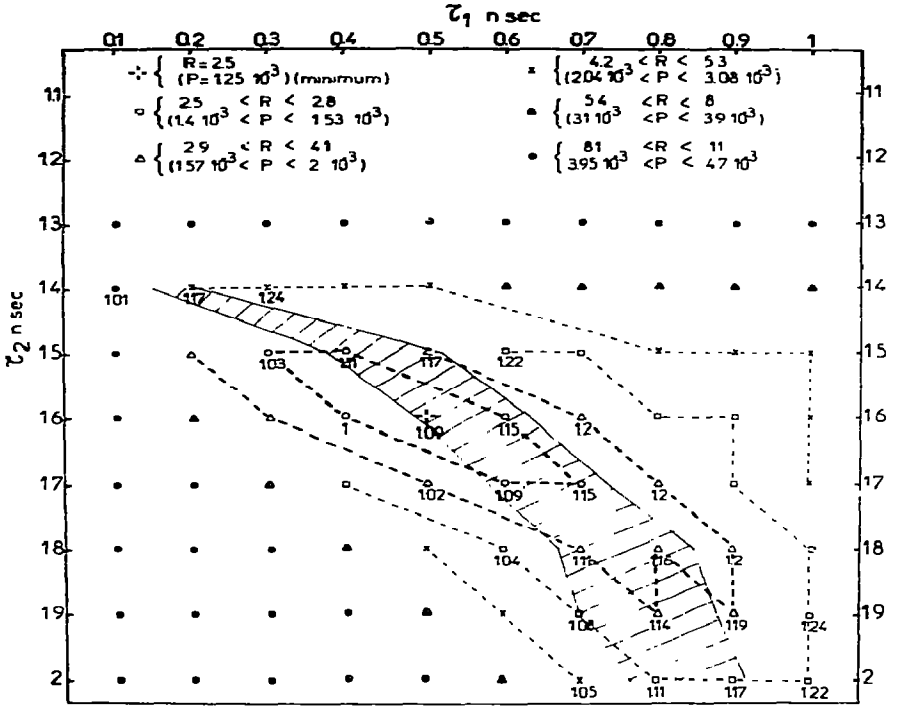

Fig. 6. Variation of the weighted mean residue R (or of parameter P) with the value of τ_1 and τ_2, in the biexponential analysis of the transient fluorescence of TyrGly, pH 5.5. Eq. (19) (see text and legend of fig. 5) is verified when the average time constant $\langle\tau\rangle = C_1\tau_1 + C_2\tau_2$ takes a value of 1.13 ± 0.05 (shadowed area); the values of $\langle\tau\rangle$ for a number of pairs (τ_1, τ_2) appear in the figure.

of complex compounds depends on the chemical groups which are in the neighbouring of the chromophore and can modify its fluorescence, in the various conformations of their molecules, and on the rates of exchange between these conformations [12–15] in the excited state (which may differ from the rates of exchange in the ground state). Three cases can be distinguished according to the relative values of these rates of exchange and of the rates of deactivation of the excited state. If the rates of exchange between the conformations are small by comparison with the deactivation rates, the decay is a sum of exponential functions; the time constants characterize the conformations and the associated amplitudes are proportional to their relative population. Inversely, if the rates of exchange are much greater than the deactivation rates, the decay is monoexponential and its time constant takes an intermediate value between the values of the time constants characteristic of the chromophore in the various conformations. At last, if the rates of exchange and the rates of deactivation are comparable, the decay is multiexponential. The time constants and the amplitudes of the exponential terms are function of all the rate constants of the system. The amplitudes (but not the time constants) also depend on the relative populations of the various conformations.

In other respect, it was emphasized in the experimental section (and this appeared in the analysis of the transient fluorescences) that the possibility to resolve the exponential terms of a complex decay depends greatly on the relative values of the time constants and of the amplitudes.

In a preceding paper, we reported that the fluorescence decays of tryptophan diketopiperazines were complex, and could be fitted to sums of two decreasing exponentials. We attributed this behaviour to the existence of folded and unfolded conformations of these molecules, which was supported by theoretical

conformational calculations and nuclear magnetic resonance measurements. Quantitative analysis showed that the rates of exchange between these conformations were of the same order of magnitude as the rates of deactivation of the excited state.

These considerations will be taken into account to explain the decay kinetics of the tyrosine derivatives. The explanation will be set up primarily in the case of dipeptides zwitterions, and then extended to other compounds.

4.1. Emission of GlyTyr and TyrGly zwitterions in relation to their conformation

The work of Cowgill [6] strongly suggests that the carbonyl of peptide or amide groups is directly responsible of the quenching of the phenol fluorescence, by means of an intramolecular interaction with the chromophore.

Tournon et al. [32] gave evidences that in aromatic carboxylic acids fluorescence quenching occurs through a charge transfer complex between the aromatic ring and the carbonyl of the protonated carboxylic group. If we adopt this explanation for the quenching by peptide or amide carbonyl, we expect that in the compounds studied here the molecules in which quenching occurs must have their carbonyl groups in close contact with the aromatic ring. Furthermore, the nature of the adjacent chemical group can modify the electron acceptor properties of the peptide carbonyl group, and consequently its quenching power. Specially, the fluorescence quenching by ammonium groups in tyrosine oligopeptides has been attributed by Cowgill to an indirect effect of these groups on the quenching ability of the carbonyl: this point will be discussed later.

In the compounds under study, the position of the carbonyl of the amide or peptide groups borne by the C^{α} of the tyrosyl residue itself, primarily depends on the torsional angle χ_1, characterizing the rotation about the $C_{\alpha}-C_{\beta}$ bond of this residue. Three rotamers can be defined (fig. 7) in which the values of χ_1 differ by 120°. The torsional angle χ_2 about the $C_{\beta}-C_{\gamma}$ must take its value near 90° [33, 34].

Data on the relative populations of rotamers A, B, C are lacking in the case of tyrosine derivatives. However, Kainosho et al. [35] showed that rotamer A is favored in free tyrosine, as well as in phenylalanine. Nuclear magnetic resonance studies suggested that this

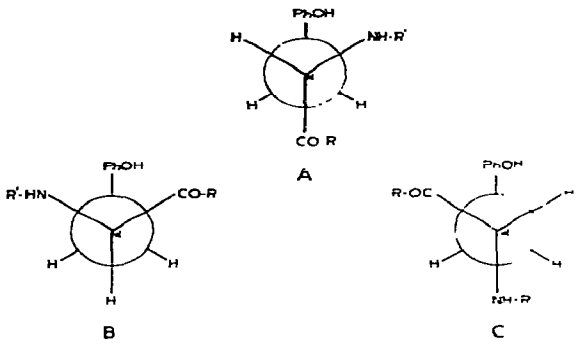

Fig. 7. Newman projections about the $C_{\alpha}-C_{\beta}$ bond for the three rotamers of the tyrosyl residue. PhOH represents the phenol group; the values of R' and R are, for instance for dipeptides containing a glycyl residue, in series I: R' = $-COCH_2NH_3^+$, R = $-O^-$; in series II: R = $-NHCH_2COO^-$, R' = $\lessdot^{H}_{H^+}$.

is also the case for the derivatives of this latter amino acid. Newmark et al. [38] stated that B is unfavored but did not provide definite evidence on the relative populations of rotamers A and C. More accurate results [36, 37] suggest that rotamer A is favored, with a relative population greater than 50%. It seems not unreasonable to assume that this is also the case for tyrosine peptides, since free tyrosine and phenylalanine show the same behaviour. Especially, in the case of dipeptides of series I the existence of a strong electrostatic repulsion between the negative charge of the carboxylate and the π electron shell of the aromatic ring must indeed decrease the relative populations of rotamers B and C.

In the zwitterions of the dipeptides containing a glycyl residue, the conformation of the peptide backbone which brings the oxygen atom of the peptide carbonyl (bearing a partial negative charge) close to the positively charged ammonium group, should be favored [36]. As a consequence, for dipeptides of series I, the carbonyl could come in close contact with the phenol group only in the preferential rotamer A (fig. 7). In contrast, for series II, only in the unfavored rotamer B is the carbonyl in the right position for quenching. It must be emphasized that in this rotamer the carbonyl group must remain in close contact with the fluorophore, since rotation about the $C^{\alpha}-C'$ bond (angle ψ) of the tyrosyl residue is sterically hindered (fig. 7). In contrast, in rotamer A of series I, the two groups can

assume rather remote positions, by rotation about the $C^{\alpha}-N$ bond (angle ψ). The intramolecular collisional quenching by the carbonyl will thus certainly be weaker for rotamer A of series I than for rotamer B of series II. This entail that the differences between the deactivation rate of the excited state of the chromophore in the various rotamers will be smaller in series I than in series II.

From all that precedes, a biexponential decay of TyrGly indicates that the rate of exchange between the various rotamers A, B and C and thus the rotation rate about the $C_{\alpha}-C_{\beta}$ bond is smaller than or of the same magnitude order as the deactivation rates of the excited state of the chromophore. In the first case, the short time constant would characterize the rotamer in which the fluorescence is quenched (namely rotamer B), and the long time constant, the other rotamer (namely A and C). Furthermore, the amplitudes of the exponential terms would be proportional to the relative concentrations of the rotamers: since we attributed the short time constant to the non preferential rotamer B, the corresponding preexponential term should be appreciably smaller than 0.5, which is not the case. Then we conclude that the rotation rate about the $C_{\alpha}-C_{\beta}$ bond has a magnitude comparable to that of the deactivation rate [‡]. In contrast, the monoexponential decay of GlyTyr could be explained assuming that the rate of exchange between the conformers is much greater than the deactivation rate. Since the average rate of deactivation of both peptides are similar, this would imply that the rotation rates about $C_{\alpha}-C_{\beta}$ differ in these compounds. However another explanation could also be found in order to explain the occurrence of a monoexponential decay in the case of GlyTyr. As discussed above, the difference between the deactivation rates of the fluorophore in the various rotamers is relatively smaller for GlyTyr (see above). Then the values of the time constants characteristic of the various environments of the chromophore would be too close to each other to allow the resolution of the exponential terms with the method used here. In contrast, since the quenching is assumed to be much larger in rotamer B of TyrGly, the resulting time constants would be different enough to give a biexponential decay.

[‡] Furthermore, this result suggests that rotamer B should be more populated in the excited state than in the ground state, though the new equilibrium should not be reached before deactivation of the excited state.

This interpretation could seem inconsistent with the finding that the quantum yield of GlyTyr is also slightly higher than that of TyrGly; however, this must be related to the assumption that the quenching occurs in the favored conformer for GlyTyr, but not for the TyrGly. Indeed, whereas the time constant of the exponential terms are independent from the relative population of the conformers, this is not the case for the amplitudes, and thus for the quantum yield [15].

4.2. Emission of series II dipeptides zwitterions

The fluorescence of these zwitterions present characteristics similar to that of TyrGly fluorescence: in particular little side chain effect is observed. So the above interpretation can reasonably be applied to these peptides (see also next section). The study of the variation of fluorescence decays with temperature permitted to show that in the decay kinetics of TyrAla and TyrVal, the amplitude of the faster exponential term increases above 35°, to take a value of 0.80 for TyrVal at 68°. In agreement with our general scheme, this can be interpreted assuming that at this temperature the rates of exchange between the rotamers are increased with respect to the deactivation rates enough to yield a nearly monoexponential decay.

4.3. Influence of amino groups on the emission of dipeptides

For tyrosyl derivatives of series II as well as for the dipeptides of series I, deprotonation of the α amino group induces an increase of the fluorescence quantum yield, and some changes in the fluorescence decay kinetics. Cowgill [2] suggested that the protonated amino group has no proper quenching activity, but enhances the quenching by the carbonyl group. It is not likely that this is due to a modulation of the population of rotamers A, B, C: indeed in the case of tyrosine, phenylalanine and phenylalanylglycine, nuclear magnetic resonance studies show that deprotonation of the α-amino group does not lead to a great variation of these populations [36–38]. Our results can be interpreted in a coherent manner if one assumes that the quenching ability of the carbonyl is greatly reinforced by its direct interaction with the charged ammonium group, due to electrostatic or electronic effect. Thus deprotonation (or substitution, see below) of the adja-

cent α amino group should decrease the quenching ability of the carbonyl; as a consequence, the difference between the deactivation rates of the chromophore in the various conformers should be less in the anion of the dipeptides than in their zwitterions. This could explain, in particular, that the divergence of the decay kinetics from the monoexponential behaviour disappears for the anions of TyrGly and TyrVal. It must be noticed that, in contrast with what was assumed for the zwitterions, some quenching might occur in rotamer C of the anions: here indeed, the carbonyl can approach the aromatic ring in rotamer C, since the interaction with the charged ammonium group has disappeared.

In the same way, the monoexponential decay of AcTyrNHEt would originate in the substitution of the amino group by the acetyl. The fact that the quantum yields of AcTyrNHEt and TyrGly (or TyrVal) at basic pH are rather close to each other suggest that the N-terminal peptide bond in AcTyrNHEt has nearly no effect on the fluorescence of the chromophore. This is confirmed by the high value of the quantum yield of N-acetyl-L-tyrosine.

4.4. Emission of series I zwitterions

Substitution of the C^{α} of the glycyl residue by aliphatic side chains of increasing hydrophobic character induces a progressive raise of the quantum yield and of the time constant of the dipeptides of series I, whereas as already seen, almost no effect is observed in series II. The occurrence of an hydrophobic interaction between the aromatic ring and the aliphatic groups could provide an explanation to these findings. Inspection of CPK molecular models show that in conformer A of series I, setting the side chain in close contact with the aromatic ring removes the ammonium group away from the carbonyl which, in agreement with our previous hypothesis, would then partially lose its quenching ability. The fact that the value of the quantum yield does not reach that of, for instance, N-acetyltyrosine suggests that there is still a given probability that the ammonium remains in the neighbouring of the carbonyl. This could be related to the low directional effect of hydrophobic interactions. At the opposite, it appears that, for dipeptides of series II, the interaction between the carbonyl and the ammonium groups is not hindered by the building of the hydrophobic interaction.

4.5. Emission of the other compounds

The carbonyl of the non-substituted amide bond of $TyrNH_2$ appears to be a stronger quencher than that of the peptide bond, or of the alkyl substituted amide bonds (see also Edelhoch et al. [5]). In addition, the influence of the deprotonation of the amino group on the quenching power of the amide carbonyl is smaller than with peptide carbonyl, as shown by the small enhancement of the quantum yield and the persistence of a biexponential decay at basic pH.

Quite surprising is the behaviour of $GlyTyrNH_2$ the fluorescence of which, though it shows the lowest quantum yield among all compounds studied, decays as a monoexponential function. In fact this can be rather easily explained as long as one notice that quenching should occur not only in the conformer A (in which the quencher is the carbonyl) of the N-terminal peptide bond, interacting with the NH_3^+ of the glycyl residue) but also in conformer B and C (quenching by the carbonyl of the amide bond). The rates of deactivation of the excited state would thus be high and the resulting time constants too low and too close to each other to allow the resolution of the decay in its exponential terms.

In tripeptides of series I and II and amides of series II dipeptides, the positions of the amide or of the second peptide group relative to the aromatic ring are determined by rotations about several bonds. Thus the probability that the carbonyl is in a favourable position for quenching, for each conformation of the side chain of the tyrosyl residue, is less than for the carbonyl of the first peptide bond. Furthermore, rotations about $N-C^{\alpha}$ and $C^{\alpha}-C'$ bonds of the aminoacyl residue adjacent to tyrosine are most likely little hindered, so that the exchange between the various position of the carbonyl can occur during the lifetime of the excited state of the chromophore.

GlyGlyTyr and AlaAlaTyr have quantum yields and lifetimes higher than that of GlyTyr and AlaTyr. This would be due to the cancelling of the quenching by the first peptide group (owing to the substitution of the adjacent amino group) and the appearance of the less efficient quenching by the carbonyl of the second peptide group.

In the case of $TyrGlyNH_2$ and $TyrLeuNH_2$, the quenching by the peptide carbonyl should persist, which is in agreement with the low value of the quantum

yield. Furthermore the amide bond can approach the aromatic ring not only in conformer B, but also in conformer C: with regard to the dipeptides, this could explain the decrease of the quantum yield, and the fact that the decay remains clearly biexponential even when the quenching by the peptide carbonyl is weakened by the deprotonation of the amino group.

The quantum yield of the zwitterion of TyrGlyGly is slightly smaller than that of $TyrGlyNH_2$, though for other compounds, the peptide carbonyl appeared as a weaker quencher than the amide carbonyl. In this case part of the decrease of the fluorescence might occur through the quenching by the carboxylate ion. This group was found to quench the fluorescence of tyrosine in its copolymers with glutamic acid [31], by dissociation of the phenol hydroxyl. Building a molecular model of the tripeptide show that the carboxylate could indeed come in the neighbouring of the hydroxyle, by folding of the peptide backbone.

5. Conclusion

Advances in the techniques of transient fluorescence measurements by the photoelectron counting method enables the detection of fast components in fluorescence decays. The present study shows that the fluorescence decays of aqueous solutions of several amide or peptide derivatives of tyrosine (namely those in which this amino acid is substituted on its carboxyl group) are sums of two exponential terms, and that one of this terms decays with a time constant ranging from 0.3 ns to 0.7 ns. In contrast, the fluorescence decays of other derivatives (namely those in which tyrosine is substituted on its α amino group) can be fitted to monoexponential functions. It is shown that the radiative lifetime of the phenol chromophore takes close values in all compounds studied. Assuming that the changes in the fluorescence properties of these compounds is mainly due to the intramolecular action of the carbonyl of amide or peptide groups, a tentative interpretation of the results, based on the conformational characteristics and dynamics of the molecules, is proposed. This interpretation requires further confirmation and perfection, and complementary experiments are in progress. It must be noted that the part played by solvent molecules, though important, has not been considered in our development, since very little is known in this domain.

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