



Excited state lifetimes of pyrimidines and purines in room temperature solution from fluorescence quantum yields and anisotropies
by Walter Berkett Knighton

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Chemistry
Montana State University
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Abstract:

Fluorescence excitation spectra are presented for 26 nucleic acid components in room temperature aqueous solution. The fluorescence excitation spectra and absorption spectra coincided for all the pyrimidines studied. Many of the purines, however, exhibited considerable deviations between the fluorescence excitation and absorption spectra. Discussion leads to the conclusion that discrepancies between the two spectra are not due to competition between internal conversion and vibrational relaxation, but can be attributed to the presence of a highly fluorescent minor tautomer. Fluorescence excitation spectra of methylated adenine derivations have contributed to the recognition of the tautomers responsible for the fluorescence observed for adenine, in both neutral and acid solutions. The fluorescence properties observed for adenine in neutral solution are consistent with the previously reported 7(H) tautomer model. The protonated adenines studied indicate that the fluorescence is probably dominated by a tautomeric species, which has protons residing on both the 7 and 9 positions.

Fluorescence lifetimes are predicted from radiative fluorescence lifetimes and quantum yield measurements for 10 pyrimidines (8 neutrals and 2 cations) and 12 purines (8 neutral and 4 cationic species). Reorientational times (T_{cSB}) derived from the predicted fluorescence lifetimes (T_{fSB}) and fluorescence anisotropy measurements are compared to those calculated using classical Stokes-Einstein hydrodynamic formulas (T_{cstick}). For the 13 nucleic acid components in which the quantum yield and polarization were independent of wavelength the ratio of T_{cSB}/T_{cstick} ranged from .1 to .7. The average ratio was .27 for the neutral species and .5 for the cations.

Predicted rotational correlation times. (T_{cSB}) were found to be on the order of 10 to 20 picoseconds for the neutral free bases. Of the 13 nucleic acid components in which there was no wavelength dependence of the quantum yield and polarization ratio 10 had predicted fluorescence lifetimes (T_{cSB}) which were less than 2 picoseconds.

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Signature Walter B. Knighten

Date May 19, 1980

DEDICATION

I would like to dedicate this thesis to my wife, Maryanne,
and to my mother, for without their persistent encouragement and
assistance this thesis would never have been written.

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IN ROOM TEMPERATURE SOLUTION FROM FLUORESCENCE
QUANTUM YIELDS AND ANISOTROPIES

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

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in

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ABSTRACT

Fluorescence excitation spectra are presented for 26 nucleic acid components in room temperature aqueous solution. The fluorescence excitation spectra and absorption spectra coincided for all the pyrimidines studied. Many of the purines, however, exhibited considerable deviations between the fluorescence excitation and absorption spectra. Discussion leads to the conclusion that discrepancies between the two spectra are not due to competition between internal conversion and vibrational relaxation, but can be attributed to the presence of a highly fluorescent minor tautomer. Fluorescence excitation spectra of methylated adenine derivations have contributed to the recognition of the tautomers responsible for the fluorescence observed for adenine, in both neutral and acid solutions. The fluorescence properties observed for adenine in neutral solution are consistent with the previously reported 7(H) tautomer model. The protonated adenines studied indicate that the fluorescence is probably dominated by a tautomeric species, which has protons residing on both the 7 and 9 positions.

Fluorescence lifetimes are predicted from radiative fluorescence lifetimes and quantum yield measurements for 10 pyrimidines (8 neutrals and 2 cations) and 12 purines (8 neutral and 4 cationic species). Reorientational times (τ_c^{SB}) derived from the predicted fluorescence lifetimes (τ_f^{SB}) and fluorescence anisotropy measurements are compared to those calculated using classical Stokes-Einstein hydrodynamic formulas (τ_c^{stick}). For the 13 nucleic acid components in which the quantum yield and polarization were independent of wavelength the ratio of $\tau_c^{SB}/\tau_c^{stick}$ ranged from .1 to .7. The average ratio was .27 for the neutral species and .5 for the cations. Predicted rotational correlation times (τ_c^{SB}) were found to be on the order of 10 to 20 picoseconds for the neutral free bases. Of the 13 nucleic acid components in which there was no wavelength dependence of the quantum yield and polarization ratio 10 had predicted fluorescence lifetimes (τ_c^{SB}) which were less than 2 picoseconds.

INTRODUCTION

Interaction of molecules, with radiation in the visible or ultraviolet region of the spectrum, usually results in an electronic transition. Figure 1 shows a schematic of the possible electronic deactivation processes after absorption. The approximate rate constants for each process are also shown. The nonradiative processes include vibrational relaxation, internal conversion and intersystem crossing. The radiative processes are fluorescence and phosphorescence. Vibrational relaxation involves the transfer of excess molecular vibrational energy to the solvent. Internal conversion is the radiationless process, whereby molecules go from an excited singlet electronic state to the ground state, converting excitation energy to heat. Intersystem crossing is internal conversion involving a change in multiplicity. The radiative processes involve emission of a photon from the lowest vibrational level of the excited state to some vibrational state of the electronic ground state. If the emission is from the excited singlet state, it is referred to as fluorescence; whereas, emission from the lowest triplet state is phosphorescence.

The excited state lifetime of a molecule is the inverse of the sum of all the rate constants involved in the electronic deactivation process. The excited state lifetime, in terms of these rate constants is

$$\tau_f = \frac{1}{k_f + k_{IC} + k_{ISC}} = \tau_{rad}^0 \cdot \phi_f \quad (1)$$

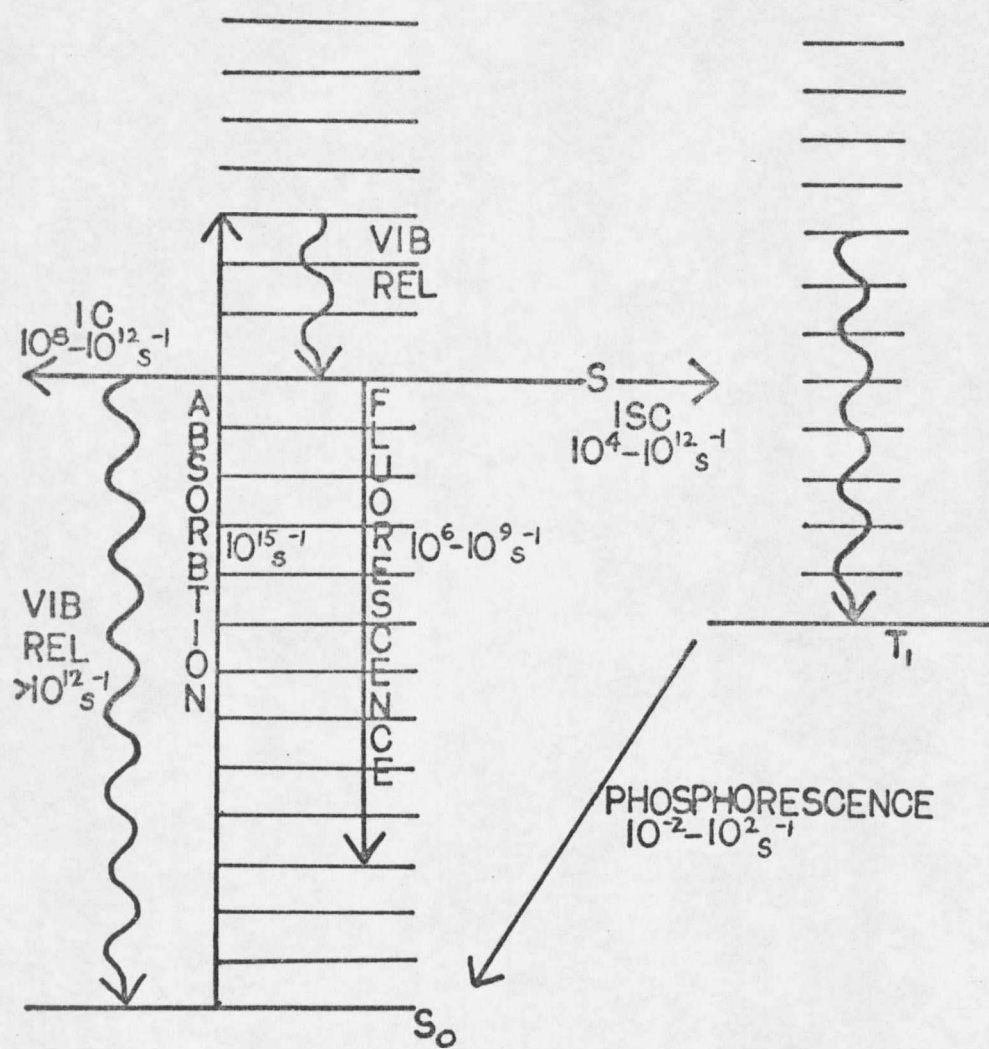


Figure 1

Electronic state diagram for a typical molecule

where τ_{rad}^0 is the radiative lifetime and ϕ_f is the quantum efficiency (1). Several research groups have reported the fluorescence of the nucleic acid components to be very weak at room temperature, having quantum yields on the order of 10^{-4} (2-4). Morgan and Daniels (5) have estimated the radiative lifetimes, using the method described by Strickler and Berg, to be ca. 10^{-8} for the nucleic acid components. This infers excited singlet lifetimes on the order of a picosecond. Daniels and Hauswirth (2) have suggested that if the excited state lifetimes are indeed this short, then the molecule would be able to undergo little reorientation in the excited state and should exhibit highly polarized emission. Two authors have found the nucleic acid components to exhibit considerable fluorescence anisotropy in room temperature aqueous solution (5, 6).

The results of photoselection theory are utilized heavily in any study involving fluorescence anisotropy measurements. Albrecht (7) has published an excellent comprehensive review of photoselection methods, so a brief version will suffice here.

Illumination of an isotropic solution, with either polarized or unpolarized light, leads to photoselection of an ensemble of molecules whose absorption dipoles are properly oriented. Figure 2 shows the typical instrumental configuration employed for measuring polarized emission. Excitation is directed along the x axis, with viewing along the y axis. The probability that a molecule will undergo photoselection is

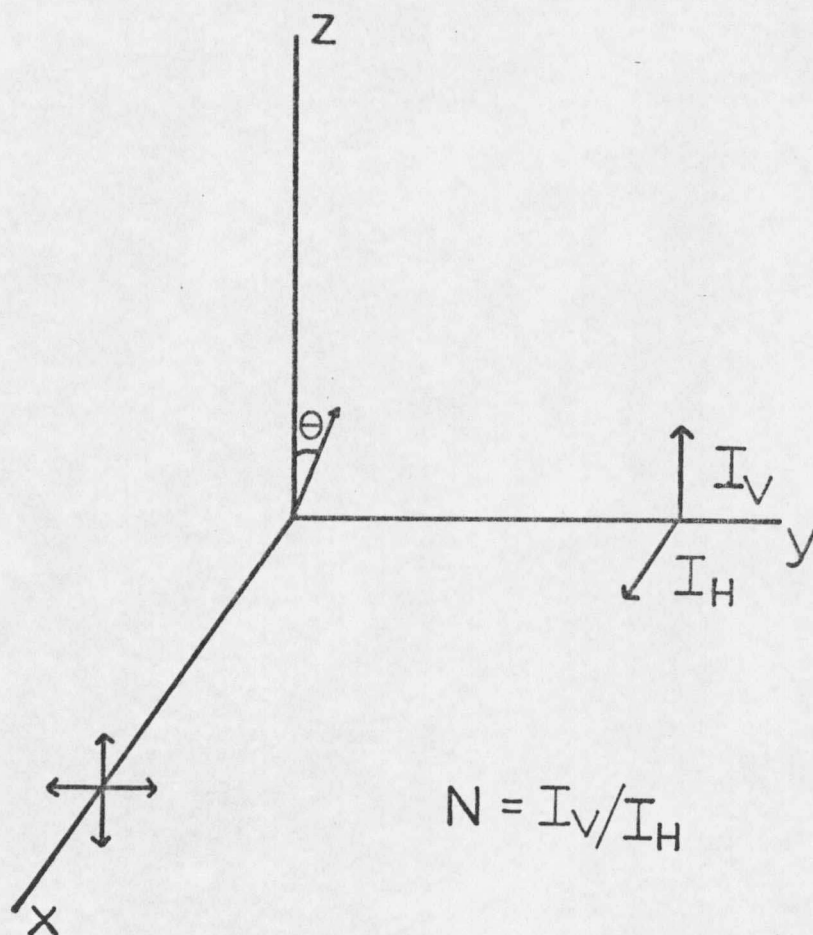


Figure 2

Instrument geometry for measuring polarized emission

given as $\cos^2\theta$ where θ is the angle between the absorption dipole and the direction of the electric vector of the light. The degree of polarization measured I_V/I_H is dependent on the angle subtended by the emission dipole, with respect to the absorption dipole. If only one electronic transition is involved, the emission will be from the same state as the absorption, with the theoretical maximum of I_V/I_H being 2.0 for the case when only one polarizer is used. The theoretical minimum is envisaged when there are two absorption dipoles which are perpendicular to each other. Then I_V/I_H is .75. The latter represents a case where absorption is into one state and the emission emanates from the other. The limits of the polarization ratio $I_V/I_H = N$ range from .75 to 2.0, when only one polarizer is used. Unfortunately, even under ideal conditions these theoretical limits are never observed. The major causes for deviation from ideality are energy transfer to neighboring molecules and rotational Brownian motion. It would be expected that, if the polarization was measured using dilute solutions in a rigid media, then the above mechanisms would be inoperative. Even under these conditions some intrinsic depolarization is observed with $N = 1.81$ being the highest polarization ratio observed in photoselection studies (8). Albrecht (7) has instituted the use of a randomization parameter to correct for these deviations from ideality.

In a non-rigid medium the emitted radiation can become depolarized by rotational Brownian motion of the molecule while in the excited state.

The polarization measured for molecules in a non-rigid environment is proportional to the ratio of $(\tau_f/\tau_c)^{-1}$ where τ_f is the fluorescence lifetime and τ_c is the rotational correlation time associated with the decay of the fluorescence polarization (9). In 1933, Perrin (10) formulated a relationship between fluorescence depolarization and rotational Brownian motion for the case of a hydrodynamically special molecule

$$1/p + 1/3 = (1/p_0 + 1/3) (1 + \tau_f/\tau_c)$$

where P is the measured polarization and P_0 is the limiting value in the absence of rotational motion. The polarization P can be related to the polarization ratio N by the expression:

$$P = \frac{N-1}{N+1}$$

Chuang and Eisinger (9) have formulated a general theory for fluorescence depolarization due to rotational Brownian motion for an asymmetric rotor. It is shown that the time dependent fluorescence polarization decays with a maximum of five exponential components. Unfortunately, utilization of the generality of this theory is severely limited, due to the difficulty in determining the magnitude and orientation of the diffusion tensor. Their expressions can be reduced to a more useable form, if the nucleic acid components are considered to be represented as symmetric tops. This seems justifiable due to the planarity and quasi-cylindrical shape of these molecules. Under these conditions only three relaxation times are involved, due to the degeneracy of the eigenvalues of the angular momentum components directed along the sym-

metry axis. The three relaxation times are as follows:

$$\tau_1 = \frac{1}{6D_x} \quad \tau_2 = \frac{1}{5D_x + D_z} \quad \tau_3 = \frac{1}{4D_x + 2D_z}$$

where the D_k 's are the principal diffusion coefficients for rotation about the k^{th} axis. $D_k = \frac{kT}{\xi_k}$ where ξ_k is the frictional coefficient for rotation about the corresponding axis. The z axis is assigned such that it is normal to the molecular plane yielding $D_x = D_y \neq D_z$. Using Tao's (11) equation 50, the rotational correlation time can be expressed as the weighted average of the three relaxation times

$$\tau_c = (3/2 \cos^2 \theta - 1/2) \tau_1 + (3 \cos^2 \theta \sin^2 \theta) \tau_2 + 3/4 \sin^4 \theta \tau_3 \quad (2)$$

where θ is the angle the transition dipole subtends with the symmetry axis. Polarized reflectance studies, of the nucleic acid components, found that the major absorbance in the 260 nm range is in plane polarized (12). Therefore, θ is 90° . The rotational correlation time is further simplified by substituting $\theta = 90^\circ$ into the above equation.

$$\tau_c = \frac{1}{4} \tau_1 + \frac{3}{4} \tau_3$$

Callis (6) has determined the fractional coefficients for the major bases, assuming both sticking and slipping boundary conditions and reported the corresponding rotational relaxation times. Sticking boundary conditions are known to over-estimate frictional coefficients for systems where solute size approaches that of the solvent (13). There is little reason to believe that frictional coefficients determined using slipping boundary conditions (no interaction between solute and solvent) are reasonable for hydrogen bonding molecules in aqueous

solution. It is the belief of this author that sticking boundary conditions are a better approximation of the hydrodynamic conditions utilized in this study, and that, although the magnitude of the frictional coefficients is too large, the ratio of the coefficients is representative. From Callis' (6) results it is seen that $\tau_1 = .85\tau_2$ when sticking boundary conditions are assumed. Since these two relaxation times are so similar that, within experimental error, the molecules can be assumed to isotropically rotate it then appears justifiable to use the Perrin relation. In this study, the Perrin relation will be expressed in terms of the fluorescence anisotropy R , instead of the traditional form utilizing the polarization P . In terms of the anisotropy, the Perrin relation becomes

$$R_0 = R(1 + \tau_f/\tau_c) \quad (4)$$

with R_0 and R being the anisotropies corresponding to P_0 and P . The anisotropy R can be determined from the polarization ratio N from the following expression

$$R = \frac{N-1}{N+1/2}$$

The Perrin relation gives a measure of τ_f/τ_c but no indication of the magnitude of either quantity. Obviously, direct measurement of either τ_f or τ_c would enable direct determination of the other. As direct determination of either quantity is not possible, predictive methods must be utilized. Reliable determination of τ_c hinges on the ability to calculate the principal frictional coefficients, whereas τ_f

requires measurement of the radiative lifetime and the quantum yield. As previously mentioned, there are presently no hydrodynamic formulas which adequately predict rotational behavior of small molecules in aqueous solution. Fluorescence lifetimes will be calculated using the radiative lifetimes determined from Strickler-Berg formulas and will be referred to as Strickler-Berg fluorescence lifetimes $\tau_f^{SB}(1)$.

The adequacy by which τ_f^{SB} represents the actual τ_f is based critically upon two criteria: the emitting state carries the entire oscillator strength contained within the first apparent absorption band and the major absorbing species is the major emitting species. The validity of these assumptions has been challenged in just about every paper dealing with the excited state lifetimes of nucleic acid components. The fact that the nucleic acid components are nitrogen-containing heterocycles which contain at least one pair of non-bonding electrons, leads to the possibility of tautomerization or degenerate $\pi-\pi^*$ and $n-\pi^*$ transitions. Several sets of results have caused considerable skepticism with regard to the validity of these assumptions.

The greatest source of skepticism is the slightly red shifted corrected fluorescence excitation spectrum, with respect to the absorption spectrum observed for the major bases (2,3). This is distressing, as the constancy of the quantum yield across the absorption band is so common as to be called Vavilov's law (14). However, these bases are found to obey Kasha's rule, which states that only one fluorescence

should be observed because the nonradiative processes, leading to the lowest vibrational level of the excited state, are much faster than the nonradiative and radiative processes leading to the ground state (15). These deviations from Vavilov's law may just be manifestations of the difficulty in measuring quantum yields as low as 10^{-4} . However, at low temperatures even greater red shifts are observed, where quantum efficiencies are much higher, hence, difficulties in experimental measurements can not be attributed to the deviations observed (2, 16, 17). These results have led to speculation of the origin of this anomalous behavior (6, 12, 16).

Daniels (12) has postulated that the presence of a highly fluorescent minor tautomeric species is responsible for the red shifted corrected fluorescence excitation spectrum, with respect to the absorption spectrum. This argument appears plausible for the purine system, as there is considerable evidence supporting the presence of tautomers in the case of adenine (17, 18). Pfeleiderer (19) has proposed that a similar tautomeric system is also present in guanine, based on its room temperature absorption spectrum. Although the argument has been applied to the pyrimidines, there are results contradictory to the presence of a highly fluorescent minor tautomer. Temperature jump kinetic studies have shown that cytosine only tautomerizes to the extent of .25% (20). If a minor tautomer is responsible for the fluorescence of cytosine, it must have a quantum yield of .01 or greater. This infers

a fairly long excited state lifetime, hence, the fluorescence should be considerably depolarized. However, several authors have reported room temperature polarizations for cytosine and thymine to be nearly that found in a rigid glass at low temperatures (5, 6). Recently Vigney et al. (21) found that thymine did not violate Vavilov's law.

Wilson et al. (16) have proposed that the origin of the wavelength dependence of the quantum yields is based on a competition between the internal conversion and vibrational relaxation deactivation processes. Their argument is based on the results found for isolated molecules in the gas phase. In low pressure gas phase experiments, the rate of internal conversion was found to increase with increasing excitation energy above the 0 - 0 band (22). These results are explained in terms of the coupling between the ground and the excited Born-Oppenheimer states with the nuclear kinetic energy operator (23, 24).

Another result which affects the validity of τ_f^{SB} , is the presence of weakly allowed transitions. These weakly allowed transitions are often obscured by the much more intense allowed transitions. Photo-selection results are often very helpful in identifying fluorescence emanating from a slightly forbidden state. The polarization ratio of AMP has been found to change markedly across the first apparent absorption band (8).

Although all of the above discussions question the validity of τ_f^{SB} 's representation of the actual τ_f , it should be noted that τ_f^{SB}

represents a lower limit of the actual fluorescence lifetime. That is, that all of the latter arguments lead to the possibility of longer lifetimes, but never shorter. The radiative lifetime, as determined by the Strickler-Berg formalism is inversely proportional to the area under the first absorption band. In the case of a tautomer or fluorescence from a slightly forbidden transition, the emitting state carries less oscillator strength than that determined by the integration of the first apparent absorption band. This leads to a predicted radiative lifetime which is too short. If the violations of Vavilov's law are due to competition between internal conversion and vibrational relaxation then the quantum yield measured for the 0 - 0 transition should be used, instead of that measured using excited light corresponding to the absorption maximum. In the absence of mixed polarization and deviations of Vavilov's law, τ_f^{SB} should be an adequate representation of the actual τ_f . When these conditions prevail the rotational correlation time predicted, τ_c^{SB} from τ_f^{SB} and the polarization ratio, should represent the real τ_c .

In this study, the excited state lifetimes for 26 nucleic acid components in room temperature aqueous solution are tabulated. Their corresponding reorientational times are also compiled and subsequently compared to reorientational times calculated from the Stokes-Einstein hydrodynamic formulas. The validity of these excited state lifetimes are discussed in light of the results of their corrected fluorescence

excitation spectra and photoselection studies. It will be shown that the pyrimidines have corrected fluorescence excitation spectra which coincide with their absorption spectra. Many of the purines, however, are shown to exhibit considerable deviation between the two spectra. Discrepancies between the corrected fluorescence excitation spectra and the absorption spectra are shown to be attributed to the presence of a highly fluorescent minor tautomer and are not due to competition between internal conversion and vibrational relaxation. Tautomeric structures are predicted for adenine, on the basis of corrected fluorescence excitation spectra, which explain the fluorescent properties observed in both neutral and acid solutions.

EXPERIMENTAL

The nucleic acid components utilized in this study were obtained from commercial sources as follows: 7-methyladenine from Cyclo Chemical Corporation, 5-hydroxythymine from Pacific Chemical Company, 3-methyladenine from Vega Chemical Corporation, adenosine and N⁶, N⁶-dimethyladenine from Calbiochem, with the remainder being purchased from Sigma Chemical Corporation. All compounds were used without further purification, as their fluorescence spectral band shape was virtually independent of excitation wavelength, with the exception of 2-methyladenine and 5-hydroxythymine. In both of the latter, spectral bandshapes remained constant when exciting from 250 to 280 nm. However, excitation beyond 280 nm led to a large red shift in the fluorescence maximum. Neither compound was re-purified, so data reported within corresponds to excitation up to 280 nm. Water, once distilled, proved to be adequate as its background emission was roughly 20 times less than the prominent O-H stretch Raman line.

All measurements reported here were for aqueous solution (ca. 4-.1 mM) in nucleic acid components. The instrumentation was housed in a constant temperature laboratory at $20 \pm 2^{\circ}\text{C}$, in which solution temperatures were assumed to be that of the ambient room temperature. Neutral solutions buffered to pH 6.7 were .01 molar in phosphate. Acidic solutions pH 1.5 were .15N in H_3PO_4 .

The luminescence apparatus used in this study was a collection of components which were arranged such that viewing was 90° to the propagation of the exciting light. An Osram XBO 150 W/1 high pressure xenon lamp was employed as an excitation source. This excitation radiation was then passed through a 500 mm Bausch and Lomb grating monochromator. The fluorescence cell was mounted in the center of a stage which had movement capabilities along the excitation axis. The emitted light then passed through a second monochromator identical to the first. Detection was performed by an EMI9558QC photomultiplier tube, in which the photocurrent produced was measured by a Pacific Laboratory Model II photometer. Spectra were recorded on a Hewlett Packard Model 7030A x-y recorder.

Absorbance measurements were performed using either a Cary 14 recording spectrometer or a Varian Tektron series 634 spectrometer with a A25 model strip chart recorder.

The pH of the solution used were measured on a Corning model 12 pH meter. A fluorescence spectrum of the blank was recorded each time to determine the extent of any fluorescing impurities. A few crystals of the nucleic acid component were then immediately added to the fluorescence cell, except for those with low solubilities. Compounds with low solubilities were preconcentrated in 25ml glass stoppered Erlenmeyer

flasks. Quantum yield and excitation spectrum measurements were then done immediately, without the use of the polarizer. For the quantum yield determination, the excitation wavelength corresponded to that of the absorption maximum. The excitation spectrum was recorded by setting the viewing monochromator to the approximate wavelength of the fluorescent maximum and varying the excitation wavelength from 250 nm to the onset of absorption. The polarization ratio was measured by using the same excitation wavelength as the quantum yield determination and then setting the viewing monochromator to the fluorescence maximum. With the instrument in this configuration, the x-y recorder was operated in a time scan mode of .5in/min. This allowed the vertical and horizontal components of the emission to be measured by rotating the polarizing film. The background current could also be monitored by manually operating the shutter of the excitation monochromator. An entire polarized fluorescence spectrum was also recorded for use in determining any background corrections.

Spectral variations in the intensity of the excitation source were determined by using a Rhodamine B quantum counter. This entailed taking a 640 nm fluorescence excitation spectrum of a 3g/l solution of Rhodamine B in ethylene glycol (25). At high solute concentrations, the fluorescence intensity becomes independent of concentration and the signal becomes a function of the quantum yield and the lamp intensity. The

quantum yield of Rhodamine B is constant across the absorption band, therefore, the fluorescence intensity is proportional to the intensity of the exciting light. Phototube response was determined by placing a freshly prepared MgO screen in the fluorescence cell holder and measuring the intensity of the scattered light, when both the excitation and viewing monochromators were set on the same wavelength (26).

Corrections due to solvent emission were not usually needed at the concentrations employed. Minor corrections were made when needed, however, the solvent emission had to be adjusted before it could be subtracted from the overall spectrum. These adjustments were made by using the relative intensity of the Raman water line in both the blank and sample spectra. It is found that as the intensity of the water Raman line changes, the background changes proportionally. Scaling the solvent emission to the relative attenuation of the Raman line of the blank, to that of the sample spectrum, yields a background spectrum that can be subtracted directly from the overall fluorescence spectrum (6).

The nucleic acid components, for which fluorescence quantum yields were quite low, required using solutions in which concentrations were not negligible. When solutions of this nature were employed, the intensity of the exciting light was not constant through the cell. Correction for beam absorption along the excitation axis must be made, as a quantum counter only assures 'constant' intensity at the face of the sample cuvette. The detector does not view the entire face of the fluorescence cell, so

a geometry factor, depending on the effective solid angle viewed by the detector, must also be included (27). The total fluorescence signal detected by the photomultiplier tube is given as follows:

$$F = -\phi_f \int_0^a W(x) dI(x) \quad (5)$$

where ϕ_f is the quantum efficiency, $W(x)$ is a weighting factor which accounts for the geometry of the detection system, and $dI(x)$ is given by Beer's law as follows:

$$-dI(x) = 2.303 \epsilon c I_0 e^{-2.303 \epsilon \bar{c} x} dx \quad (6)$$

Integration is carried out from 0 to a where a is the effective path-length of the cell.

Translation of the sample stage allows the detector to focus on any portion of the cell along the excitation axis. In this study, concentrated samples were used exclusively, hence, the sample stage was adjusted such that the detector viewed the front of the cell better than the center. The profile seen by the detector is probably gaussian in shape. However, under the above conditions, the profile is adequately represented by a triangle, with its apex at the front wall of the cuvette. It was found that the geometry of the detection system could be accounted for by the following weighting factor:

$$W(x) = 1 - x/a \quad (7)$$

Substitution of equations (6) and (7) into equation (5) and integrating from 0 to a gives the total fluorescence signal detected by the

photomultiplier:

$$F = \phi_f I_0 G \quad (8)$$

where

$$G = 1 - \left(\frac{1 - e^{-2.303 \epsilon c a}}{2.303 \epsilon c a} \right) \quad (9)$$

which is the correction factor that accounts for the geometry of the detection system and absorption properties of the solution.

The effective pathlength a was found to be .21 cm. This effective pathlength was determined by measuring the 350 nm fluorescence intensity of tryptophan at different absorbances. The a value was determined by taking the ratio of the fluorescence intensities of the two tryptophan samples and finding a effective pathlength, which makes the ratios of their respective G values equal to the ratio of their fluorescence intensities.

Tryptophan has been shown by several authors to have an excitation spectrum which coincides with its absorption spectrum (4, 16). This work has utilized tryptophan as a standard to check that when the G factor is employed the corrected fluorescence excitation spectrum of tryptophan is superimposable on its absorption spectrum. The corrected fluorescence excitation spectrum (FES) at any given wavelength is the product of the absorbance and the quantum yield measurement at that wavelength. The quantum yield was determined relative to that of Rhodamine B as follows:

$$\phi_{fNAC} = \frac{F_{NAC} \cdot \phi_{fRB} \cdot G_{RB}}{F_{RB} \cdot G_{NAC}} \quad (10)$$

$$G_{RB} = 1$$

where F_{NAC} and F_{RB} are the fluorescent intensities at the wavelength of interest for the nucleic acid component and Rhodamine B, respectively. The quantum yield of Rhodamine B was taken to be 1 (28). Figure 3 shows that the corrected fluorescence excitation spectrum of tryptophan, using the G factor, coincides with its absorption spectrum.

Quantum yields were determined by comparing the relative areas under the fluorescence curves of the nucleic acid components to that of tryptophan, for which the quantum yield was taken to be .15 (29).

The quantum yield determined from the following expression

$$\phi_{fNAC} = \frac{AR_{NAC} F_{MAXNAC} SNT_{NAC} G_{tryp} I_{tryp} \phi_{ftryp}}{AR_{tryp} F_{MAXtryp} SNT_{tryp} G_{NAC} I_{NAC}} \quad (11)$$

where AR is the area under the corrected fluorescence curve after the fluorescence maximum has been normalized to one, F_{MAX} is the height of the fluorescence curve at the fluorescence maximum, SNT is the photo-current required for full scale deflection and I is the relative intensity of the radiation field, as found by the 640 nm excitation spectrum of Rhodamine B.

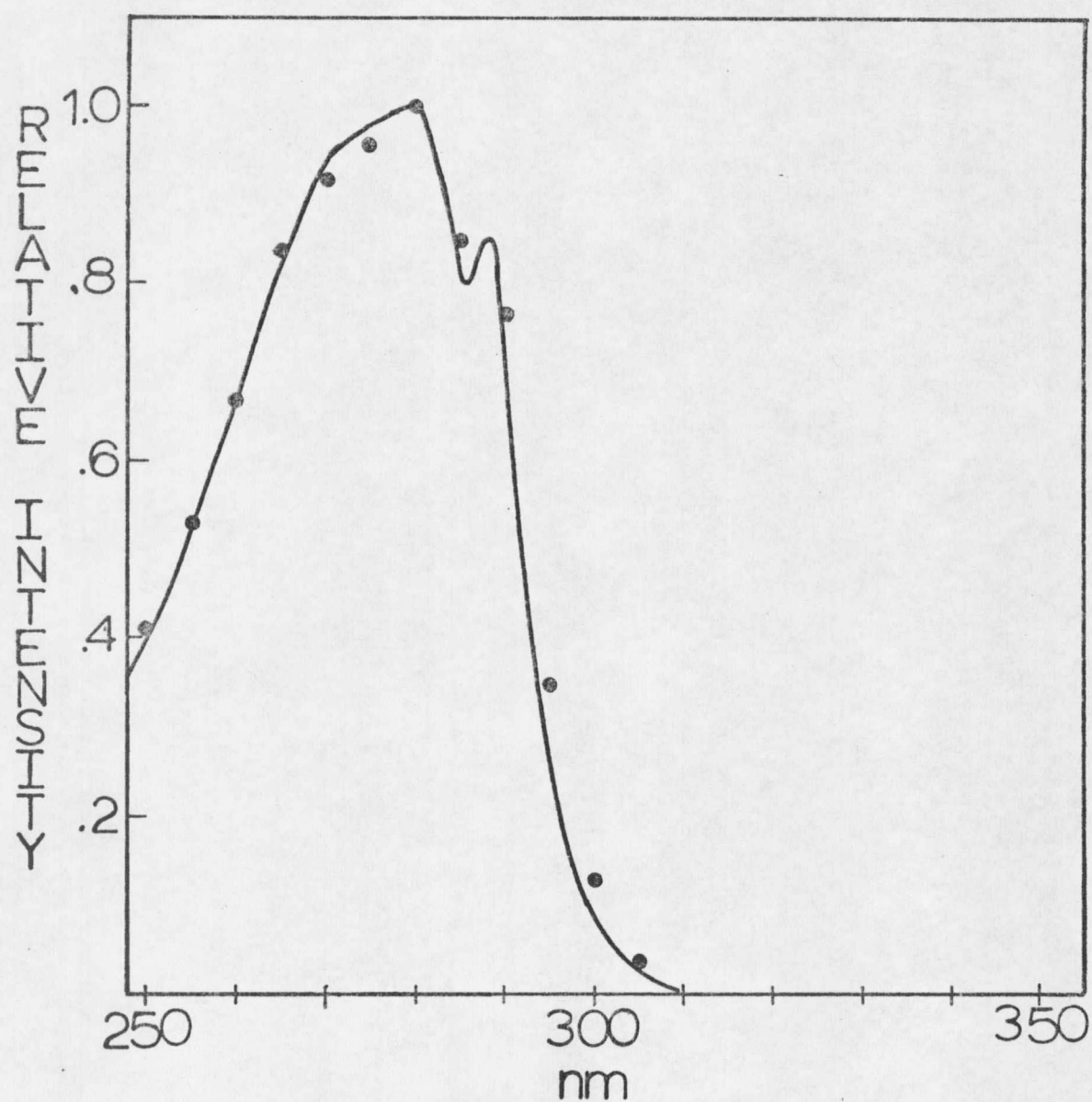


Figure 3

Corrected fluorescence spectrum (filled circles) and absorption spectrum (—) of tryptophan in pH 6.7 aqueous solution

RESULTS

Table 1 is a summary of all the pertinent experimental or derived parameters for the hydrodynamic study of the nucleic acid components. All the values in Table 1 were determined by this author, except for those indicated by an asterisk or dagger. The values for cytosine and guanine were taken from Callis (6) and all the 5-methylcytosine data from Anderson and Callis (30). The low temperature polarizations reported were taken from other sources, as indicated. It should be noted that low temperature polarization ratios were not available for all the nucleic acid components studied. Values for the components, on which no experimental work has been done, were taken to be the same as the parent fluorophore under similar conditions.

The anisotropy is quite pronounced in all of the nucleic acid components, indicating fluorescence lifetimes much shorter than their rotational correlation times (with the exception of the 5-methylcytosine anion and the 7-methylguanosine cation). In general, the pyrimidines exhibit much higher polarization ratios than the purines. The protonated adenines, which were substituted at the 2, 7 and 9 positions, were not included in Table 1 because they exhibited totally depolarized fluorescence spectra. Table 3 lists the quantum yields for these compounds, which are seen to be smaller than those of the anion of 5-methylcytosine and the cation of 7-methylguanosine. This is interesting since the latter exhibited some fluorescence anisotropy. The ambient temperature polarization

TABLE 1

Polarizations, fluorescence yield (ϕ_f), fluorescence lifetimes (τ_f) and rotational correlation time (τ_c) parameters for the nucleic acid components in room temperature aqueous solution. τ_o^{SB} is the radiative lifetime; $\tau_f^{SB} = \tau_o^{SB} \phi_f$; $\tau_c^{SB} = \tau_f^{SB} (\frac{R_o}{R} - 1)^{-1}$; τ_c^{stick} is from Stokes-Einstein formula

Nucleic Acid Component	N	No ⁺	R	Ro ⁺	$\phi_f \times 10^4$	$\tau_o^{SB}_{ns}$	$\tau_f^{SB}_{ps}$	$\tau_c^{SB}_{ps}$	$\tau_c^{stick}_{ps}$
Ade	1.46±.01	1.76	.235	.336	3.32±.35	4.2	1.38	3.2	36
3-MeAde	1.56±.04	1.76	.272	.336	1.17±.03	4.5	.53	2.2	46
7-MeAde	1.51±.02	1.76	.254	.336	8.2±.24	4.8	3.9	12.1	43
6-MeAde	1.59±.04	1.76	.282	.336	.7±.02	4.0	.28	1.5	43
6-MeAde ⁺	1.49±.04	1.76	.246	.336	1.2±.04	4.9	.59	1.6	43
6-Me ₂ Ade	1.44±.04	1.76	.227	.336	.99±.14	4.6	.45	.93	52
6-Me ₂ Ade ⁺	1.55±.04	1.76	.268	.336	.47±.09	4.7	.22	.87	52
Ado	1.47±.08	1.53	.239	.261 ^(a)	.49±.02	5.1	.25	2.7	89
6-MeAdo	1.51±.04	1.53	.254	.261	.91±.06	5.0	.45	15.5	99
6-Me ₂ Ado	1.46±.02	1.53	.235	.261	1.51±.01	4.1	.62	5.6	109

TABLE 1 (continued)

Nucleic Acid Component	N	No [†]	R	Ro [†]	$\phi_f \times 10^4$	$\tau_o^{SB}_{ns}$	$\tau_f^{SB}_{ps}$	$\tau_c^{SB}_{ps}$	$\tau_c^{stick}_{ps}$
Cyt*	1.65±.03	1.70	.302	.318	.76	7.7	.59	11	26
3-MeCyt ⁺	1.77±.02	1.79	.339	.345	.61±.02	6.2	.38	21	36
5-MeCyt*	1.40±.01	1.75	.211	.333	5±1	10±1	5	8.7	40
5-MeCyt ⁺⁺	1.69±.01	1.79	.315	.345	3±5	7±.7	2.1	22.2	40
5-MeCyt ^{-*}	1.04±.01	1.70	.026	.318	140±10	11±1	154	13.8	40
5-HmCyt	1.47±.03	1.75	.239	.333	2.15±.04	13.4	2.87	7.3	41
5-HmCyt ⁺	1.70±.02	1.79	.318	.345	1.88±.05	7.6	1.41	16.3	41
Cyd	1.74±.02	1.80	.330	.350	.68±.06	7.4	.51	8.4	69
Cyd ⁺	1.78±.03	1.80	.342	.350	.9±.05	5.0	.45	19.6	69
CMP	1.73±.03	1.80	.327	.350 ^(a)	.84±.03	7.4	.62	8.9	94
Gua*	1.50±.02	1.72	.250	.324	2.8	7.8	2.18	7.4	35.5
7-MeGua	1.47±.03	1.72	.239	.324	5.28±.6	9.7	5.14	14.4	48
7-MeGuo ⁺	1.13±.02	1.65	.08	.304 ^(a)	139	17.1	238	78	108
5-MeUra	1.77±.02	1.81	.339	.351 ^(b)	1.01±.21	8.2	.83	23.7	36

TABLE 1 (continued)

Nucleic Acid Components	N	No [†]	R	Ro [†]	$\phi_f \times 10^4$	$\tau_o^{SB_{ns}}$	$\tau_f^{SB_{ps}}$	$\tau_c^{SB_{ps}}$	$\tau_c^{stick_{ps}}$
5-HmUra	1.71 $\pm$.04	1.81	.321	.351	1.06 $\pm$.21	8.6	.91	9.7	40
5-MeUrd	1.78 $\pm$.03	1.81	.342	.351	1.02 $\pm$.08	6.5	.66	25.4	83
TMP	1.78 $\pm$.03	1.81	.342	.351	1.14 $\pm$.04	6.3	.72	27.7	111

a Wilson and Callis (8)

b Callis (6)

*All values were taken from other sources; cytosine and guanine, Callis (6), 5-methylcytosine, Anderson and Callis (30).

[†]All low temperature values were taken from references a and b. Values which were not referenced were taken to be the same as the parent fluorophore.

ratios of the pyrimidines, in many cases, approaches the value of the low temperature polarization, indicating that there is little, if any, reorientation in the excited state.

The quantum yields reported in Table 1 were determined relative to tryptophan, so the uncertainty of these values is in the precision of the measurements. The accuracy of the values is dependent on the value of the quantum efficiency taken for tryptophan. The quantum yields reported here are comparable with those reported by other authors utilizing different standards. The quantum yield reported for thymine is in good agreement with that reported by Hauswirth and Daniels (3) and the values for adenosine, cytidine 5'-monophosphate, and thymidine 5'-monophosphate are within experimental error of those reported by Vigney and Duquesne (4).

Also shown in Table 1 are Strickler-Berg radiative lifetimes, which were determined by integration of the spectral curves. The τ_f^{SB} (theoretical fluorescence lifetimes) tabulated are the product of the quantum yield and the radiative lifetime. There are two rotational correlation times, τ_c 's shown; τ_c^{SB} is found using equation 4 and τ_c^{stick} from Stokes-Einstein formulas (see Appendix). As noted, none of these derived quantities have error limitations associated with their reported values. This is not to insinuate that the values are exactly known, but reflect the fact that the uncertainties of these values are not directly measur-

able. These derived quantities have uncertainties of, probably, ten percent (10%). Deviations of greater than twenty percent (20%) would require a correlation of the errors. What should be stressed is that these results represent the most probable values and any conclusions reported in this study are based on the most probable results.

Figures 4 and 5 show plots of $\frac{R_0}{R} - 1$ vs $\tau_f^{SB}/\tau_c^{stick}$ for selected neutral and cation nucleic acid components, respectively. Based on the assumption that τ_f^{SB} is equal to the actual τ_f , the slope yields the coefficient for how much greater τ_c^{stick} is than the actual rotational correlation time. The solid line in each figure represents the average behavior of the points plotted. The neutrals appearing in figure 4 have τ_c 's which range from 10 times faster for CMP to 1.5 times faster for thymine than their corresponding τ_c^{stick} . The mean behavior shown by the solid line in figure 4 indicates τ_c 's which are approximately four times smaller than the correlation time predicted from sticking boundary conditions for the neutrals. The cations shown on figure 5 vary from 4 times faster for cytidine to 1.4 for 7-methylguanosine than the τ_c^{stick} , with an average behavior of roughly one-half that predicted by the Stokes-Einstein formulas. Overall, the cations appear to rotate 2 times slower than the neutrals. Table 2 shows the ratios of $\tau_c^{SB}/\tau_c^{stick}$ for all the nucleic acid components in a monotonically decreasing order. The ratio $\tau_c^{SB}/\tau_c^{stick}$ is the inverse of the slope of figures 4 and 5. As has

Figure 4

Plot of $R_0/R - 1$ versus $\tau_f^{SB}/\tau_c^{stick}$ for the neutral
nucleic acid component in room temperature aqueous
solution; $(\text{---}) \tau_c^{ave} = .27\tau_c^{stick}$

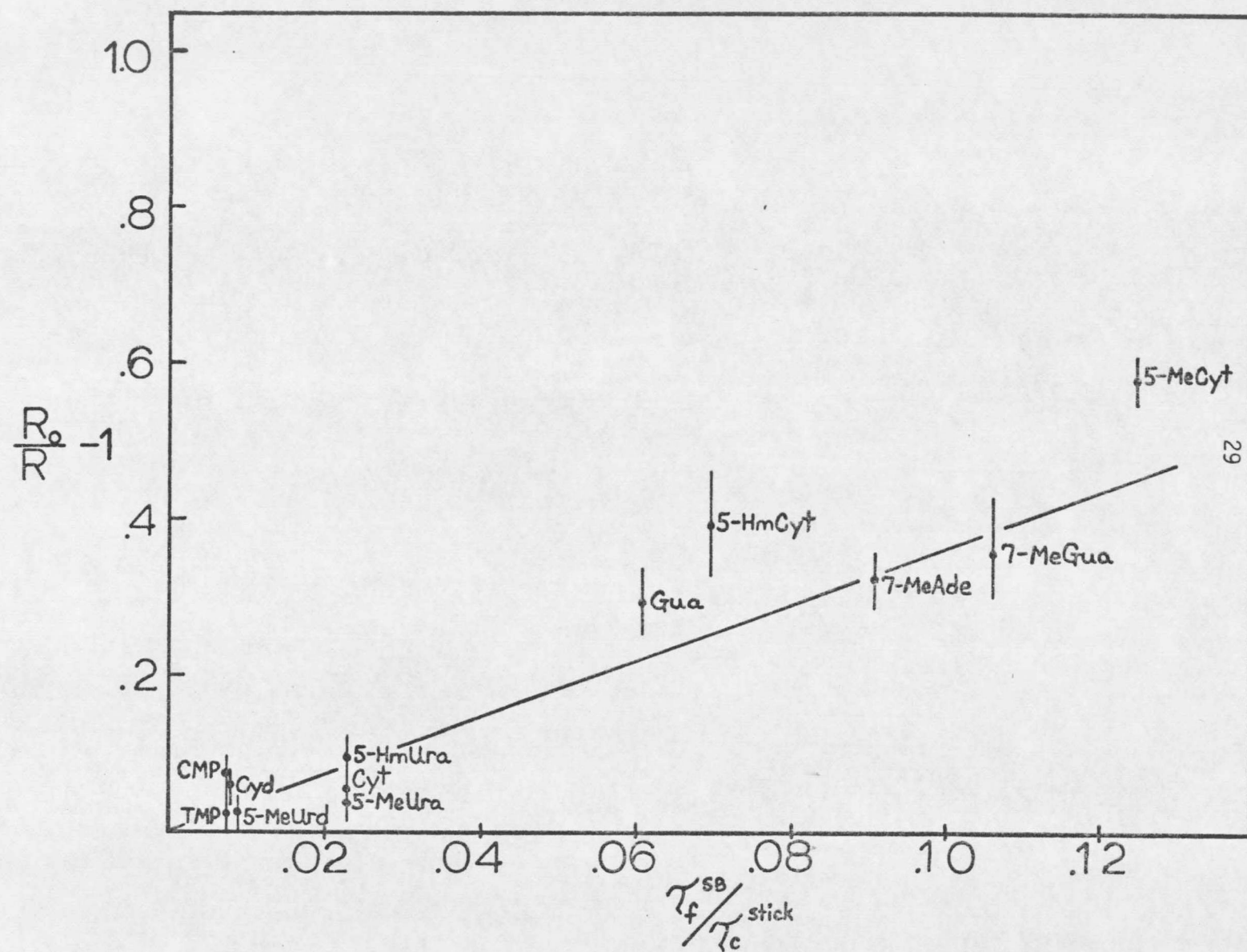


Figure 5

Plot of $R_0/R-1$ versus $\tau_f^{SB}/\tau_c^{stick}$ for the protonated
nucleic acid components in room temperature aqueous
solution; (—) $\tau_c^{ave} = .5 \tau_c^{stick}$

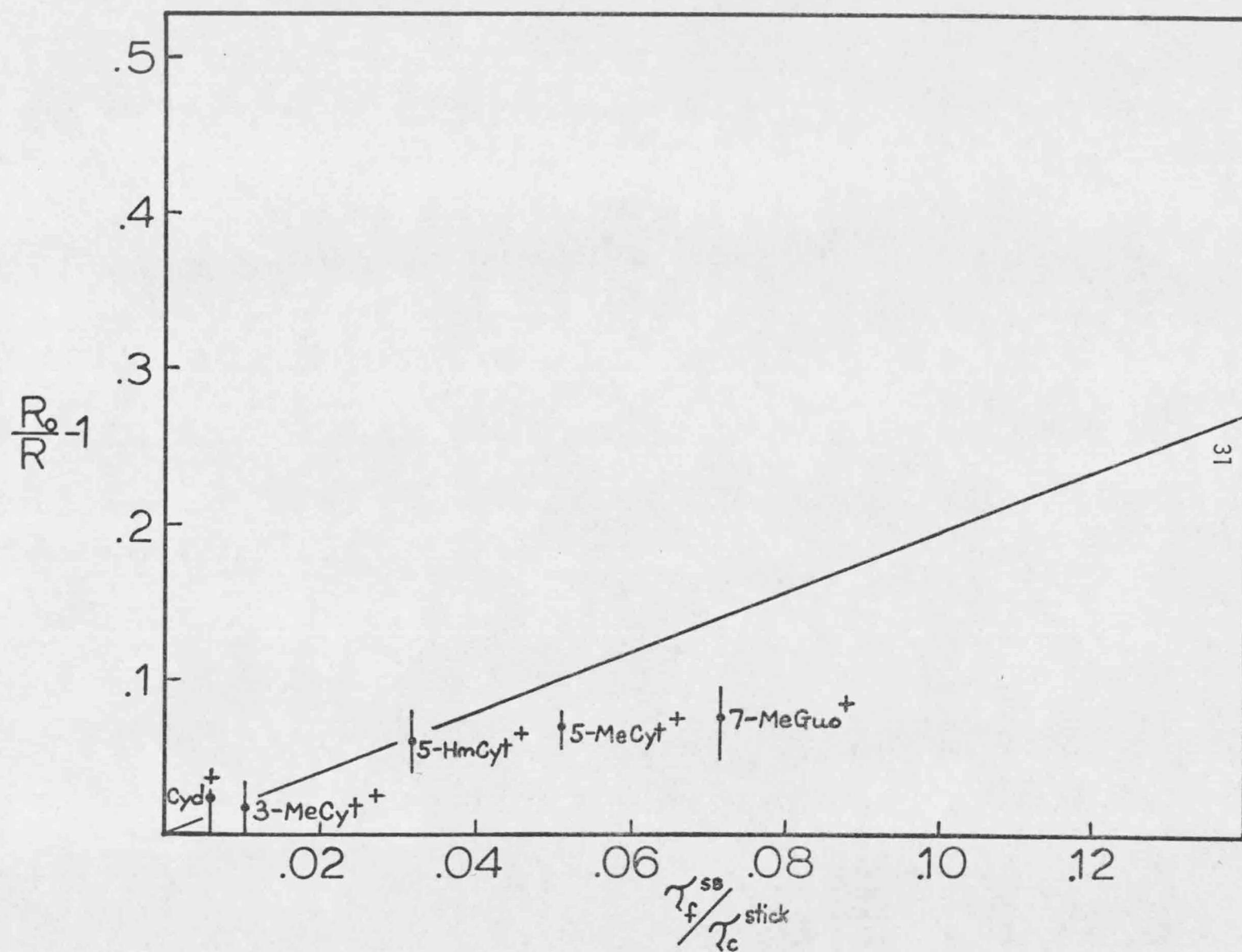


TABLE 2
 RATIOS OF $\tau_c^{SB}/\tau_c^{stick}$ FOR THE NUCLEIC ACID COMPONENTS
 IN A MONOTONICALLY DECREASING ORDER

NUCLEIC ACID COMPONENTS	$\tau_c^{SB}/\tau_c^{stick}$	NUCLEIC ACID COMPONENTS	$\tau_c^{SB}/\tau_c^{stick}$
7-MeGuo ⁺	.72	Gua	.21
5-MeUra	.66	5-HmCyt	.18
3-MeCyt ⁺	.59	6-MeAdo	.16
5-MeCyt ⁺	.55	Cyd	.12
Cyt	.43	CMP	.095
5-HmCyt ⁺	.4	Ade	.09
5-MeCyt ⁻	.34	3-MeAde ⁺	.05
5-MeUrd	.31	6-Me ₂ Ado	.05
7-MeGua	.3	6-MeAde ⁺	.04
Cyt ⁺	.28	6-MeAde	.035
7-MeAde	.28	Ado	.03
TMP	.25	6-Me ₂ Ade	.018
5-HmUra	.24	6-Me ₂ Ade ⁺	.017
5-MeCyt	.22		

been predicted, none of the molecules have τ_c^{SB} 's which are longer than their corresponding τ_c^{stick} 's. With the exception of 7-methyladenine, none of the other adenines were included in figures 4 and 5. These molecules were excluded on the basis of their apparent anomalous excitation spectra or the results of photoselection studies.

Figures 6 - 31 show the absorbance spectra solid line, corrected fluorescence excitation spectra filled circles, and the corrected fluorescence spectra broken line for the the nucleic acid components studied by this author. There is very good agreement between the absorbance and fluorescence excitation spectra for all the non-adenine nucleic acid components.

Table 3 shows a summary of the experimental data for all the adenine derivatives studied. Within the table are given the quantum yields, approximate excitation spectrum maximum and whether their excitation spectrum coincides with their absorbance spectrum. Only one of the cations, N^6, N^6 -dimethyladenosine, showed any agreement between its fluorescence excitation and absorption spectra. There is a large disparity amongst the quantum yields of the cationic forms, with the N^6 methylated derivatives and 3-methyladenine being very low (ca. 10^{-4}). Whereas, the other cations are very high (ca. 10^{-2}). In contrast, most of the neutrals showed good agreement between their excitation and absorbance spectra, except adenine and N^6 -methyladenine. Also, the quantum yields do not show the

Figure 6

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for adenine in pH 6.7 aqueous solution.

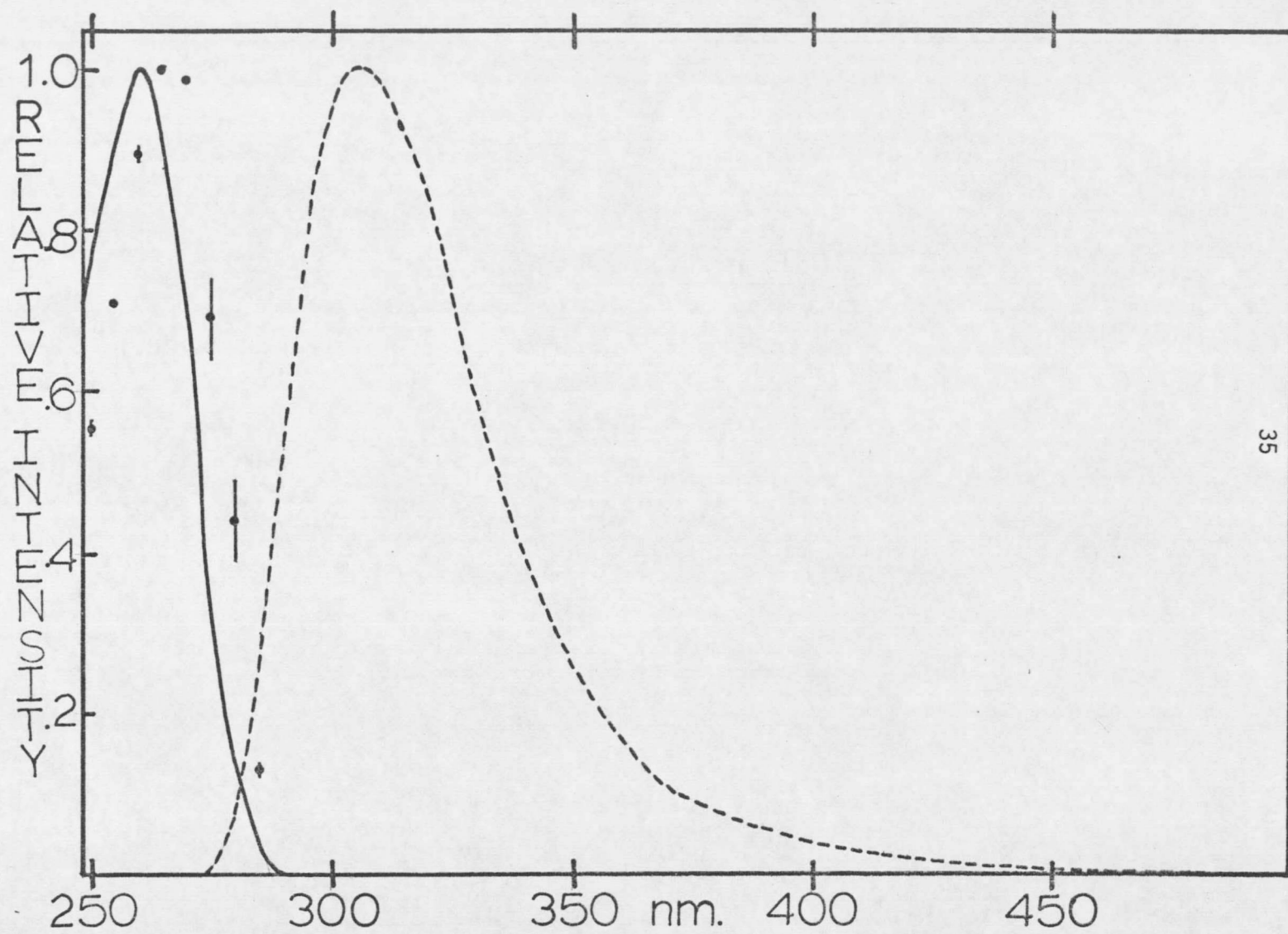


Figure 7

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for adenine in pH 1.5 aqueous solution.

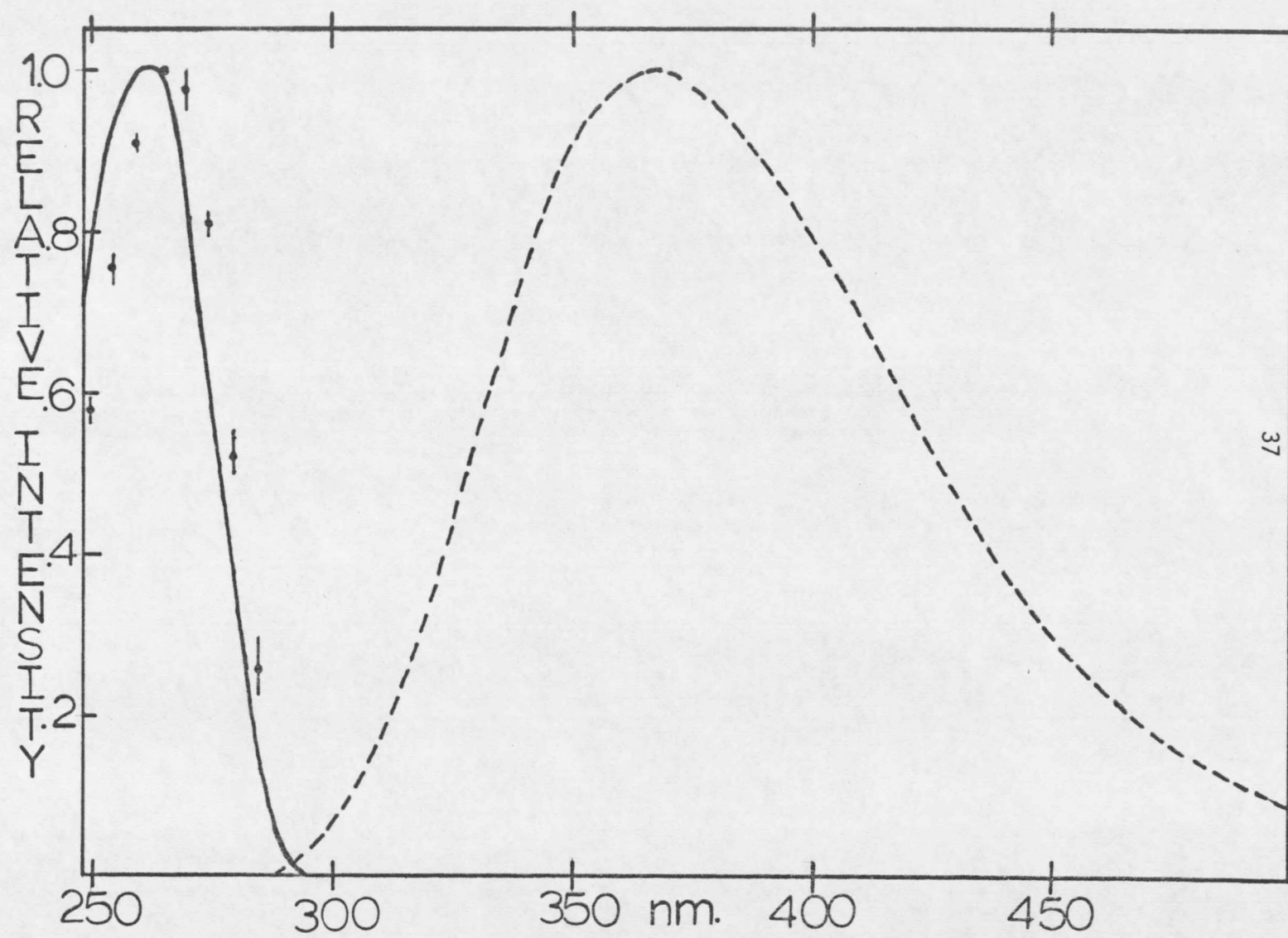


Figure 8

Absorption (—), fluorescence (-----), and corrected
fluorescence excitation (filled circles) spectra for
2 - methyladenine in pH 1.5 aqueous solution.

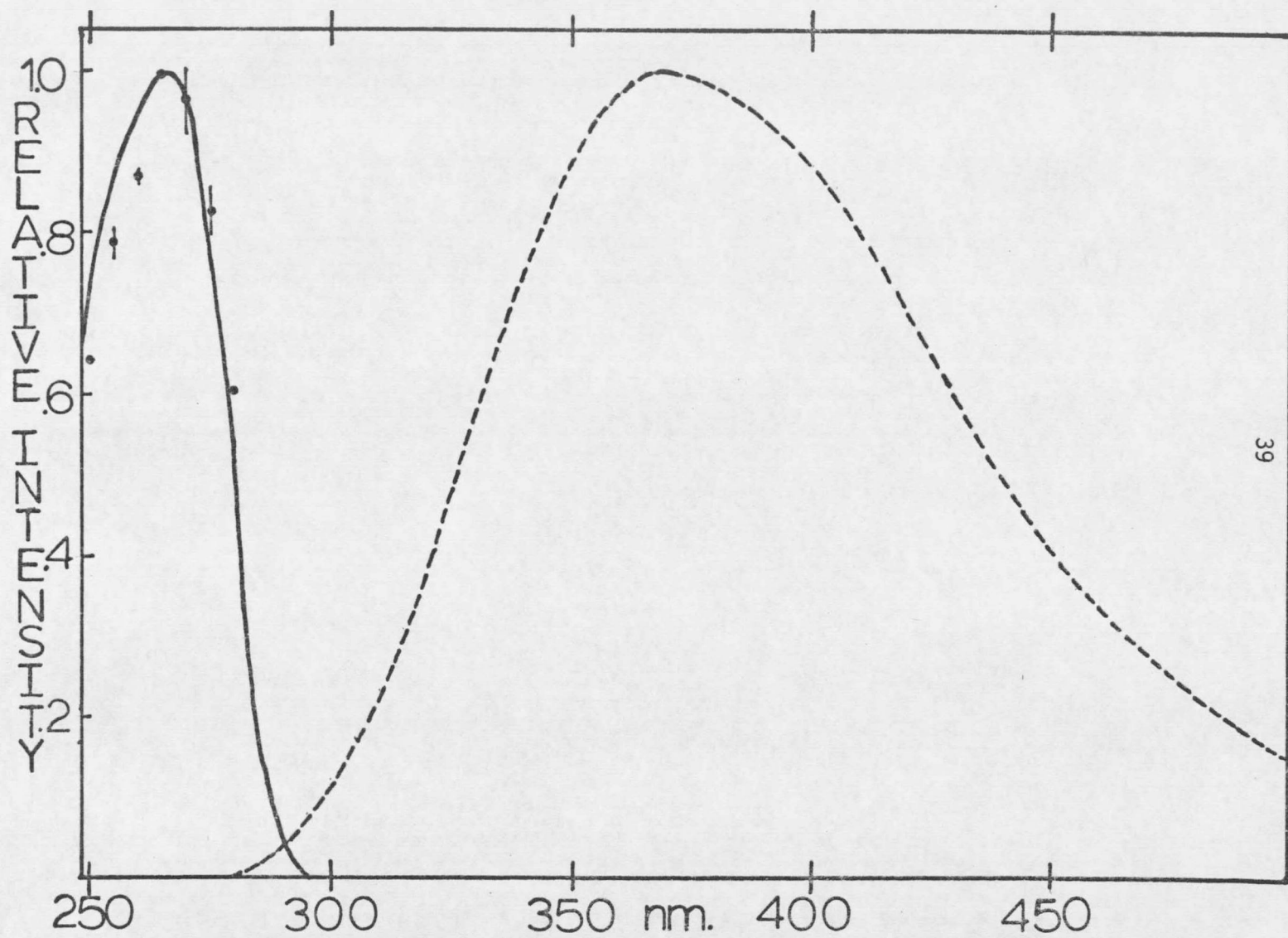


Figure 9

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for 3 - methyladenine in pH 1.5 aqueous solution.

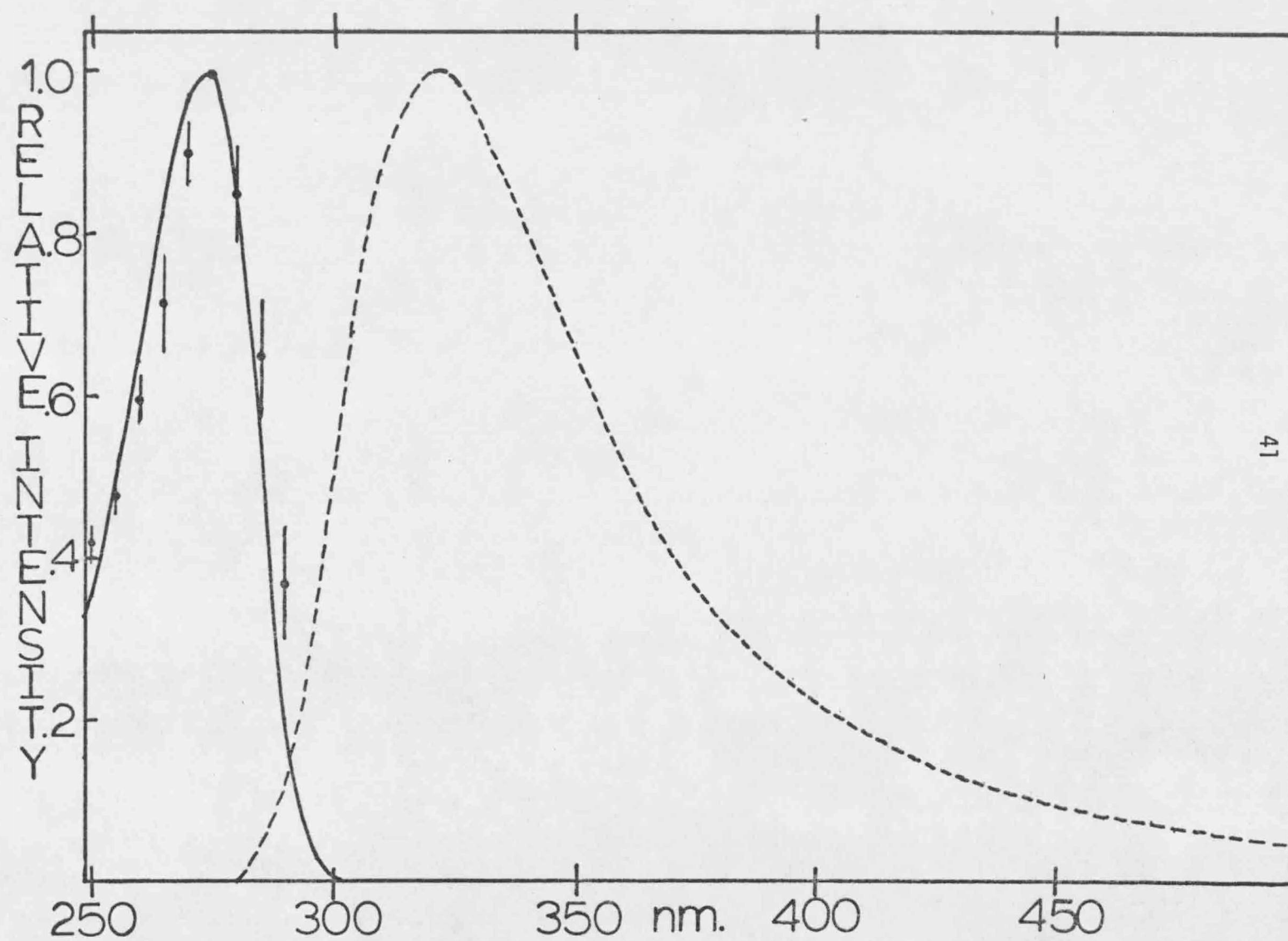


Figure 10

Absorption (—), fluorescence (-----), and corrected
fluorescence excitation (filled circles) spectra for
N⁶ - methyladenine in pH 6.7 aqueous solution.

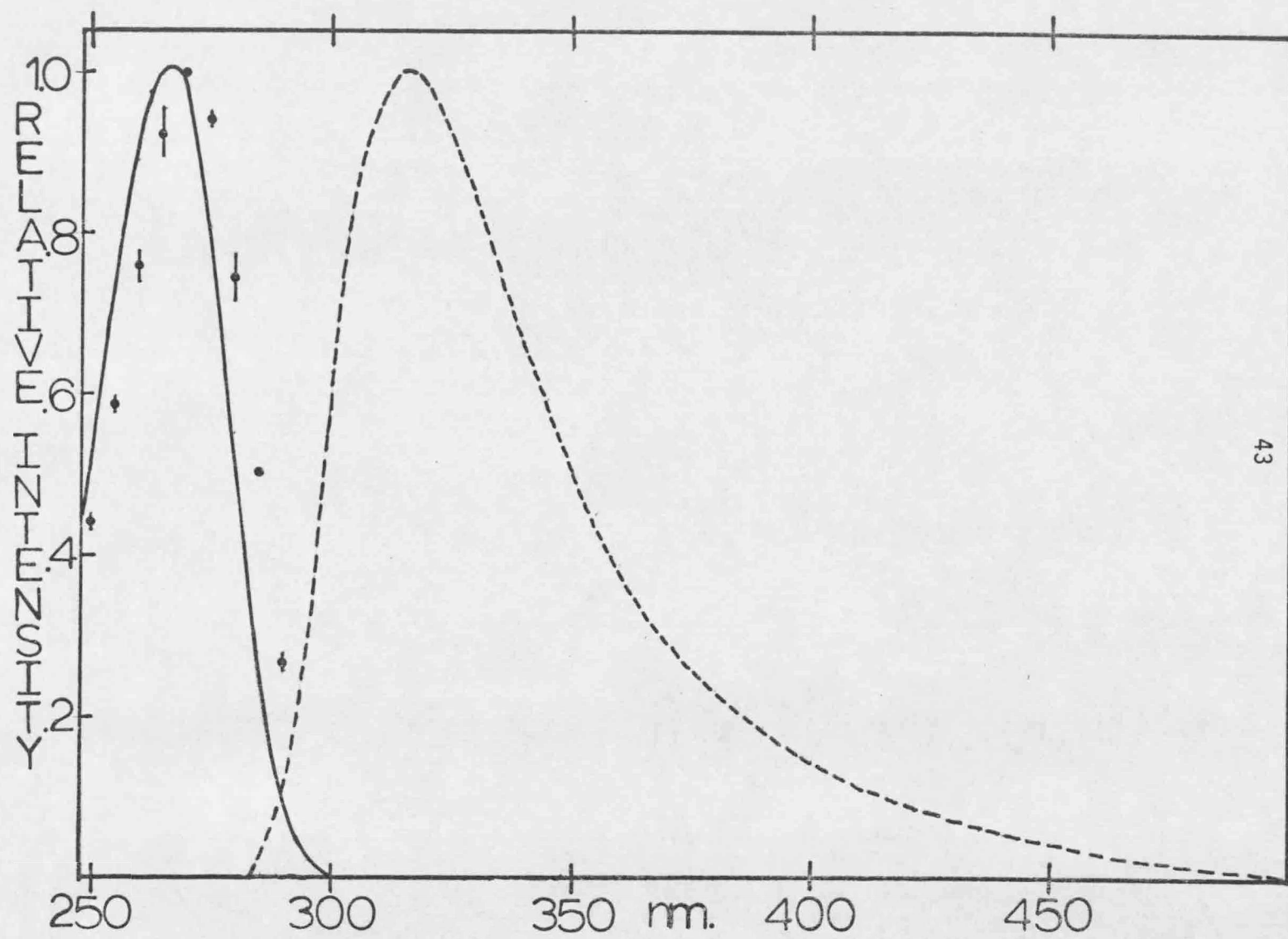


Figure 11

Absorption (—), fluorescence (-----), and corrected
fluorescence excitation (filled circles) spectra for
⁶
N⁶ - methyladenine in pH 1.5 aqueous solution.

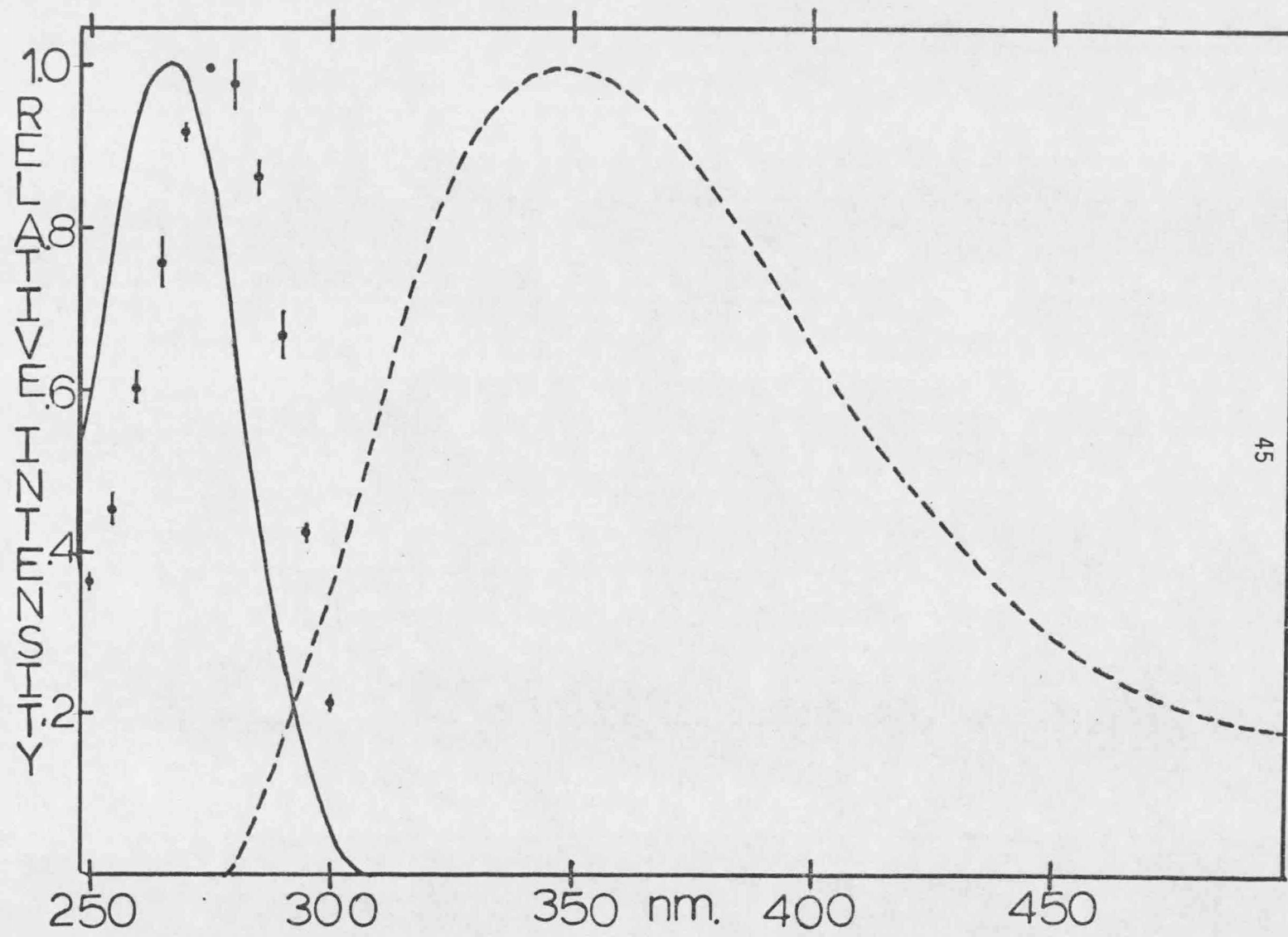


Figure 12

Absorption (—), fluorescence (-----), and corrected
fluorescence excitation (filled circles) spectra for
 N,N - dimethyladenine in pH 6.7 aqueous solution.

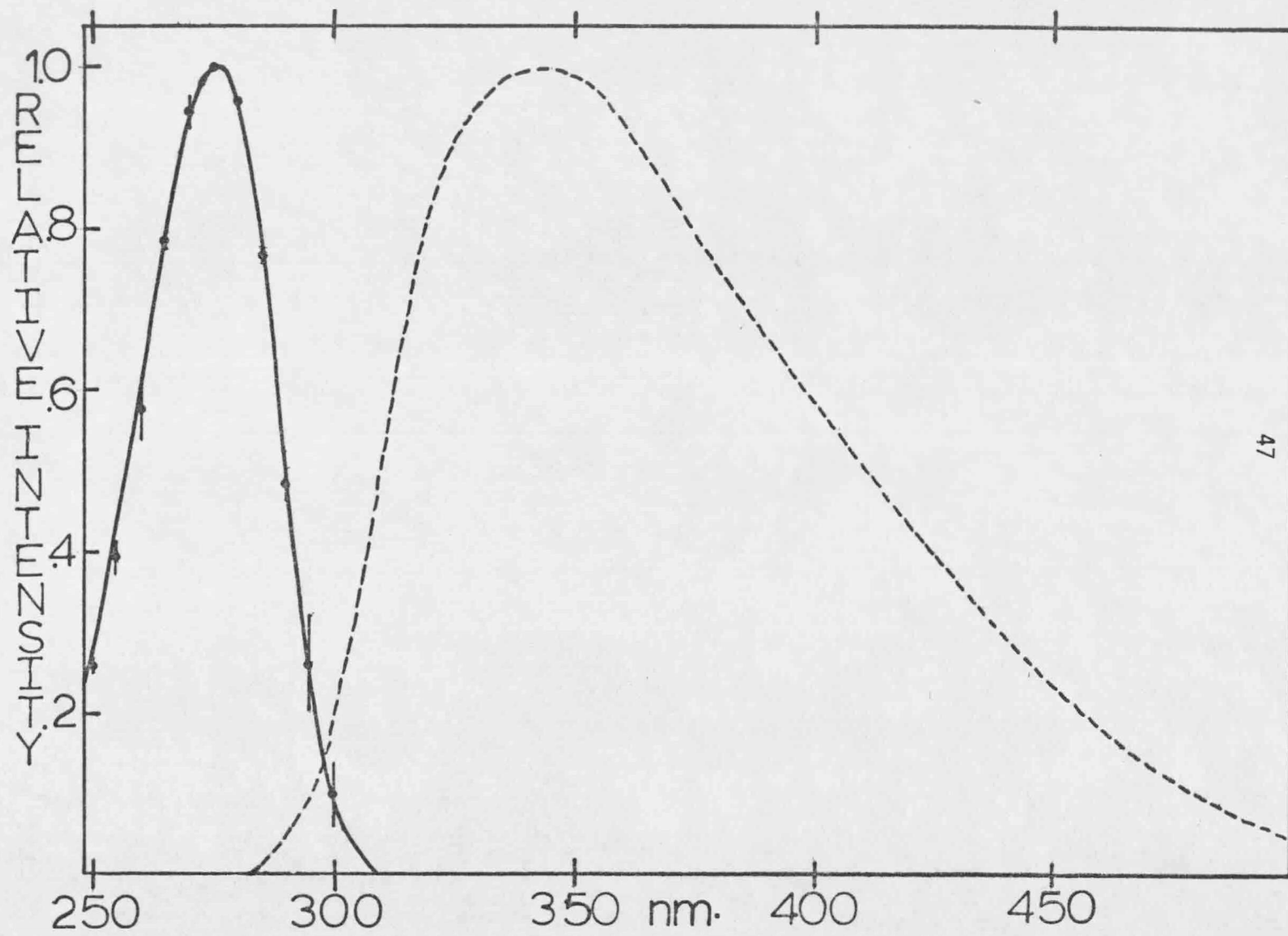


Figure 13

Absorption (—), fluorescence (-----), and corrected
fluorescence excitation (filled circles) spectra for
 N,N -dimethyladenine in pH 1.5 aqueous solution.

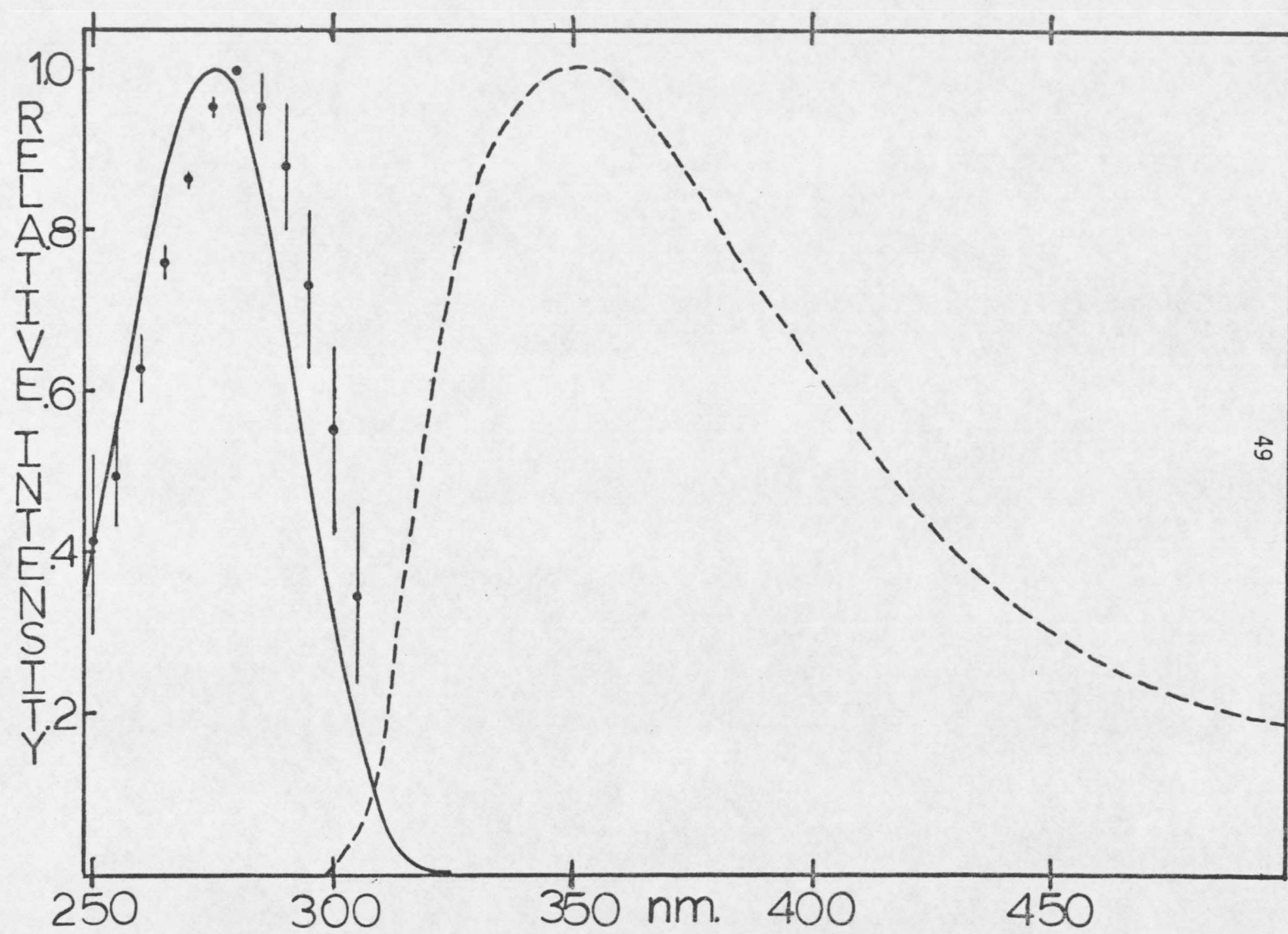


Figure 14

Absorption (——), fluorescence (-----), and corrected
fluorescence excitation (filled circles) spectra for
7 - methyladenine in pH 6.7 aqueous solution.

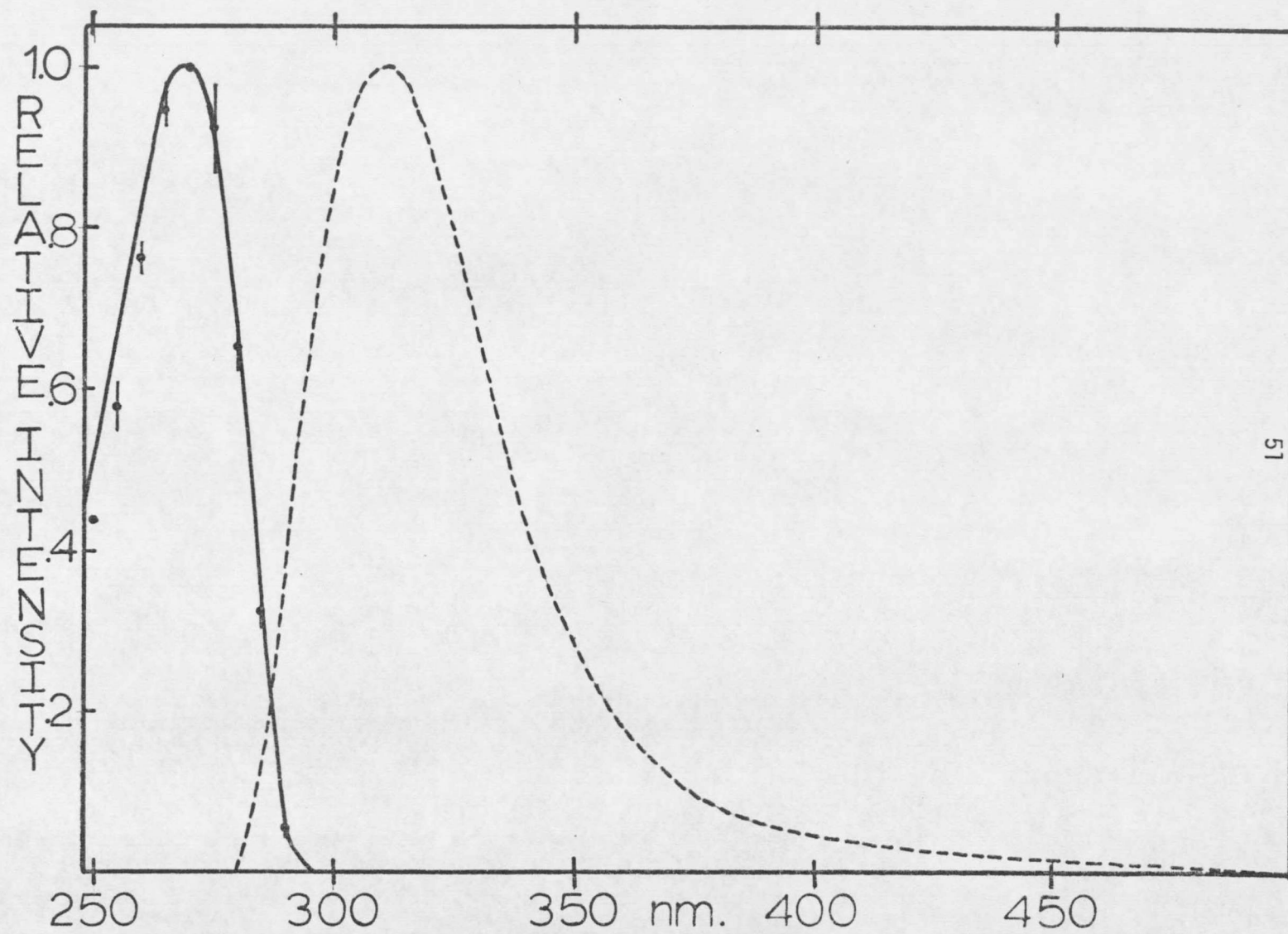


Figure 15

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for 7 - methyladenine in pH 1.5 aqueous solution.

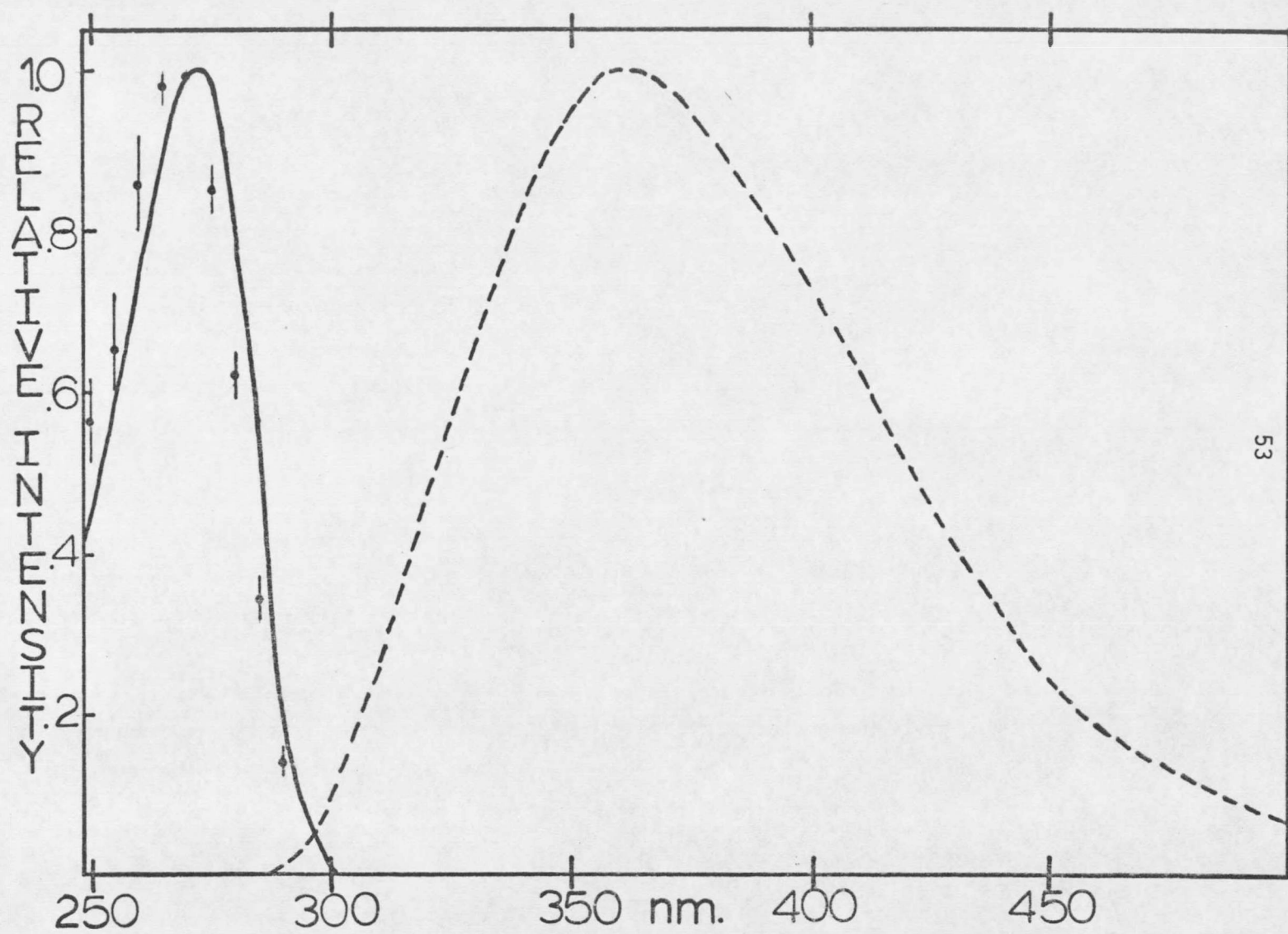


Figure 16

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for adenosine in pH 6.7 aqueous solution.

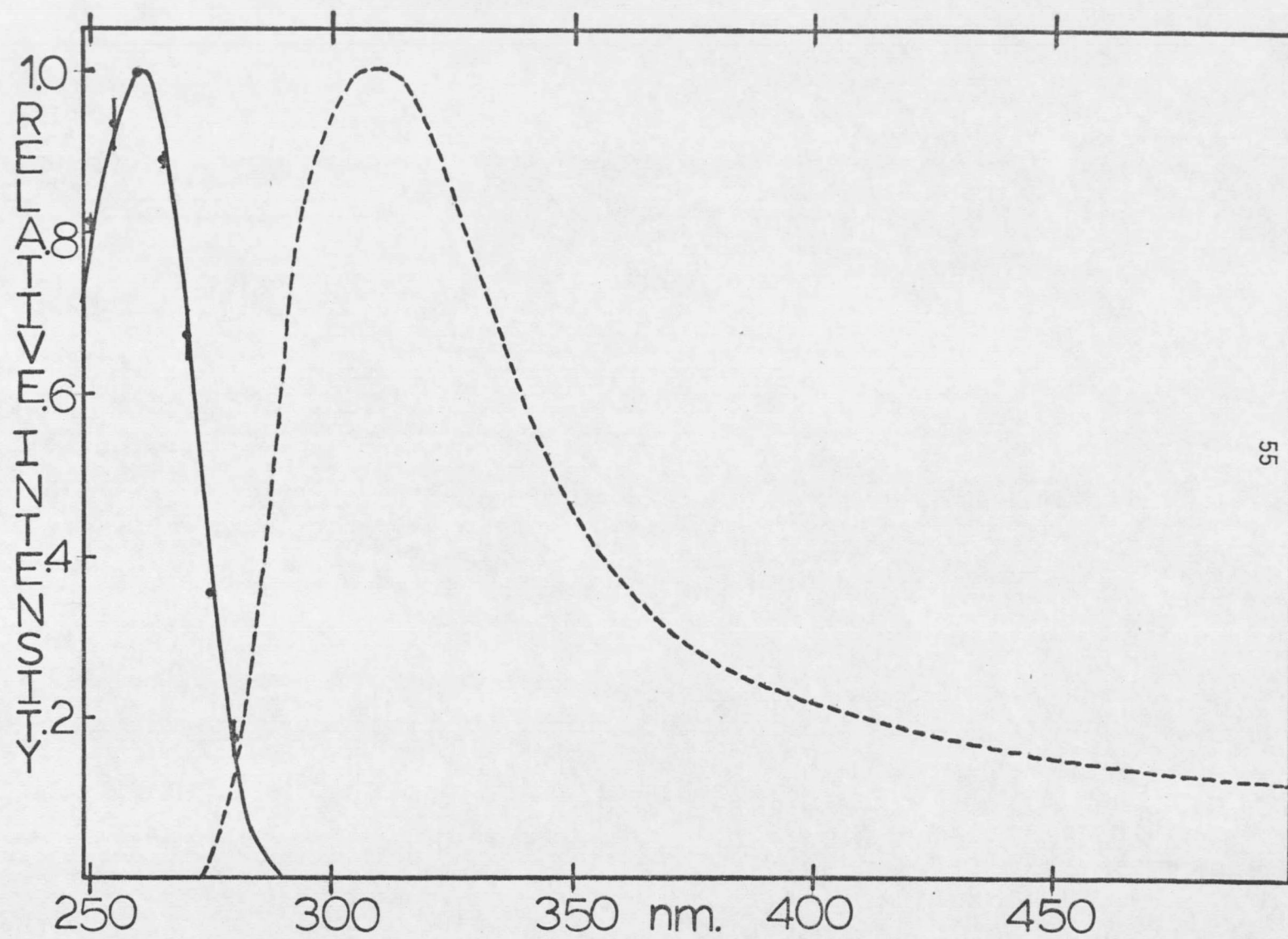


Figure 17

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for adenosine in pH 1.0 aqueous solution.

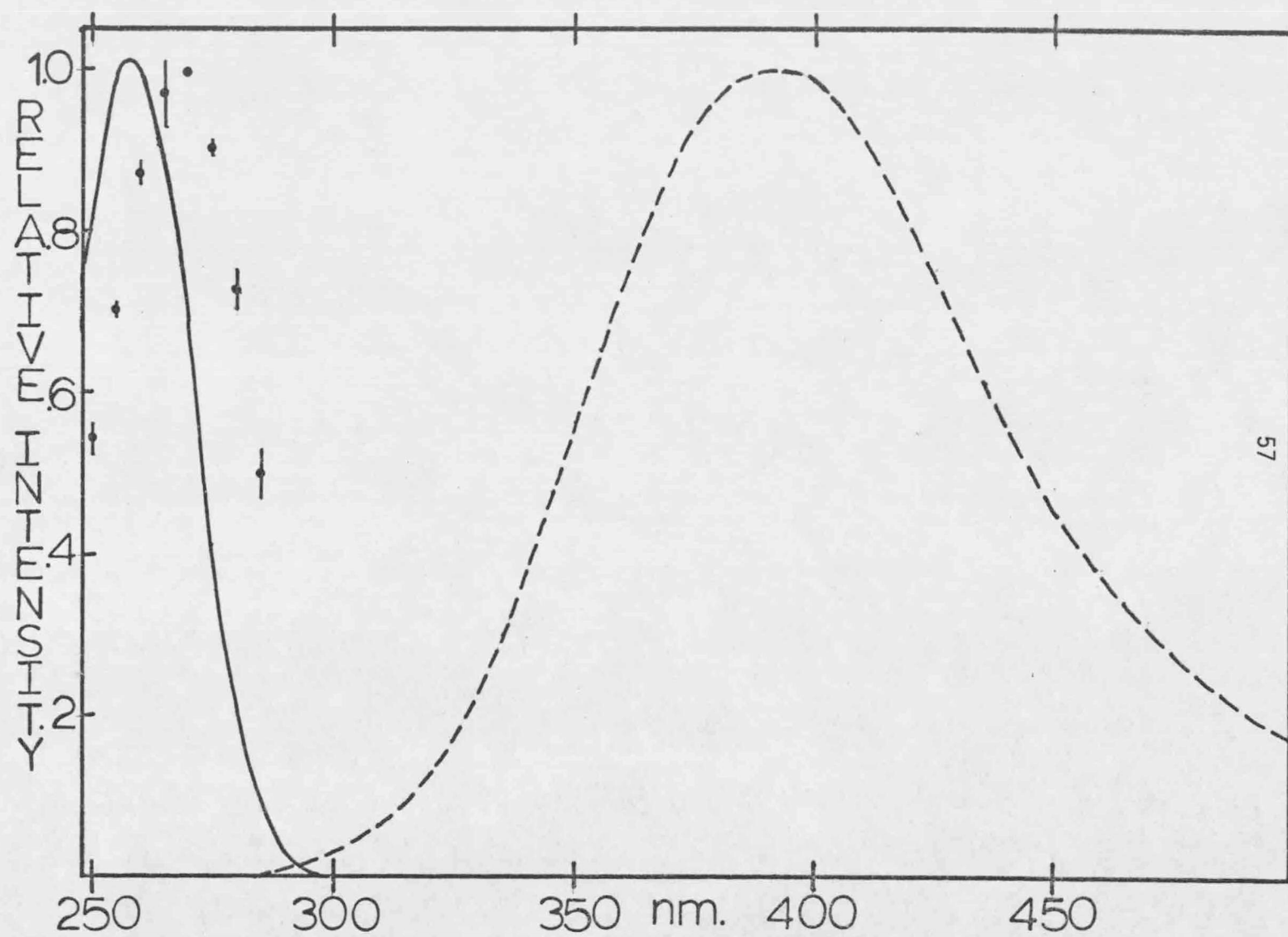


Figure 18

Absorption (—), fluorescence (-----), and corrected
fluorescence excitation (filled circles) spectra for
⁶N - methyladenosine in pH 6.7 aqueous solution.

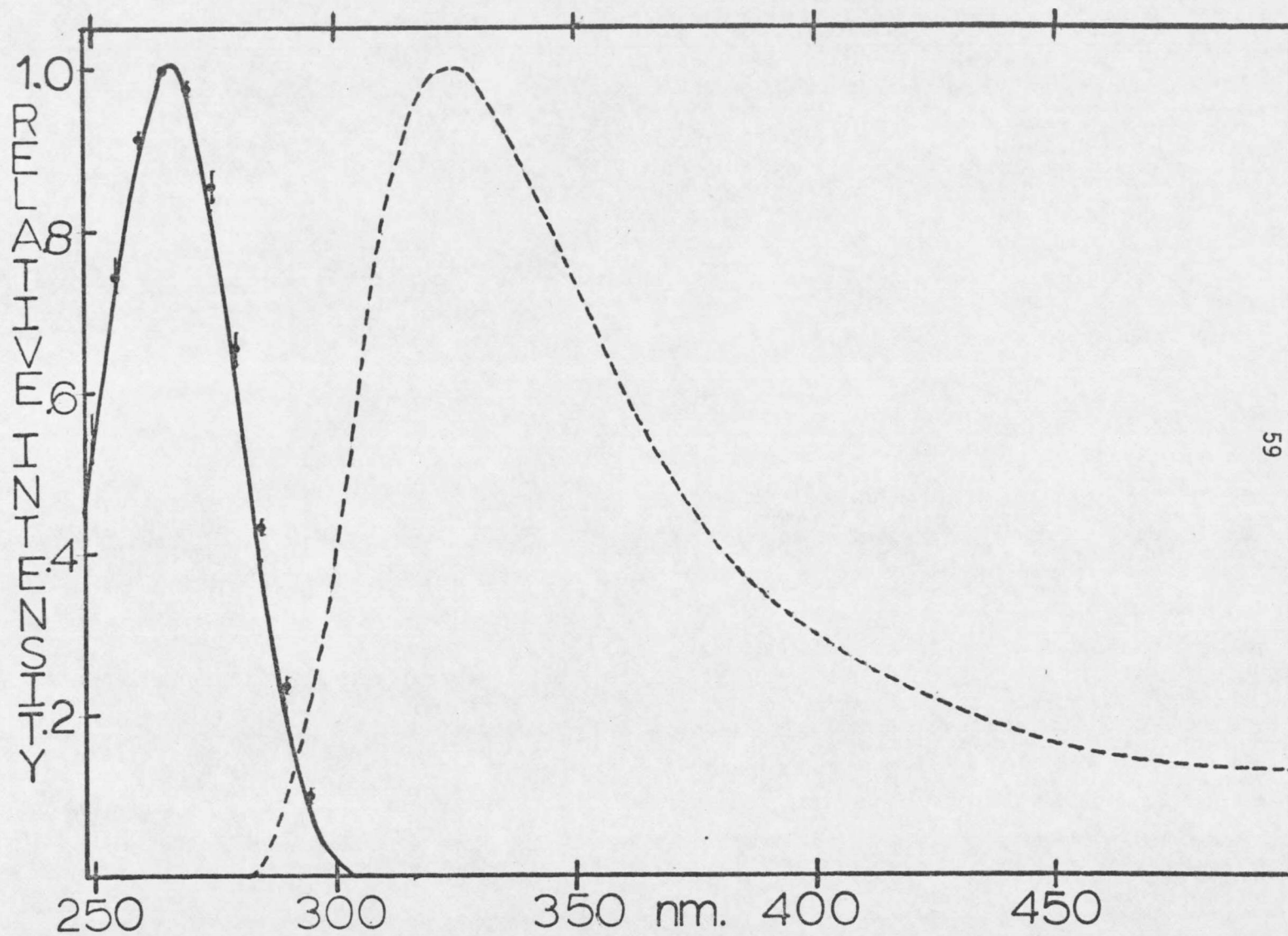


Figure 19

Absorption (—), fluorescence (-----), and corrected
fluorescence excitation (filled circles) spectra for
 N,N -dimethyladenosine in pH 6.7 aqueous solution.

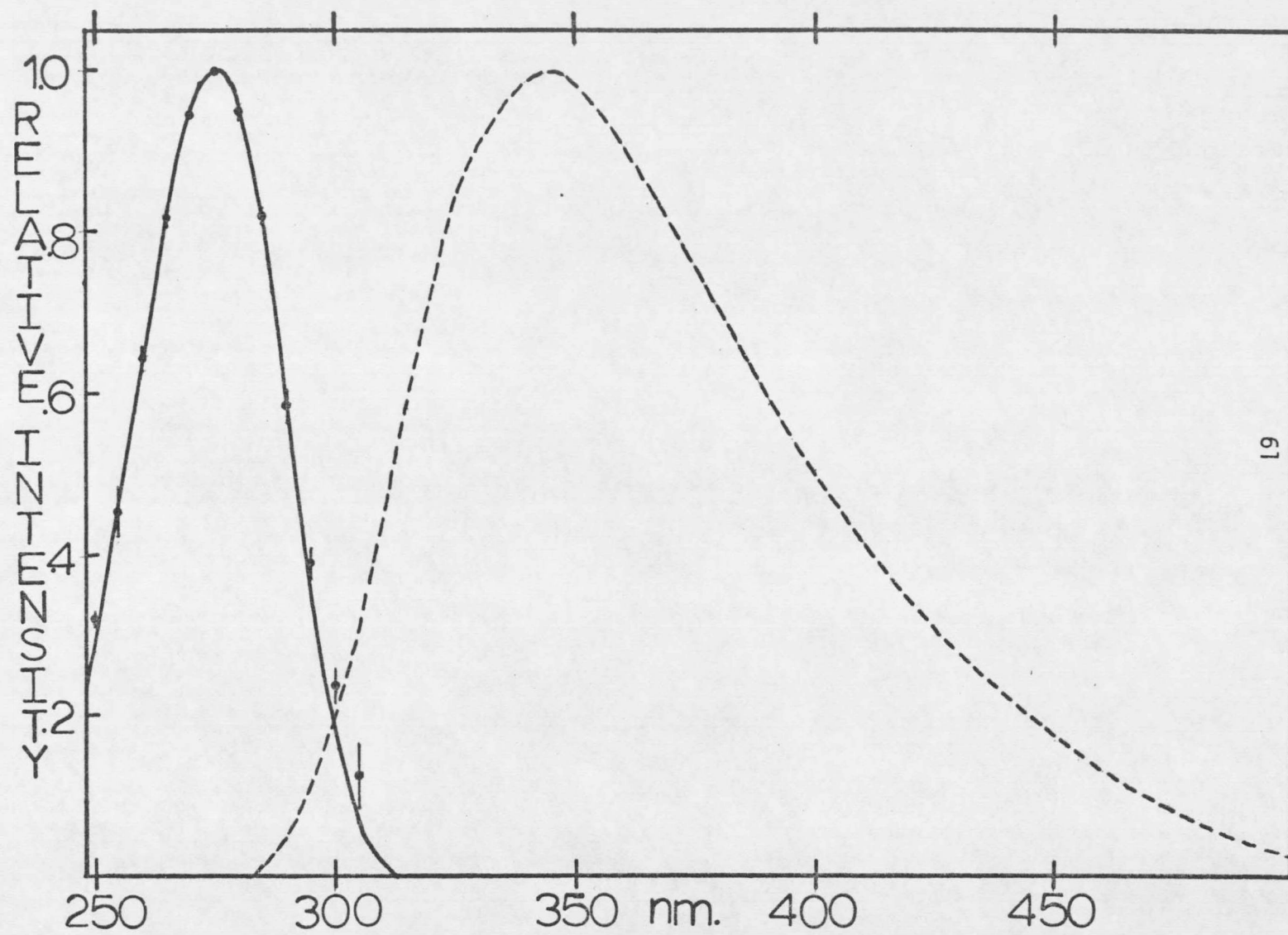


Figure 20

Absorption (—), fluorescence (-----), and corrected
fluorescence excitation (filled circles) spectra for
 N_6N_6 - dimethyladenosine in pH 1.5 aqueous solution.

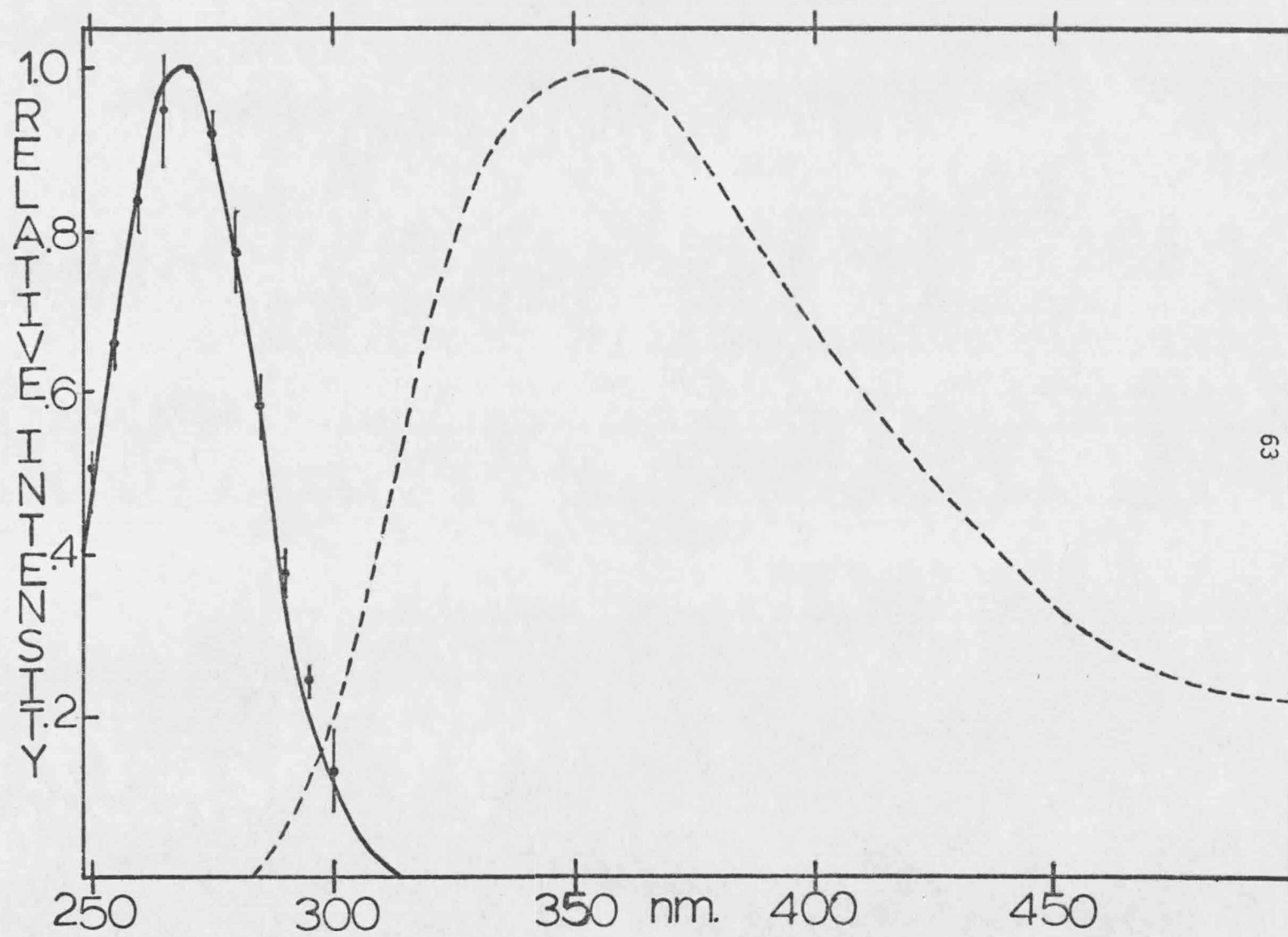


Figure 21

Absorption (—), fluorescence (-----), and corrected
fluorescence excitation (filled circles) spectra for
3 - methylcytosine in pH 4.4 aqueous solution.

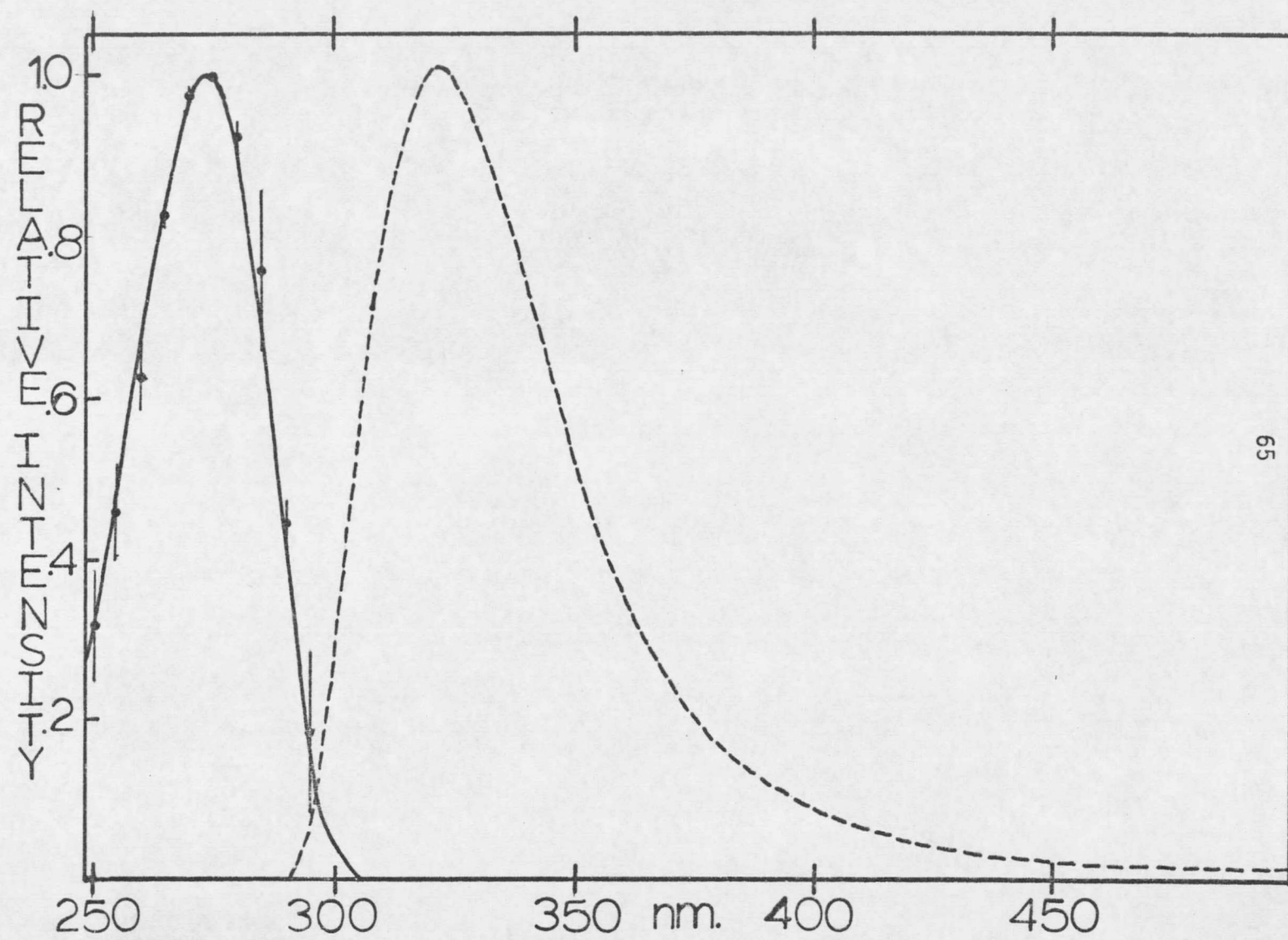


Figure 22

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for 5 - hydroxymethylcytosine in pH 6.7 aqueous solution.

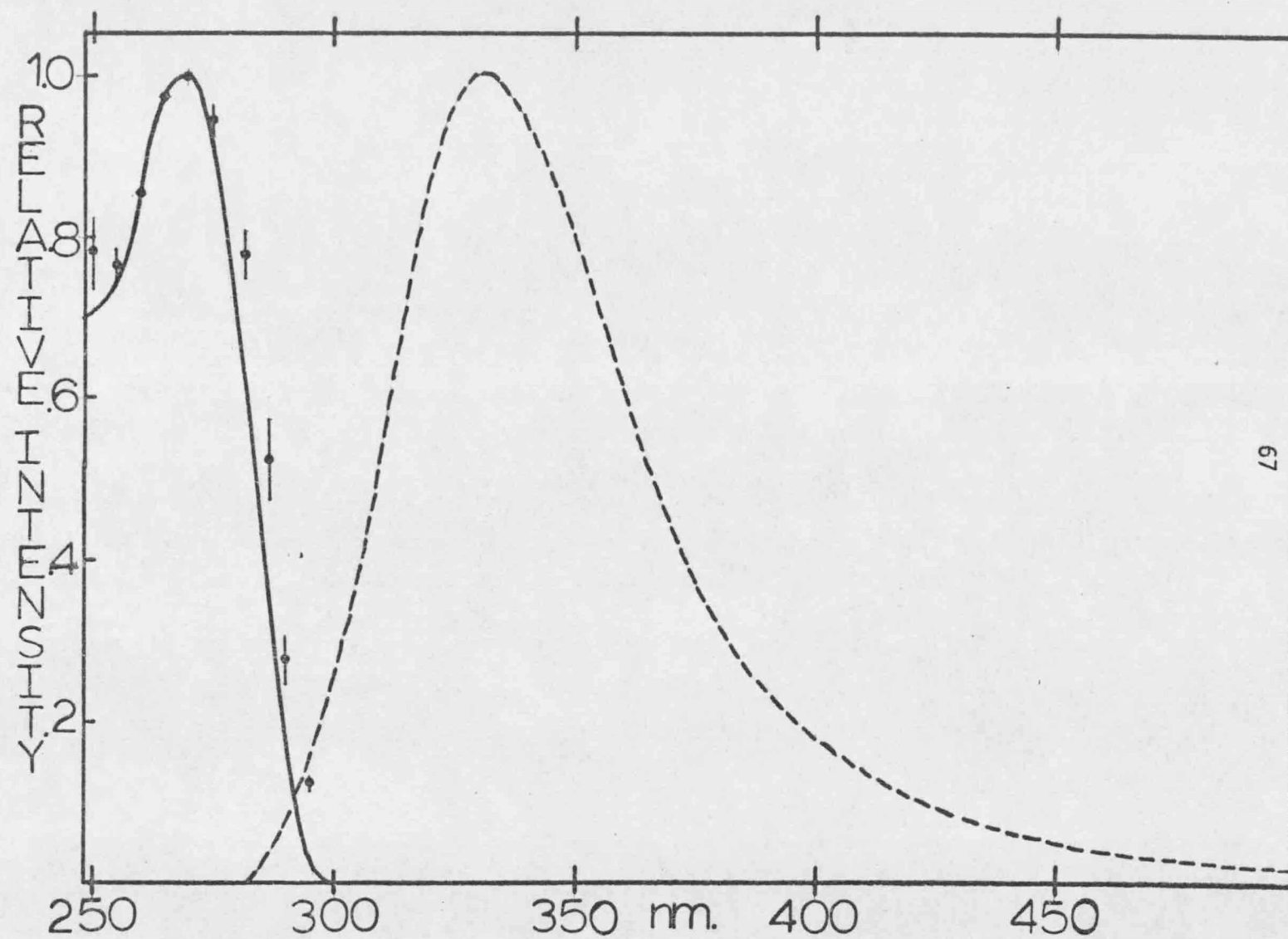


Figure 23

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for 5 - hydroxymethylcytosine in pH 1.5 aqueous solution.

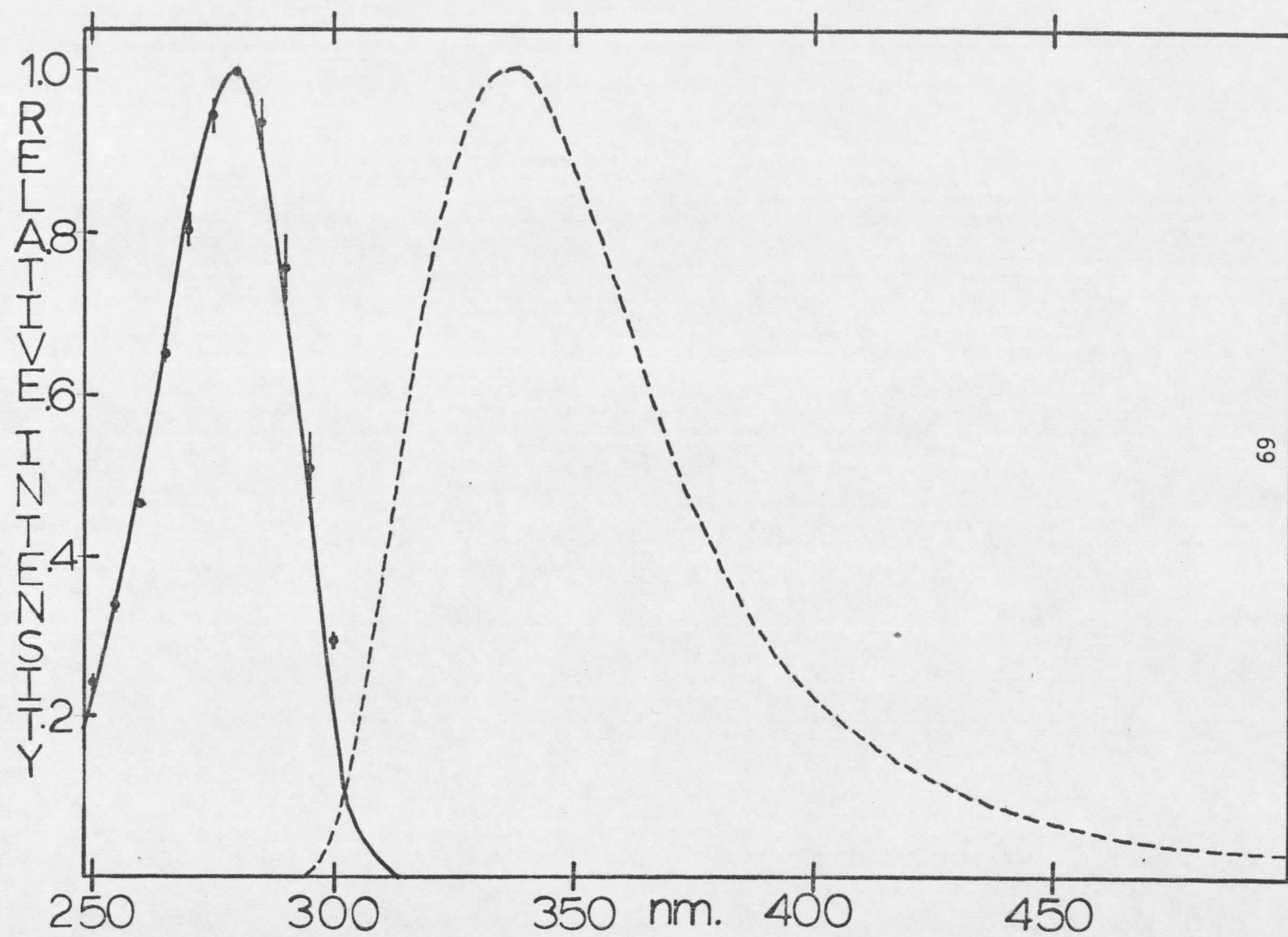


Figure 24

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for cytidine in pH 6.7 aqueous solution.

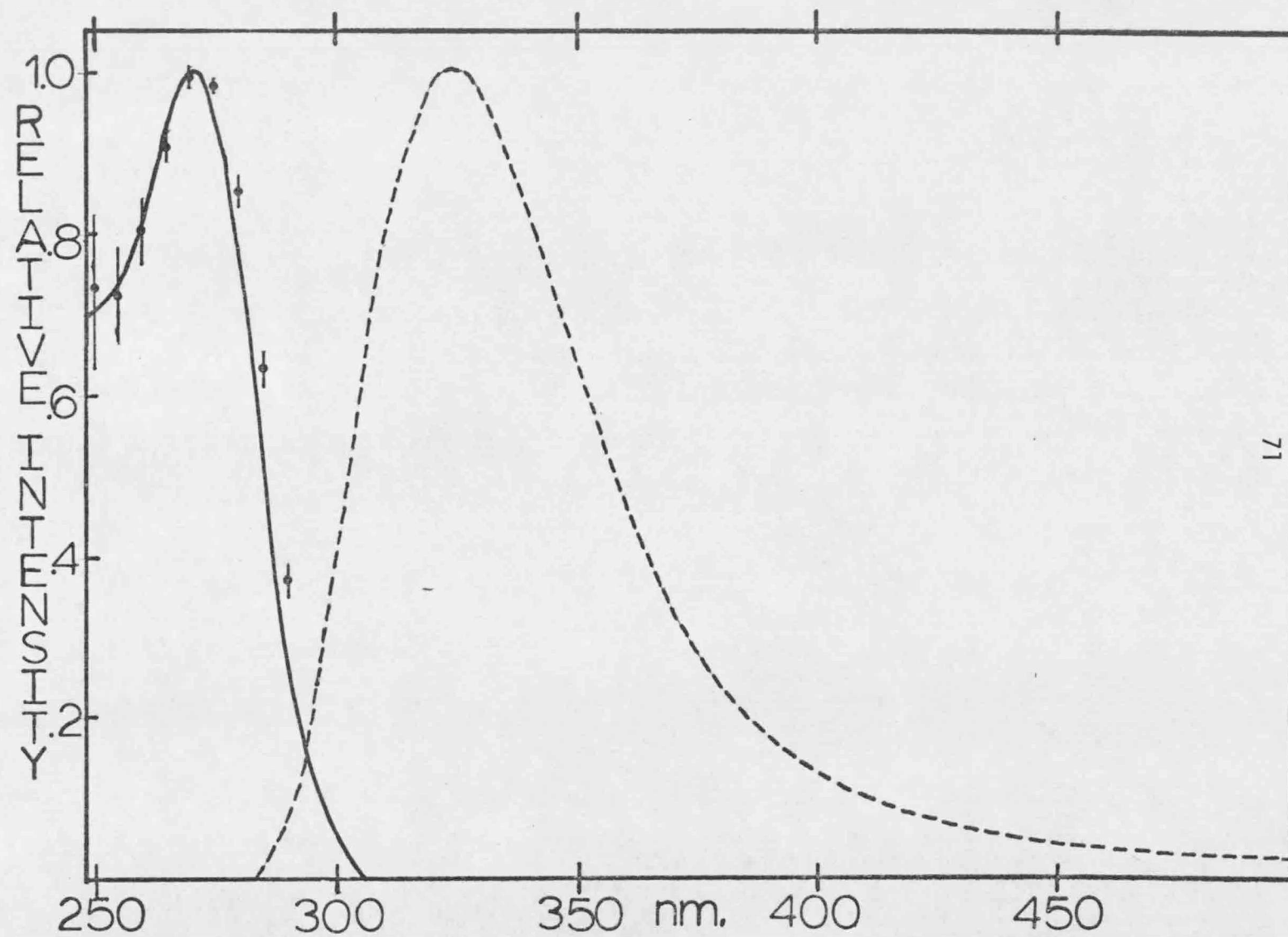


Figure 25

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for cytidine in pH 1.5 aqueous solution.

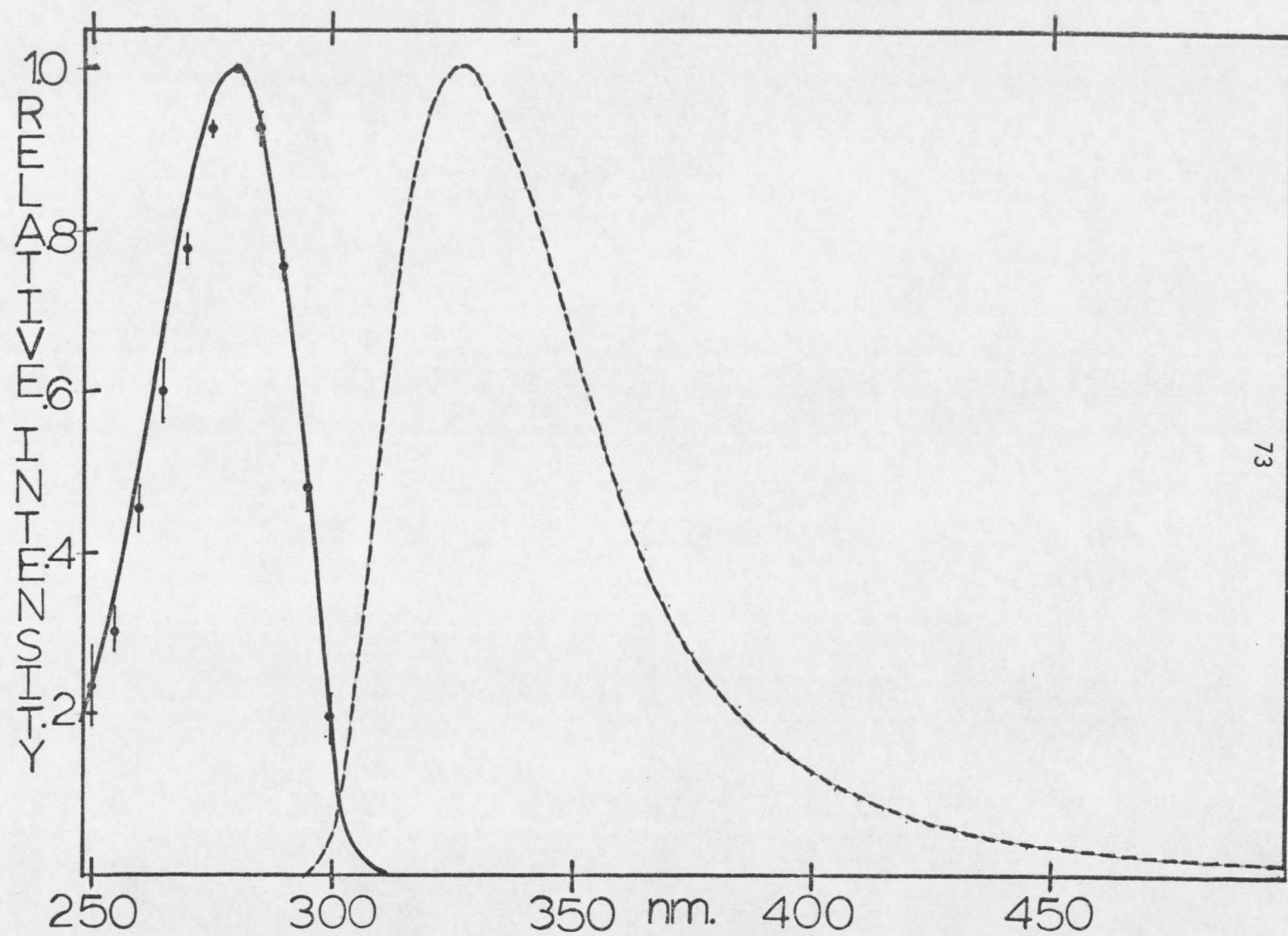


Figure 26

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for cytidine 5'-monophosphate in pH 6.7 aqueous solution.

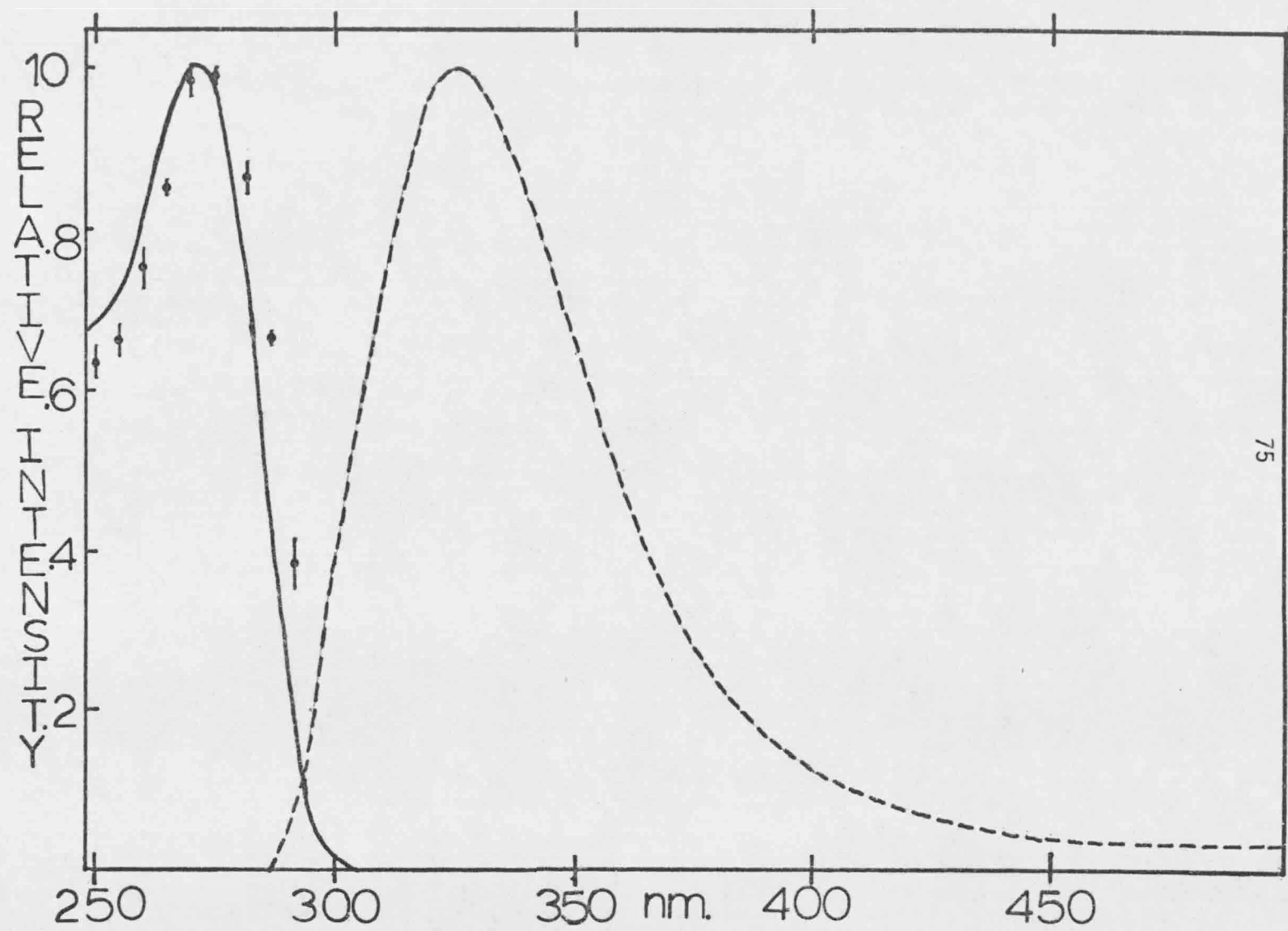


Figure 27

Absorption (—), fluorescence (-----), and corrected
fluorescence excitation (filled circles) spectra for
7 - methylguanine in pH 6.7 aqueous solution.

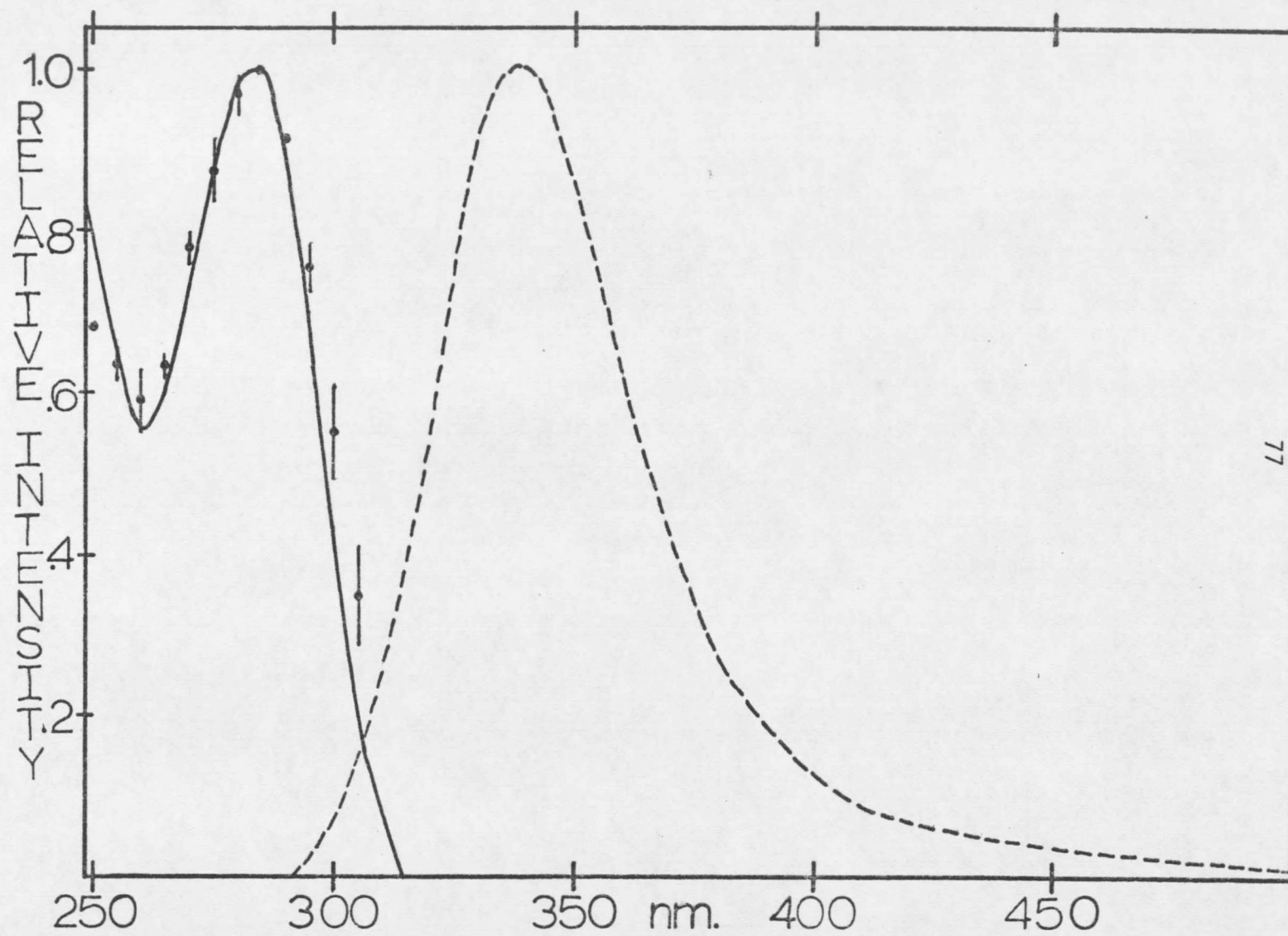


Figure 28

Absorption (—), fluorescence (-----), and corrected
fluorescence excitation (filled circles) spectra for
7 - methylguanosine in pH 1.5 aqueous solution.

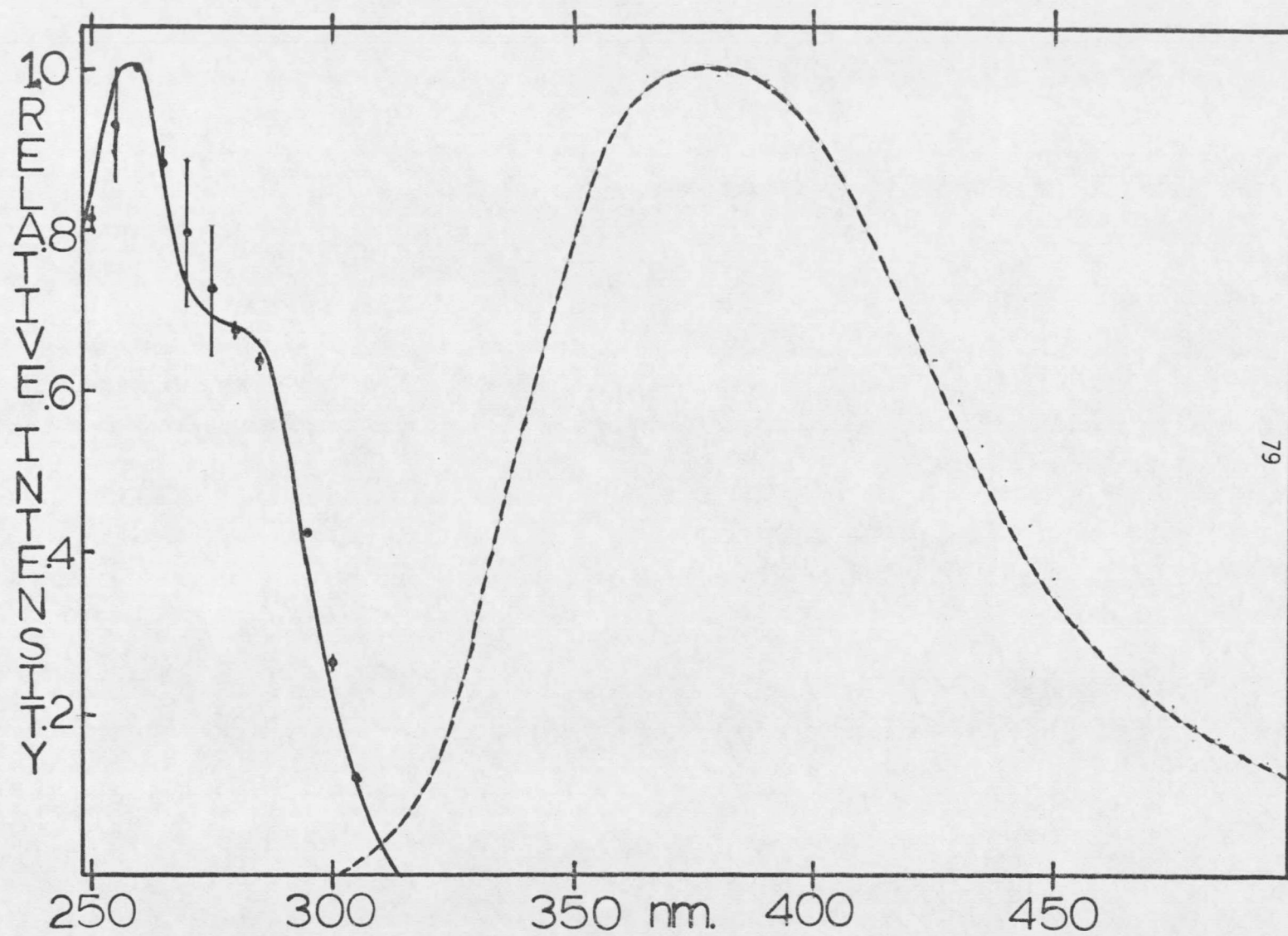


Figure 29

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for thymine in pH 6.7 aqueous solution.

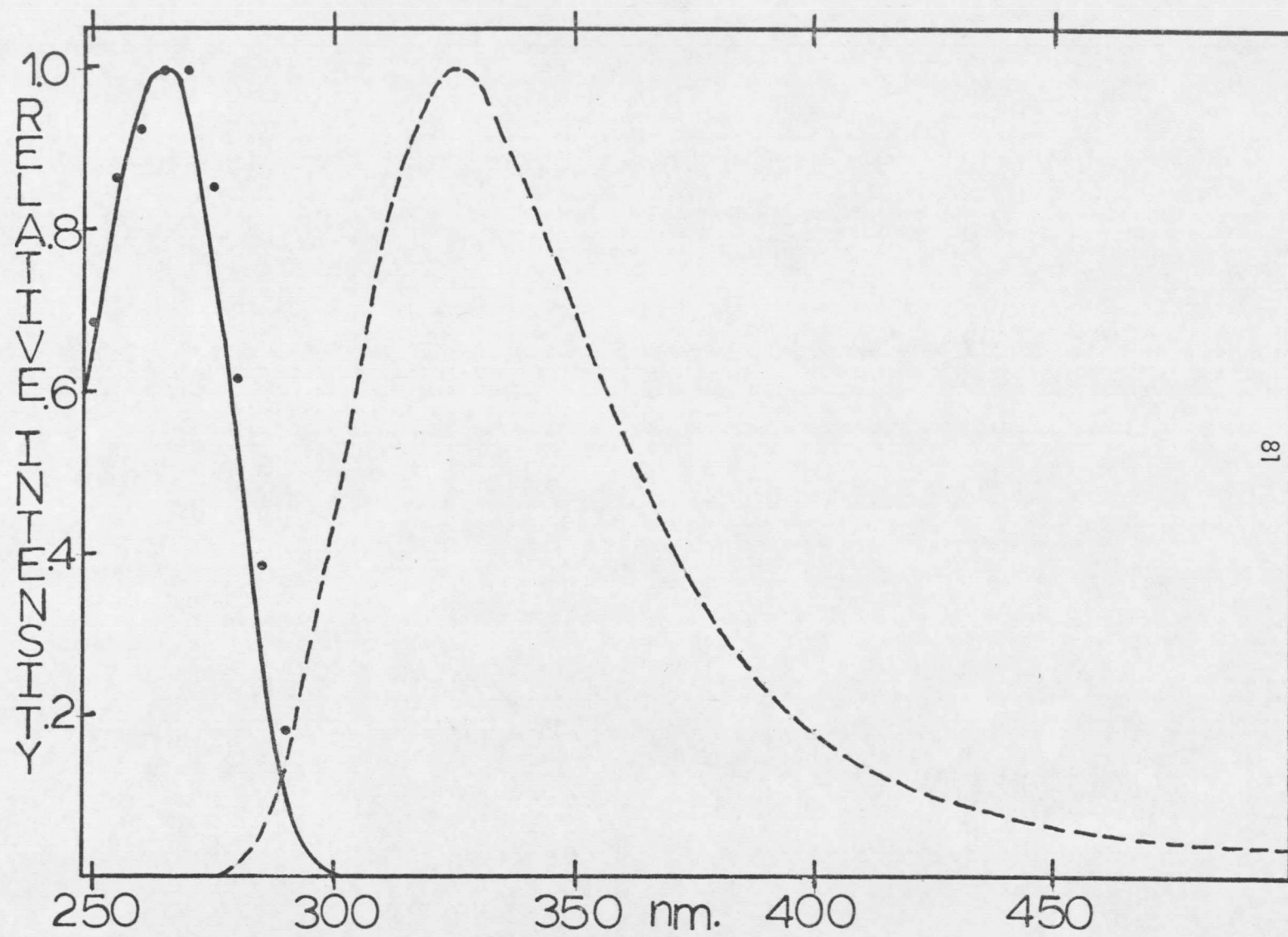


Figure 30

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for thymidine in pH 6.7 aqueous solution.

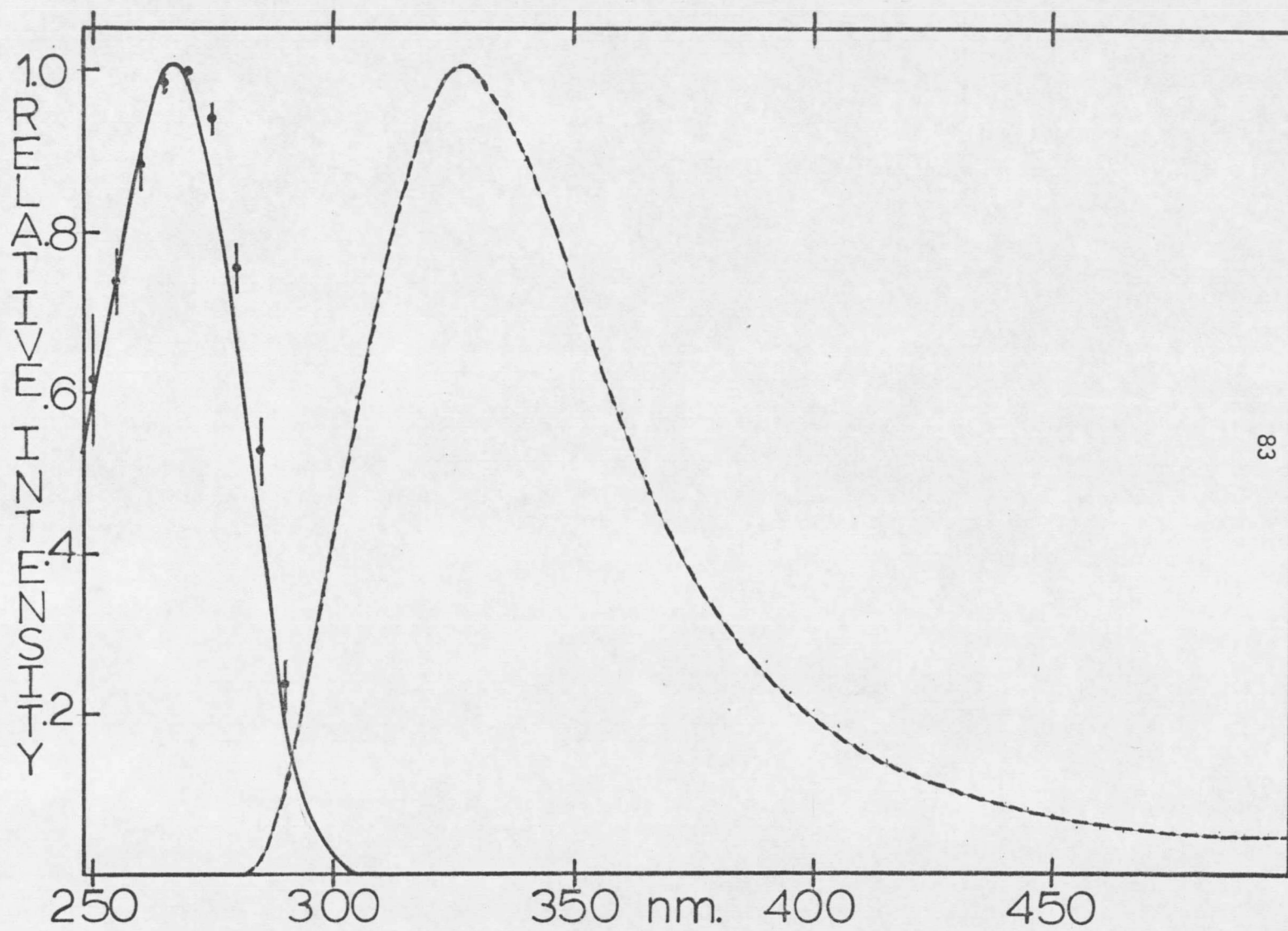


Figure 31

Absorption (—), fluorescence (-----), and corrected fluorescence excitation (filled circles) spectra for thymidine 5' - monophosphate in pH 6.7 aqueous solution.

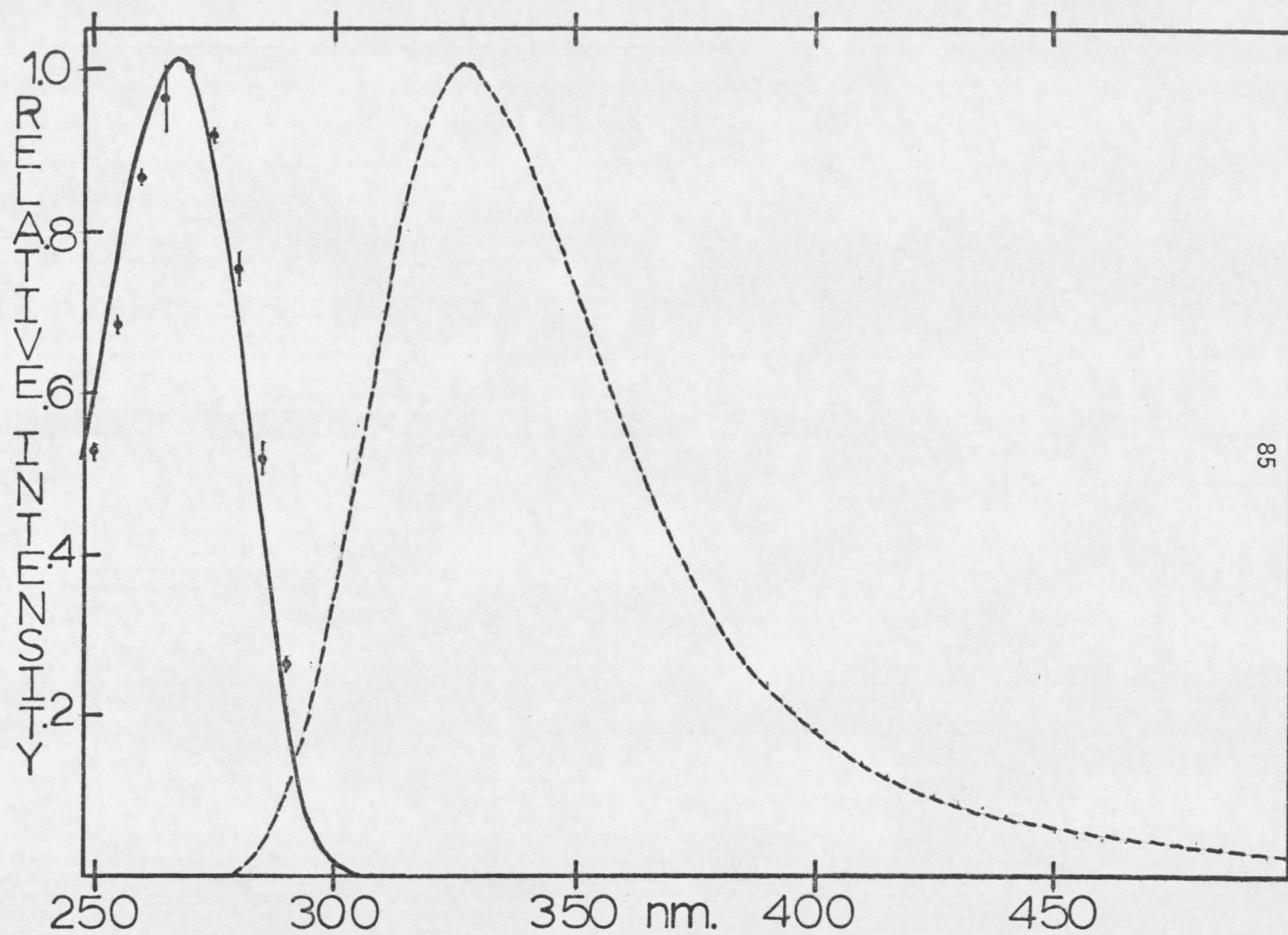


Table 3

Nucliec Acid Component	$\phi_f \times 10^4$	Coincidence of Excitation Spectrum with Absorbance Spectrum	Approximate Excitation Spectrum Maximum
Ade	$3.32 \pm .35$	No	265 nm
7-MeAde	$8.2 \pm .24$	Yes	270 nm
6-MeAde	$.7 \pm .02$	No	270 nm
6-Me ₂ Ade	$.99 \pm .14$	Yes	274 nm
Ado	$.49 \pm .02$	Yes	260 nm
6-MeAdo	$.91 \pm .06$	Yes	266 nm
6-Me ₂ Ado	$1.51 \pm .01$	Yes	275 nm
Ade ⁺	$20.7 \pm .4$	No	265 nm
2-MeAde ⁺	$9.9 \pm .1$	No	265 nm
3-MeAde ⁺	$1.17 \pm .03$	No	275 nm
7-MeAde ⁺	$11.9 \pm .1$	No	268 nm
6-MeAde ⁺	$1.2 \pm .04$	No	275 nm
6-Me ₂ Ade ⁺	$.47 \pm .09$	No	280 nm
Ado ⁺	$7.7 \pm .1$	No	270 nm
6-Me ₂ Ado ⁺	$.4 \pm .02$	Yes	270 nm

great variation observed among the cations. Although no data appears for adenines substituted at one position, a cursory check was performed on these compounds. The cations of both 1-methyladenine and 1-methyladenosine were found to be very weakly fluorescent, having quantum yields much lower than ca. 10^{-4} . Borresen (31), however, has reported the cation of 1-methyladenosine to be highly fluorescent. He was not able to document the authenticity of the fluorescence of this compound. This is the only explanation for this non-systematic discrepancy, as the quantum yields reported within for the other protonated adenines are consistent with those reported here.

DISCUSSION

Analysis of the excited state behavior of the nucleic acid components relies on the ability of Strickler and Berg's method to adequately predict the actual fluorescence lifetimes. Assuming this method is valid, fluorescence lifetimes determined using the Strickler-Berg method are taken to be adequate representations of the actual fluorescence lifetimes of the nucleic acid components when these molecules adhere to the following criteria:

- (1) The quantum yield is independent of excitation wavelength;
- (2) The emitting state carries the entire oscillator strength represented by the area contained within the first apparent absorption band.

The adherence of the nucleic acid components to the above criteria can be assessed on the basis of their corrected fluorescence excitation spectra and the results of photoselection studies.

Fluorescence excitation spectra. The corrected fluorescence excitation spectra coincided with the absorption spectra of 17 of the 26 nucleic acid components studied. Previously, thymine was the only nucleic acid component which had been reported to obey Vavilov's law (4). The 9 nucleic acid components which exhibited deviations between their corrected fluorescence and absorption spectra all were adenines. Present theories lead to the conclusion that the deviations from Vavilov's law

are due to one of the following:

- (1) The wavelength dependence of the quantum yield is due to competition between internal conversion and vibrational relaxation; or
- (2) The fluorescence observed is not from the major absorbing species, but due to the presence of a highly fluorescent minor tautomer.

Adherence of any of the nucleic acid components to Vavilov's law discredits the possibility that the discrepancies observed can be attributed to competition between internal conversion and vibrational relaxation. Other evidence against this mechanism is the result of the cation of 7-methyladenine, in which the corrected fluorescence excitation spectrum is blue shifted, relative to the absorption spectrum. This would indicate that the quantum yield increases with increasing excitation energy above the 0 - 0 band, which is direct contradiction to this theory. The above evidence indicates that deviations from Vavilov's law, by the nucleic acid components in room temperature aqueous solution, are not due to competition between nonradiative deactivation processes.

Deviations between the corrected fluorescence excitation and absorption spectra are attributed to the presence of a highly fluorescent minor tautomer. This explanation has been cited many times to explain the violation of Vavilov's law and sometimes indiscriminately (2, 3, 12, 17, 31). Caution must be used in invoking this argument, that the dis-

crepancies observed are real and not due to improper interpretation of the data. The tautomer model appears very general; however, it is rather stringent. If the fluorescence is due to a minor tautomer, it must adhere to the following criteria:

- (1) The minor tautomer must have pK values similar to the main tautomer; and
- (2) The minor must be present in substantial quantity because of the extreme temperature dependence of the quantum yield (16); and
- (3) The polarization measured should be somewhat depolarized, owing to the longer lived excited state of the minor tautomer.

The purines studied here, which violate Vavilov's law, are consistent with this model. Protonated adenine, 7-methyladenine and adenosine exhibited totally depolarized fluorescence spectra. The apparent quantum yields for these compounds are not large enough to account for this degree of depolarization. The remaining purines exhibited polarization ratios which are lower than would be predicted on the basis of their apparent quantum yields and radiative lifetimes. None of the pyrimidines studied here showed any deviations from Vavilov's law. This generates speculation concerning the validity of Daniels and Hauswirth (2) result that the fluorescence observed for cytosine was due to the presence of a highly fluorescent minor tautomer. Kinetic studies show that cytosine

only tautomerizes to an extent of .25% in room temperature aqueous solution. If the quantum yield reported for cytosine is only apparent, then the quantum yield reported of the tautomer must be greater than .01. In the absence of some phenomenally long reorientational time, the fluorescence should be significantly depolarized. Callis (6), however, has shown cytosine exhibited highly polarized fluorescence. On this evidence, and the behavior observed for the other pyrimidines, cytosine would be predicted to obey Vavilov's law. Discussion here leads to the conclusion that the nucleic acid components usually adhere to Vavilov's law and deviations from this behavior can be attributed to the presence of a highly fluorescent minor tautomer.

Fluorescent lifetimes predicted from the Strickler-Berg formulas for the purines, which have deviations between their corrected fluorescence excitation and absorption spectra, are not representative of the actual fluorescence lifetimes. The τ_f^{SB} 's reported for these molecules are much too short, as their behavior is inconsistent with the criteria previously mentioned. The quantum yields not only changed across the absorption band, but the values reported represent the apparent quantum yield. The actual quantum yields are much higher. This is obvious from the fact that the minor tautomer only absorbs a small percentage of the exciting light, yet is responsible for nearly all of the emission. The radiative lifetimes are not representative, because the oscillator strength is calculated from the first apparent absorption band, which is the sum

of the absorbances of all the tautomeric species. The excited state lifetimes reported for adenine, N^6 -methyladenine, the cations of 3-methyladenine, N^6 -methyladenine, and N^6,N^6 -dimethyladenine are anomalously short and have no physical significance.

Photoselection. The validity of the predicted fluorescence lifetimes relies heavily on the results of photoselection studies. The Strickler-Berg method of determining the radiative lifetimes only remains valid if the first apparent absorption band contains one transition dipole. Under these conditions molecules exhibit polarization ratios which are independent of excitation wavelength. Mixed polarization (wavelength dependent polarization), however, indicates absorption into two states, where the lowest energy state is responsible for the emission. Radiative lifetimes determined for molecules which exhibit mixed polarization will be too short, as the total oscillator strength is represented by the area contained within the lowest energy transition. Wilson and Callis (8) have shown that AMP exhibits considerable mixed polarization. This mixed polarization has been attributed to the presence of a weakly allowed transition. This transition is lower in energy than the more intense allowed transition, and is responsible for the fluorescence. Adenine, more properly its fluorescent tautomer, does not exhibit any mixed polarization, so substitution of the 9 position of adenine leads to fluorescence from a weakly allowed transition. The actual lifetimes for adenosine, N^6 -methyladenosine, N^6,N^6 -dimethyladenosine and N^6,N^6 -dimethyladenine are considerably

larger than those reported here. N^6, N^6 -dimethyladenine is included because it is believed to have a hydrogen residing on the 9 nitrogen.

Hydrodynamic behavior. The remaining nucleic acid components, all the pyrimidines, and the 3 seven substituted purines exhibited behavior consistent with the criteria established from the Strickler-Berg formalism. The Strickler-Berg fluorescence lifetimes reported for these molecules are considered to be good representations of the actual fluorescence lifetimes. When τ_f^{SB} is equal to the actual τ_f , then the rotational correlation time calculated from the fluorescent lifetime and fluorescence anisotropy represents the actual rotational correlation time. Due to the structural similarity of the nucleic acid components, these molecules would be expected to exhibit similar hydrodynamic behavior. The validity of the τ_f^{SB} 's representation of the actual τ_f can be checked by the reasonability of the reorientational times calculated from the Perrin relation. The τ_c^{SB} 's reported in Table 1, for these molecules, are roughly equivalent, showing deviations of only about a factor of three. These deviations may be due to minor structural difference, and possibly different degrees of solvent interaction between the neutral and protonated species. These structural variations can be accounted for by comparing the τ_c^{SB} 's to the rotational correlation times determined from the Stokes-Einstein hydrodynamic formulas. The Stokes-Einstein formulas account for the differences in the volume and shape of the molecules. Figures 4 and 5 show plots of $\frac{R_0}{R} - 1$ versus $\tau_f^{SB} / \tau_c^{stick}$. The Perrin re-

lation shows that $\frac{R_0}{R} - 1$ is equal to the actual ratio of τ_f/τ_c . These figures are, therefore, actually graphs of τ_f/τ_c versus $\tau_{fSB}^c/\tau_c^{stick}$. Since τ_{fSB} is taken to be the actual τ_f , then the slope of the line yields how much larger τ_c^{stick} is than τ_c . Table 2 is a compilation of the ratios of $\tau_{fSB}^c/\tau_c^{stick}$ for all the molecules studied. The τ_{fSB}^c 's for the molecules, in which τ_{fSB}^c is taken to be the actual τ_f , range from $.1\tau_c^{stick}$ to $.7\tau_c^{stick}$. The nucleic acid components for which the τ_{fSB}^c 's were all shown to be too short, yield τ_{fSB}^c 's which are not reasonable, being up to 50 times faster than τ_c^{stick} . Table 2 shows that, on the average, the cations exhibit longer reorientational times than the neutrals. The cations, being a charged species, probably experience more solvent interaction. The hydrodynamic behavior reported for these molecules appears reasonable and is in excellent agreement to the hydrodynamic behavior reported by Bauer et al. (13) for the carboxylic acids in water. The evidence provided, either actual or consequential, lends validity to the adequate representation of τ_{fSB}^c for the actual τ_f . That is, that some of the nucleic acid components may, indeed, have fluorescence lifetimes as short as one picosecond.

Significance of fluorescence lifetimes. Existence of fluorescence lifetimes, on the order of one picosecond, is of interest from a chemical physical standpoint, as lifetimes this short confirm the presence of exceedingly fast nonradiative processes. A lifetime of 10^{-12} seconds infers a rate of at least $10^{12}s^{-1}$ for the rate of internal conversion.

As this work found Vavilov's law to be obeyed in the absence of tautomers, the rate of vibrational relaxation must be at least 10^{13} s^{-1} for vibrational relaxation (32). The results of fluorescence lifetimes this short are of considerable interest to photobiologists. If the behavior of DNA is taken to be similar to that of the individual nucleic acid components, the possibility of singlet energy transfer in DNA is improbable (2). Under the most favorable conditions, the energy transfer rate is estimated to be ca. 10^{12} s^{-1} (33).

Fluorescent tautomers of adenine. Study of the nucleic acid components is undertaken in attempt to better understand the behavior of DNA itself. Much of this study has alluded to a highly fluorescent minor tautomeric species in the adenines. It is of great interest to know what tautomeric species are responsible for the fluorescence properties observed. These tautomers have much longer fluorescent lifetimes than the major nonfluorescent component. So, if the structure of these tautomers is compatible with the structural possibilities of the adenine moiety in DNA, the consequences could be profound. These longer lived excited species could contribute greatly to photochemical reactions. Therefore, knowledge of the structure of the fluorescence tautomer is essential.

Spectroscopic studies often utilize methylated derivatives to characterize systems. Substitution of a hydrogen by a methyl group, typically, has less than an order of magnitude effect on radiative and non-radiative properties of planar conjugated molecules (14, 34). Generally

speaking, one observes an approximate shift of 5 nanometers in absorption and fluorescence maxima, with the substitution of a hydrogen by a methyl.

Table 3 shows that adenine and N⁶-methyladenine are the only neutral adenines which exhibit deviations between their corrected fluorescence excitation and absorption spectra. Previous discussion concludes these deviations are due to the presence of a minor fluorescent tautomer. Temperature jump kinetic studies indicate that in neutral aqueous solution the 9(H) and 7(H) tautomers are the only tautomeric species present in significant quantities (18). The fluorescent properties observed for adenine and N⁶-methyladenine should be compared to adenosine and 7-methyladenine, which are the substituted derivatives which represent the 9(H) and 7(H) tautomers, respectively. The apparent quantum yields of adenine and N⁶-methyladenine are both greater than the quantum yields for adenosine. The fluorescence excitation spectra of adenine and N⁶-methyladenine coincide well with the fluorescence excitation spectrum of 7-methyladenine, in both shape and position. The 5 nm shift between the fluorescence excitation maxima for adenine and 7-methyladenine is due to the substitution of a hydrogen by a methyl group. The results conclude that the 7(H) tautomer is responsible for the fluorescent properties observed for adenine and N⁶-methyladenine. This conclusion is in harmony with other studies which proposed the 7(H) tautomer is the fluorescent species, as the 9(H) tautomer is relatively nonfluorescent (12, 17, 35). Dimethylation of the extranuclear nitrogen does not show the presence of any tautomer-

ization. The fluorescence spectrum is seen to be very similar to its 9 substituted analog N^6,N^6 -dimethyladenosine. Apparently, the presence of the two methyls on the extranuclear nitrogen effectively block substitution of the 7 position. This explains the similarity between N^6,N^6 -dimethyladenine and N^6,N^6 -dimethyladenosine.

Characterization of the cationic adenine system is considerably more difficult than the neutrals. Protonation leads to many more tautomeric possibilities, since the adenine moiety can have a hydrogen on either the 7 or 9 position before protonation, and then can protonate on any of the other four remaining nitrogens. Borresen (31) studied this system some time ago and concluded that fluorescent species had three hydrogens on the exocyclic nitrogen and one on the 7 position. Unfortunately, Borresen's argument is based only on the results of adenine and 7-methyladenine and does not account for the results observed for adenosine.

Giskaas (36) has determined the fluorescent lifetime of protonated adenosine, at room temperature, from Stern-Volmer quenching experiments to be 1×10^{-9} s. The fluorescence excitations spectra of protonated adenosine does not coincide with its absorption spectrum, so this fluorescence lifetime corresponds to the tautomer responsible for the fluorescence observed. Assuming a radiative lifetime of ca. 5×10^{-9} s, yields a quantum yield of ca. .2 for this tautomer. This indicates that this tautomer needs to be present in only small amounts to account for the apparent quantum yields reported for the protonated adenines.

Figure 32 shows all the possible tautomer species of proto-

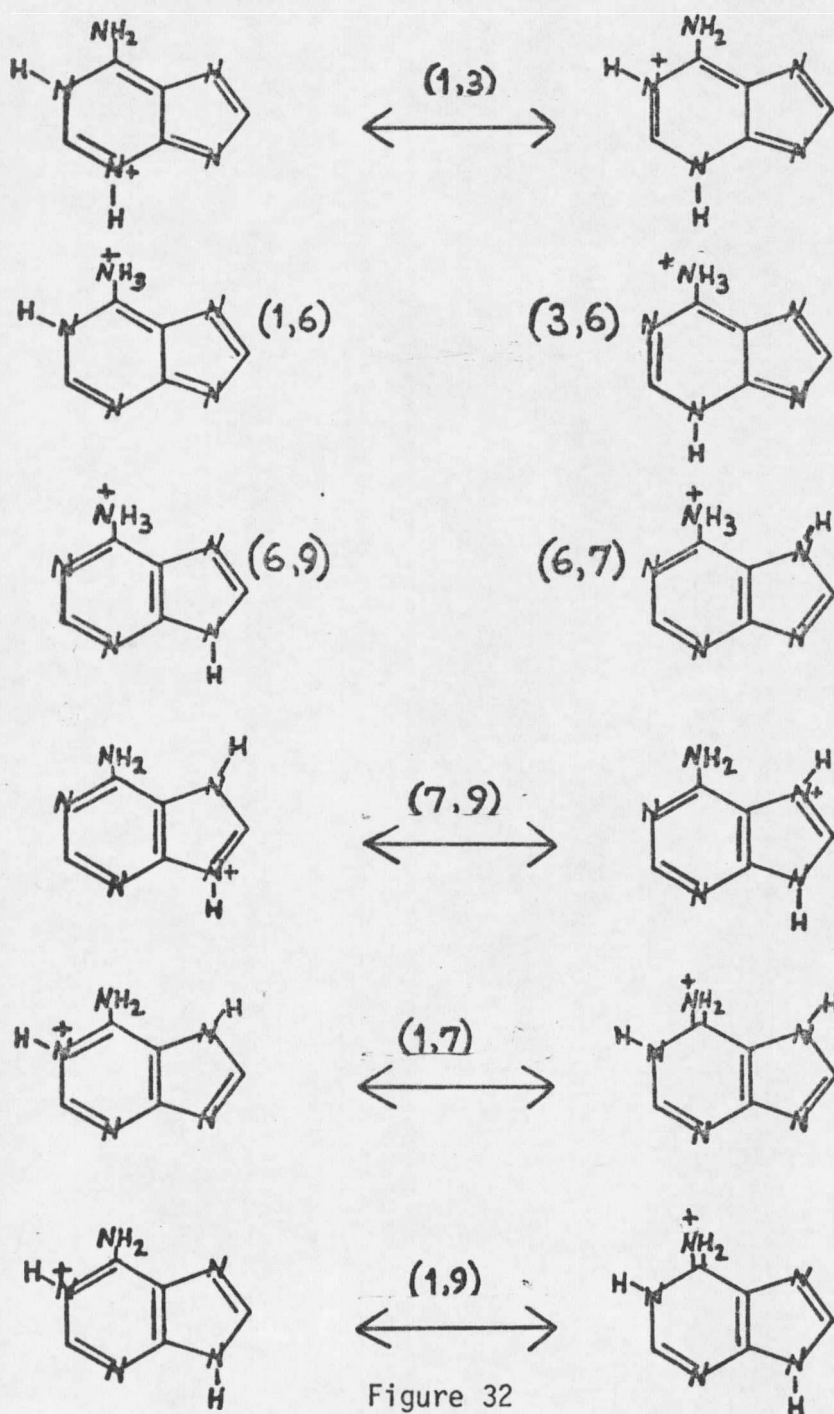


Figure 32

Possible tautomers of protonated adenine

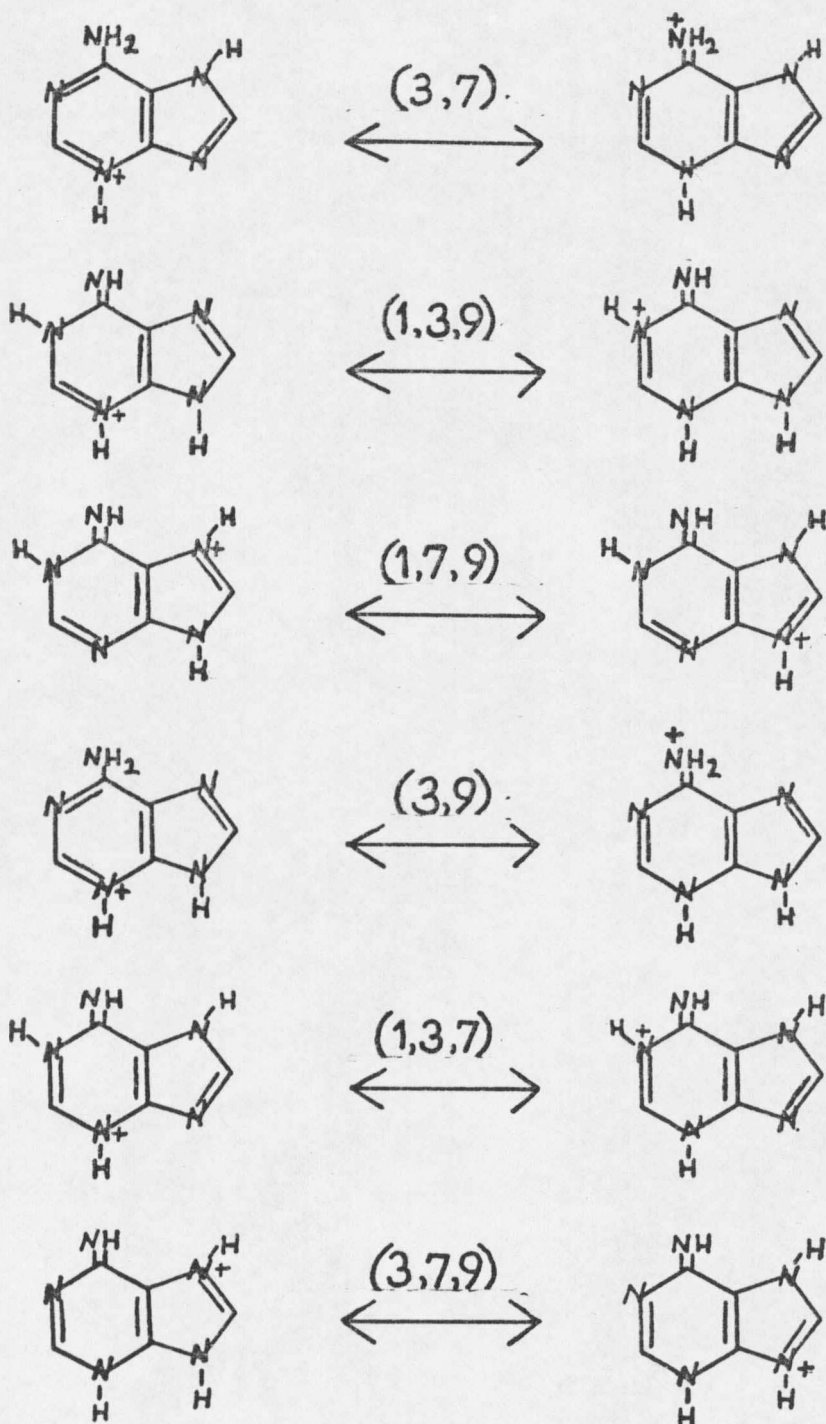


Figure 32 (continued)

nated adenine. It is not readily apparent which tautomeric structures are represented by the various methylated derivatives. All the methylated derivatives assume two or more of the structures shown. Since this is not an exhaustive study, some of the tautomers do not have corresponding substituted derivatives. Because of this, it is more useful to eliminate those structures which can not be responsible for the fluorescent properties observed. Both 1-methyladenine and 1-methyladenosine were found to be nonfluorescent having quantum yields less than 5×10^{-5} . 1-Methyladenosine is represented by the structure labeled (1,9) so this structure can be eliminated as a possible fluorescent tautomer. 1-Methyladenine has the possibility of existing as the structure labeled (1,3), (1,7) and (1,6) so these structures can be excluded as fluorescent tautomers. The exocyclic nitrogen can also exist in the imino form, so the 1-methyladenines could also exist as the structures labeled (1,3,7), (1,3,9) and (1,7,9). 3-Methyladenine is somewhat more fluorescent than the corresponding one substituted adenine. The fluorescence yield is still fairly low so that substitution of the 3 position leads to a less fluorescent species. This eliminates the tautomers labeled (3,7), (3,9), (3,6) and (3,7,9). The only structures which remain are substituted at both the 7 and 9 positions, or involved the exocyclic nitrogen. Substitution of the exocyclic nitrogen deactivated the fluorescence, which indicates this nitrogen may be involved in the fluorescent tautomer. Dimethylation of

the exocyclic nitrogen fixes the nitrogen in the amino form. The low fluorescence yields measured for these compounds appear to indicate that the exocyclic nitrogen may assume the imino form in the fluorescent tautomer. If the exocyclic nitrogen is in the imino form, then either the 1 or 3 position must be substituted. However, both 1-methyladenine and 3-methyladenine had very low quantum yields. Methylation of this exocyclic nitrogen increases the basicity of this group. It may be that this increase in basicity causes this nitrogen to become preferentially protonated over any of the other sites. This indicates that the extranuclear nitrogen is not involved in the fluorescent tautomer. It appears that if only one tautomer is responsible for the fluorescence properties of protonated adenine, it must be substituted at both the 7 and 9 position to be consistent with the results of the 7-methyladenine and adenosine. Further justification for this structure is borne out by the results reported for protonated 7-methylguanosine, which is the guanine analog of the tautomer. As shown in Table 1, the cation of 7-methylguanosine has a fairly high quantum efficiency, although not as high as that predicted for this tautomer. Although most of the evidence is consequential, it is the belief of this author that the fluorescent tautomer responsible for the fluorescence properties observed for protonated adenine has a proton residing on both the 7 and 9 position.

SUMMARY

The corrected fluorescence excitation spectra coincided with the absorption spectra for 10 pyrimidines and 7 purines of the 26 pyrimidines and purines studied. The 9 nucleic acid components which exhibited deviations between their corrected fluorescence excitation and absorption spectra were all adenines. These discrepancies, between the two spectra observed for the adenines, were attributed to the presence of a highly fluorescent minor tautomer. Fluorescence excitation spectra of the methylated adenine derivatives were utilized to identify the fluorescent tautomers of adenine, in both neutral and acid solutions. The fluorescent properties observed for adenine in neutral solution were consistent with the previously proposed 7(H) tautomer model. The protonated adenines studied indicated the fluorescence was probably dominated by a tautomer which has protons residing on both the 7 and 9 positions.

The combined results of the corrected fluorescence excitation spectra and previous photoselection studies, for the first time, gave some physical basis to the validity of fluorescence lifetimes predicted from the intrinsic fluorescence lifetimes and quantum yields for the nucleic acid components. The predicted fluorescence lifetimes of the purines and pyrimidines were found to be very short, on the order of 10^{-11} - 10^{-12} s, except for the anion of 5-methylcytosine and the cation of 7-methylguanosine. These predicted fluorescence lifetimes were taken

to be good representations of the actual fluorescence lifetimes of all the pyrimidines and the 3 purines substituted at the 7 position. The predicted lifetimes of these molecules, in conjunction with fluorescent anisotropy measurements, yielded reorientational times consistent with the results of other small molecules in aqueous solution. The neutral pyrimidines and purines appeared, on the average, to rotate four times faster than predicted from the Stokes-Einstein model. The cationic species appeared to experience more solvent interaction, having rates only twice as fast.

APPENDIX A

Determination of $\tau_{c\text{stick}}$.

As previously shown, the rotational correlation time for an oblate ellipsoid, for which the absorption is in plane polarized, is expressed as follows:

$$\tau_c = \frac{1}{4}\tau_1 + \frac{3}{4}\tau_3$$

$$\text{where } \tau_1 = 1/6 D_x ; \tau_3 = \frac{1}{4D_x + 2D_z} ; D_k = \frac{kT}{\xi_k}$$

where D_k is the principal diffusion coefficient for rotation about the k^{th} axis and ξ_k is the frictional coefficient for rotation about the corresponding axis.

The frictional coefficient for a hydrodynamically spherical molecule is given as follows:

$$\xi_{\text{ostick}} = 6\eta V$$

where η is the viscosity, was found to be 1.0 centipoise at 293° K (37), and V is the molecular volume which was determined using Van der Waals increments (38). Perrin's (39, 40) formulas enable ξ_{stick} to be found by multiplying ξ_{ostick} by a factor which relates an oblate ellipsoid to a sphere of equal volume, and depends on the axial ratios of the ellipsoid (41). The axial ratio in the xy plane was determined using the geometric mean of an ellipsoid and the thickness in the z direction was taken as 3.4 Å. The geometric mean was determined as follows:

$$V = 4/3 \pi abc \text{ (volume of an ellipsoid)}$$

$$bc = \frac{3v}{4\pi a}$$

$$bc = \text{geometric mean}$$

$\epsilon_k^{\text{stick}}$ were determined using the axial ratio defined as $\frac{a}{bc}$

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