To the Graduate Council:

I am submitting herewith a thesis written by Abulgasim M. Saim entitled "Room-Temperature Optical Properties of Methylated Calf Thymus DNA." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Radiation Biology.

Solon Georghiou, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

ROOM-TEMPERATURE OPTICAL PROPERTIES OF METHYLATED CALF THYMUS DNA

A Thesis

Presented for the

Master of Science

Degree

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Abulgasim M. Saim

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ABSTRACT

In the present study the room-temperature optical properties of calf thymus DNA, methylated at N-7 of the guanine residue, were studied and compared with those of the free fluorophore, 7-methyl GMP. It has been found that the fluorescence spectrum of methylated DNA depends strongly on the excitation wavelength, shifting to the blue as the excitation wavelength increases. The degree of fluorescence polarization exhibits a weak dependence on the excitation and emission wavelengths. The curve for the fluorescence quantum yield, q, corrected for absorbance by the A, C, and T residues, peaks at 270 nm and then drops for longer excitation wavelengths. For the excitation wavelength of 290 nm, q = 0.0017. The fluorescence decay time for that excitation wavelength has been found to be 0.11 ns.

For 7-methyl GMP at ph 5 (conditions under which the proton at N-1 is not dissociated) the fluorescence spectrum is virtually independent of the excitation wavelength. The degree of polarization exhibits a dependence on both the excitation and emission wavelengths: it increases with increasing excitation wavelength, particularly when monitoring the emission at the short wavelength region of the fluorescence spectrum, with the exception of 265 nm for which it drops dramatically. The fluorescence quantum yield, q, is weakly dependent on the excitation wavelength below 270 nm, peaks at 285 nm, drops at 300 nm and peaks again at 305 nm. For 290 nm, q = 0.024. The fluorescence decay time for that wavelength has been found to be 0.20 ns.

The observed differences between the optical properties of methylated DNA and 7-methyl GMP imply that the former cannot be explained on the basis of emission solely from monomeric methylated G residues. A scheme involving both monomeric G residues as well as G residues that have an excimer-like conformation in their ground electronic state is proposed. The data appear to favor transfer of excitation energy from A, C, and T residues to G residues.

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CHAPTER I

INTRODUCTION

Nucleic acids are very important macromolecules that have been studied extensively in the last decade. Irradiation of many biological systems with ionizing radiation leads to mutations and inactivation of cell components. The excited states of nucleic acids play an important role in those effects. When photons of energy in the ultraviolet (UV) region are absorbed by particular bonds of molecules, this absorption gives rise to the generation of excited states. The effects of UV on living organisms are attributed largely to the absorption of that light by nucleic acids (Gates, 1928; Emmons and Hollaender, 1939; McLaren and Shugar, 1964).

It has been found that irradiated solutions of DNA show a decrease of viscosity, which is linearly proportional to dose (Arena, 1971). The decrease of viscosity is believed to be caused by either of two types of alternations: (a) Radiation-induced decrease in the degree of stiffness, that characterizes the unirradiated DNA molecules, which gives more freedom to the molecule for taking up a more compact configuration by further folding up around itself; (b) Molecular degradation (either single- or double-strand breaks). Single-strand breaks are more probable and they are efficiently repaired, whereas double-strand breaks appear to be nonrepairable (except in extremely radioresistant microorganisms). In any case, it is common opinion that DNA damage is the main contributing factor to radiation injury leading to cell death.

Radiation may also delay or inhibit DNA synthesis, depending on the dose and other factors.

Nucleic acids absorb ultraviolet light strongly. The absorption takes place mainly by the purine and pyrimidine bases, but stable photoproducts are formed almost entirely by pyrimidines (Whillans et al., 1973). The formation of photodimers, their structure, and their biological consequences, have been studied extensively and in several ways (Varghese, 1972a; Fisher et al., 1973a,b; Ottensmeyer et al., 1968a,b; and the Setlows, 1967). Thymine excimers are considered as the precursors of these photodimers (Eisinger, 1968).

From this brief review it is apparent that knowledge of the excited-state properties of DNA is a prerequisite for elucidating the mode(s) of action of UV and ionizing radiations on cells. DNA is a complex system containing four different base residues and each one of them could be considered a potential emitter. It has been suggested, however, that the emission arises mainly from A-T base pairs (Guéron et al., 1977). It has been also reported that the excitation spectra of DNA and its free bases differ greatly from their absorption spectra (Daniels, 1973). Wavelength-dependent intersystem crossing and tautomeric forms of bases are the two possible explanations considered.

Energy transfer between bases, particularly from A, C, and T to G (since G absorbs at longer wavelengths than the other bases), was also suggested in the literature for explaining some of the nucleic acid optical properties (Vigny et al., 1977; Guéron et al., 1967). However, this question has not been settled yet.

Native DNA is a very weakly fluorescent molecule ($q \cong 2 \times 10^{-5}$; Daniels, 1973) and because of that room-temperature data on its excited-state properties are lacking. The only published report is that by Daniels (1973) who determined its fluorescence spectrum. In the present work we used methylated DNA (Me-DNA) as a model of DNA. Me-DNA has some of its guanine residues methylated at N-7 position. Methylation of DNA enhances its fluorescence quantum yield (q of Me-DNA $\cong 2 \times 10^{-3}$) making it possible to carry out fluorescence measurements. Methylation does not alter the nature and properties of DNA to a great extent: it does not prevent the intercalatron of dyes into DNA molecules (Ramstein et al., 1972), it does not change the molecular weight considerably, and it does not cause any double-strand breaks (Ramstein et al., 1971). However, methylation was found to reduce the thermal stability of DNA and to increase its flexibility (Pochon et al., 1967). It is of interest to note that more than twenty different methylated nucleosides have been isolated from RNA (one of which is 7-methylguanosine), but only two have been detected in DNA, 5-methyldeoxycytidine and 6-methyldeoxyadenosine (Hall, 1971).

CHAPTER II

MATERIALS AND METHODS

Calf thymus DNA was obtained from Sigma Chemical Company of St. Louis. Sodium cacodylate was obtained from BDH Chemicals Ltd. (Poole, England). Sodium chloride was a certified product of Fisher Scientific Company. Dialysis bags (Spectrapor Membrane) and cleaning agents (for sulfur and heavy metal removal) were obtained from Spectrum Medical Industries, Inc., of Los Angeles. Glycerol (free of fluorescent impurities), a product of E. Merck (Darmstadt, Germany), was obtained through MC/B Manufacturing Chemists, Inc. 7-Methyl guanosine-5'-phosphate (7-Me GMP) was a product of P-L Biochemicals, Inc., of Milwaukee, Wisconsin. Dimethylsulfate (DMS) was obtained from Aldrich Chemical Company. 7-Methyl guanine and 3-methyl adenine samples were obtained from two sources: Vega Biochemical of Tuscon, Arizona and Tridom Chemical Company (a representative of Fluka A. G.) of Hauppauge, New York. Samples from both sources gave similar results.

The buffer used in all preparations was 5×10^{-2} M sodium cacodylate, 0.01 M NaCl (ph 6.6) prepared in triply distilled water. The same buffer at pH 5.0 was used in some experiments as noted. All measurements were made at room temperature.

Instrumentation

All absorption spectra were recorded on a Cary 14 spectrophotometer using 1 cm light path cuvettes. The fluorescence spectra were taken

with a steady-state spectrofluorometer (Georghiou, 1975). A block diagram of the instrument is shown in Fig. II.1. This spectrofluorometer employs a 1000 W high pressure xenon-mercury lamp as a source of exciting light and emission and excitation Bausch and Lomb 0.5 m monochromators. The light passes through a mechanical chopper, resulting in a train of pulses of fixed repetition frequency, before entering the excitation monochromator. The emission monochromator sends a particular spectral region of the fluorescence emission to a thermoelectrically cooled EMI 9558 QB photomultiplier. The output is fed into a lock-in amplifier, which is triggered by a reference signal from the chopper. The output signal from the amplifier drives the Y-axis of a Honeywell X-Y recorder. A signal from the automatic wavelength scan device of the emission monochromator provides the corresponding X-drive. The sample holder of the spectrofluorometer is equipped with a Cryo-Tip temperature control unit allowing the temperature to be controlled from -190°C to ambient temperature.

All nanosecond measurements were carried out on a pulse sampling fluorometer (Badea and Georghiou, 1976). A block diagram of the instrument is shown in Fig. II.2. This fluorometer employs a Princeton Applied Research model 162 boxcar averager. This model is equipped with two model 163 sampled integrators which incorporate two Tektronix S-2 sampling heads. It incorporates 4 output functions (Channel A, Channel B, Channel A/B, log of Channel A/B) and a digital storage plug-in unit for infinite data holding time. Signal-to-noise ratio improvement for each channel is achieved by averaging up to 10^4 samples

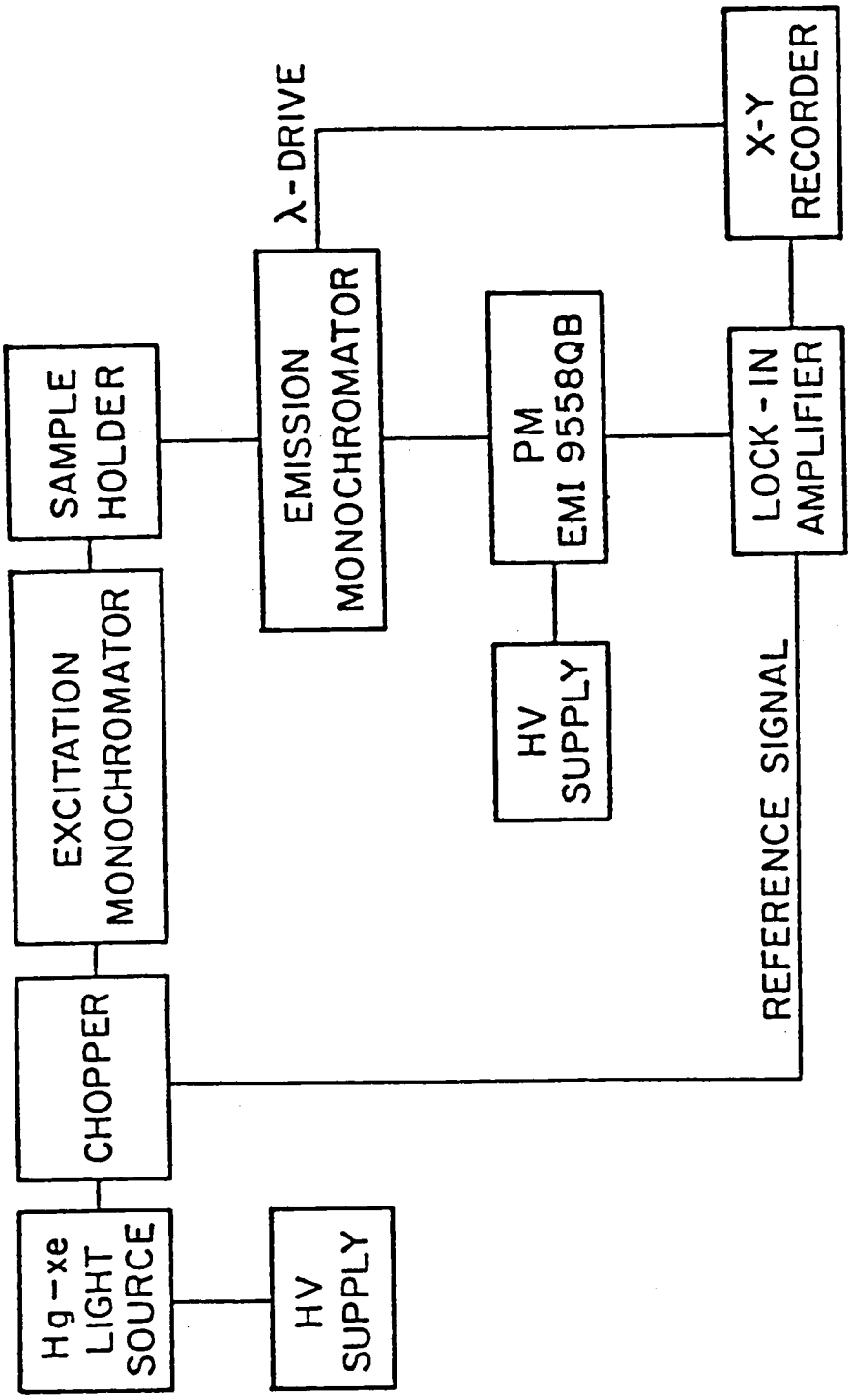

Fig. II.1. Block diagram of spectrofluorometer.

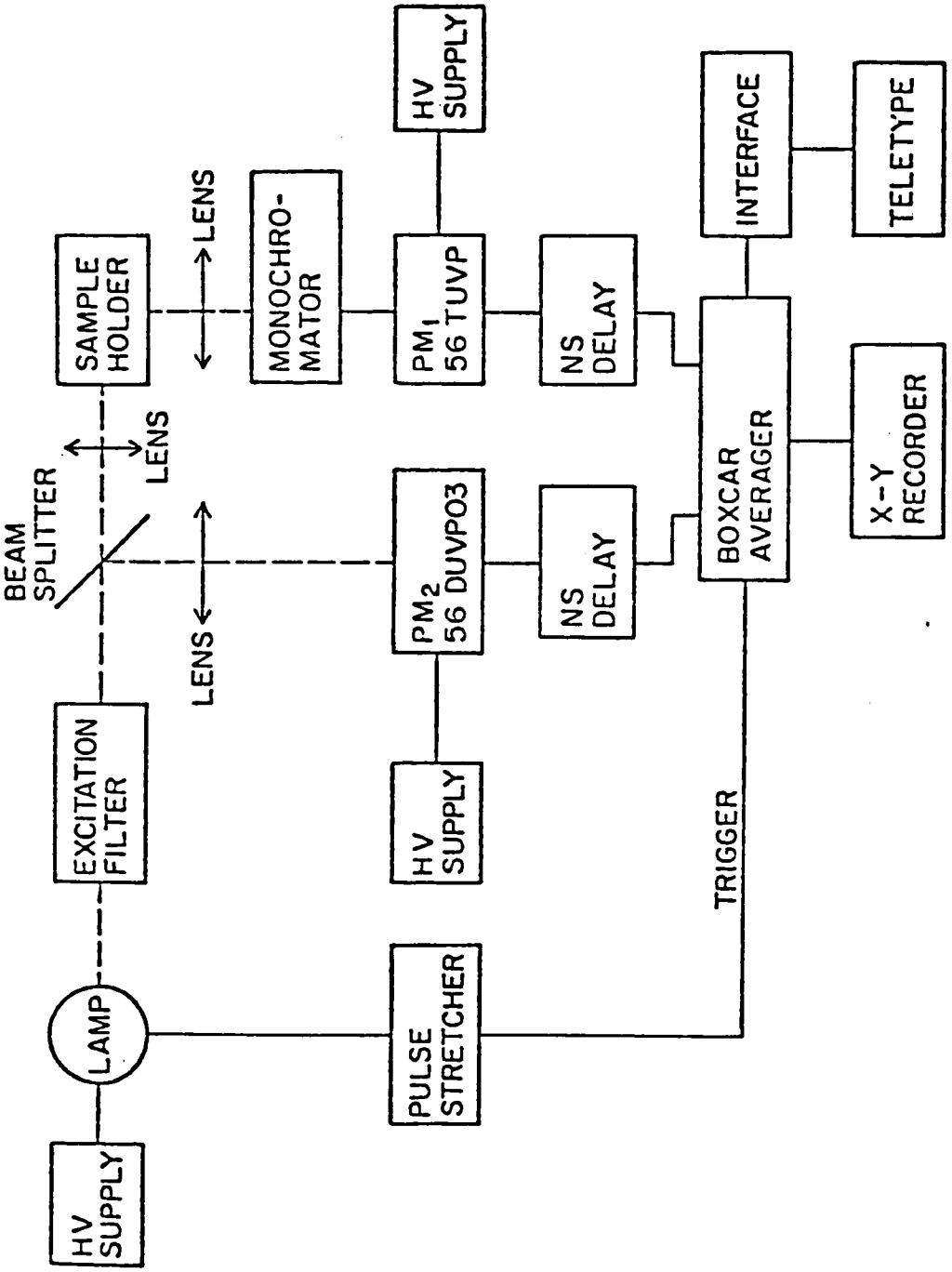

Fig. II.2. Block diagram of nanosecond fluorometer. PM_1 and PM_2 are photomultipliers of 2 ns risetime.

and further smoothing the signal with a main frame filter which has a time constant variable in decade steps from 0.1 ms to 10 s. The overall noise reduction factor is in excess of 10^3. In addition, baseline sampling allows automatic corrections for DC drifts. The exciting light source is an Optitron Model NR-1 of the free running type, with a continually variable electrode gap. A 10 kHz repetition rate is achieved for an electrode gap of about 0.5 mm at an applied voltage of 17 kV. Nanosecond pulses can be obtained over the wavelength range 200 nm-750 nm by properly selecting the type and pressure of flashing gas (purified nitrogen at 200 psi was employed). Proper shielding of the lamp results in data free of noise even at the most sensitive setting of the averager. The data were recorded on a Honeywell X-Y recorder, in which both the X and Y axes are driven by the output signal of the averager. They were also obtained on paper tape through an interface and teletype. The data were analyzed by employing a computer program based on the method of nonlinear least-squares. The sample holder is equipped with a commercial Cryo-Tip temperature control unit.

Melting profiles of DNA and methylated DNA were obtained on a Perkin-Elmer 554 spectrophotometer. This spectrophotometer is equipped with a flowchart recorder, background corrector and a scan programmer. It also has a digital printer which prints output of cell number, wavelength and ordinate values. The spectrophotometer is also connected to a heating unit which heats the cell compartment by circulating hot water through it.

Methylation of DNA

Ten mg of calf thymus DNA were soaked in 10 ml of 0.5 M sodium cacodylate, 1 M NaCl (ph 6.6) for at least 48 hours. The solution was stored at low temperature (in the refrigerator). Then the DNA solution was sheared several times (for about 5 min.) using a hypodermic syringe. For methylation, dimethyl sulfate (DMS: 60 to 100 µl) was added every 30 minutes for three hours to the DNA solution (3 to 5 ml). Then the product was dialyzed at low temperature (0°C) against 1 M NaCl, 0.5 M sodium cacodylate (pH 6.6) for two hours and then against 0.01 M NaCl, 0.05 M sodium cacodylate for two more hours. The dialysis membrane used in this process was prewashed from sulfide and heavy metals. Then, the dialysis product was divided into six to ten samples and was kept at -20°C.

It should be noted here that the reaction is not easily reproducible, confirming a previous report by Ramstein et al. (1971). Two experiments carried out under the same conditions do not yield the same percentage of methylation. Actually sometimes no methylation takes place at all, whereas other times some methylation occurs. The latter samples were not used since the fluorescence signal was too weak to measure especially after correcting for buffer contribution. It has been found (Ramstein et al., 1971; Leng et al., 1968; and Leng et al, 1968) that the amount of methylation depends on the base composition of the DNA, the amount of methylating agent (DMS) added, and the total yield of the reaction.

The percentage of methylation was determined by comparing the melting temperature of native DNA with that of methylated DNA. The difference between T_m of DNA and T_m of methylated DNA is known to be proportional to the percentage of methylation (Ramstein et al., 1971). From the straight line presented by Ramstein et al. (1971) a degree of methylation of 23% was calculated for a ΔT_m of 20°C. We found that the percentage of methylation of the samples used for all of our measurements to be about 21% with little variation (typically ±2%) from one preparation to another.

Melting Profiles of DNA and Methylated DNA

The melting transition of DNA is widely used in DNA studies for characterization and understanding of some of the properties of nucleic acids. The melting temperature of DNA (T_m) depends on many factors, such as the base composition, molecular weight of DNA and the ionic strength of the medium (Marmur and Doty, 1962).

It has been reported (Britten et al., 1974) that the optical density of EDTA at 260 nm is directly proportional to the temperature (the optical density was found to increase significantly with temperature at 260 nm). This phenomenon led to the use of EDTA as a tool for measuring the change in temperature. Dr. Stewart Riggsby (Microbiology Department, The University of Tennessee, Knoxville) used this method successfully for determinating the melting temperatures, T_m, of nucleic acids.

Determination of Melting Temperature

The steps followed in the measurements were:

1. An aqueous 0.1 M EDTA sample (in a glass-stoppered quartz cuvette having 1 cm light path) was placed in the chamber of the Perkin-Elmer 554 spectrophotometer whose temperature could be raised by circulating hot water. The absorbance of the EDTA sample was measured against water in an identical cuvette.

2. A sample of DNA and another of methylated DNA (in 5×10^{-2} M sodium cacodylate, 0.01 M NaCl, ph 6.6) were also placed in the spectrophotometer chamber. The absorbances of all the samples (at 260 nm) were measured and printed by the digital printer (the absorbance of DNA and methylated DNA at room temperature were usually between 0.2 and 0.3 at 260 nm).

3. The temperature of the chamber was raised by raising the temperature of the water bath in the heating unit connected to the spectrophotometer. The rise in temperature changed the absorbance of the samples. The digital printer printed wavelength, sample number, and the absorbance of each sample, every minute.

4. The experiment was stopped when the absorbance of DNA leveled off. Then, the temperature was determined at each absorbance from the calibration curve made for EDTA. The T_m of DNA and methylated DNA were determined from a temperature vs. absorbance plot. Figure II.3 gives an example of DNA and methylated DNA profiles.

Fluorescence Measurements

The fluorescence spectra were taken with a steady-state spectrofluorometer (Fig. II.1) and were corrected for the variation of the

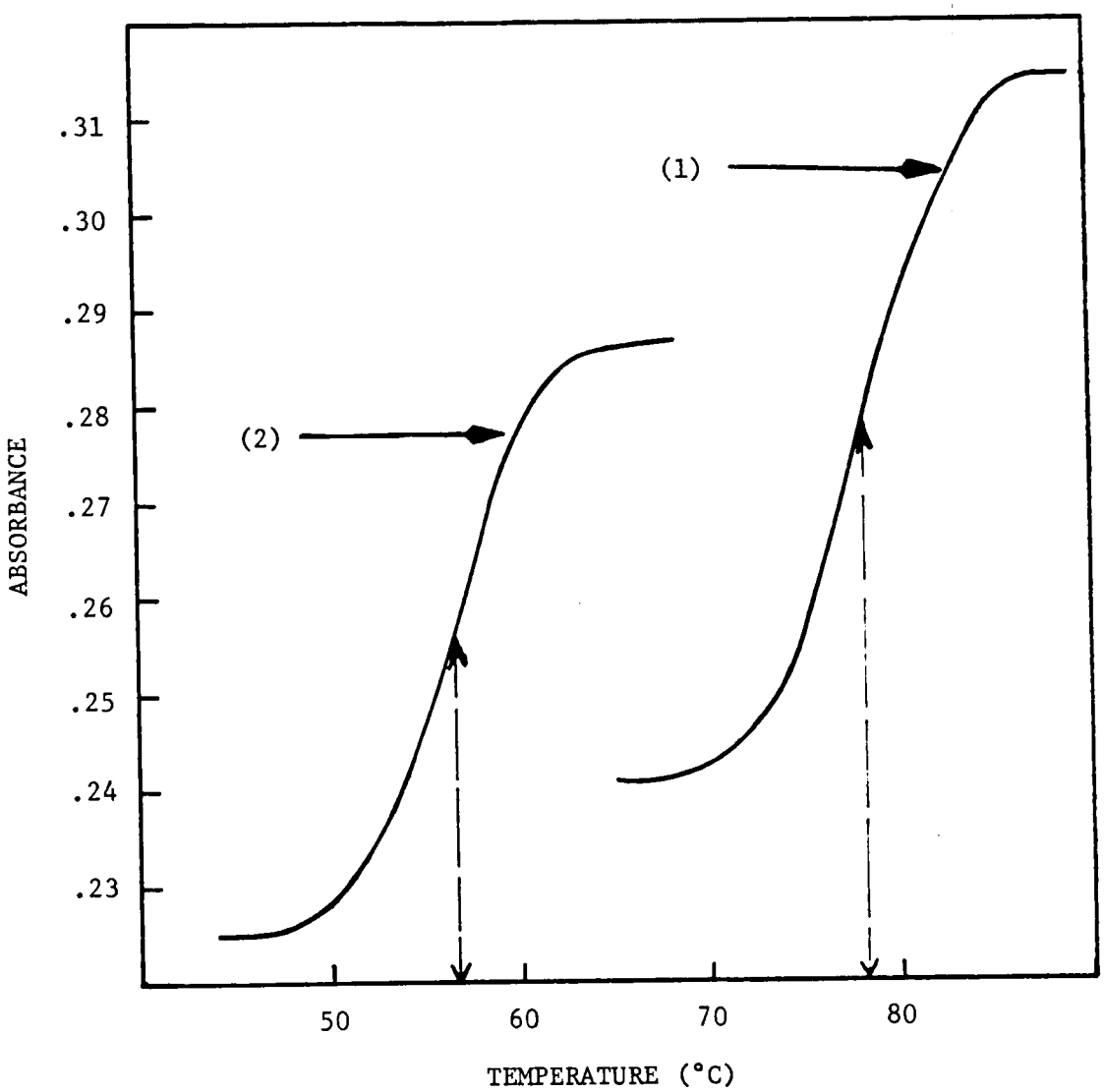

Fig. II.3. Melting profiles of DNA (1) and Me-DNA (2) in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 6.6). The arrows indicate the transition temperatures.

sensitivity of the photomultiplier--monochromator combination with emission wavelength by employing a standard lamp. The spectra were also corrected for buffer contribution. Details regarding the excitation and emission wavelengths employed are given in the individual figure captions in the Results.

Fluorescence Spectra of Methylated DNA

A sample of methylated DNA, with an absorbance of 0.3 at 260 nm measured on a Cary 14 spectrophotometer, was used for the fluorescence spectral measurements. The emission bandwidth was 8.25 nm.

Fluorescence Spectra of 7-Me GMP

In Buffer: A sample of 7-Me GMP with an absorbance of 0.3 at 258 nm was prepared in buffer and its fluorescence was measured using an emission bandwidth of 6.6 nm.

In Glycerol: A sample of 7-Me GMP with an absorbance of 0.1 was prepared in Glycerol and its fluorescence as a function of emission wavelength was recorded. The emission bandwidth was 6.6 nm.

Fluorescence Spectra of 7-Me Guanine and 3-Me Adenine

A sample of 7-Me guanine and another of 3-Me adenine were prepared in buffer having the same absorbance (0.1). The emission bandwidth was 9.9 nm. This was done in order to compare the fluorescence intensities of the two compounds (7-Me G and 3-Me A) under the same conditions.

Fluorescence Quantum Yield Measurements

The q's of methylated DNA and 7-Me GMP were measured by using a dilute solution of P-terphenyl in cyclohexane as a reference. For dilute concentrations we have:

$$\frac{q_1}{q_{ref}} = \frac{I_{F_1}}{I_{F_{ref}}} \quad \frac{\text{Absorbance}_{ref}}{\text{Absorbance}_1}$$

The q_{ref} was taken as 0.77 (Birks, 1970).

Measurement of Fluorescence Excitation Spectra

Fluorescence excitation spectra were measured by determining the intensity of the exciting light (I_{ex}) at each wavelength through the use of a dilute P-terphenyl solution in cyclohexane. Concentrations were kept low so that the absorbance was 0.05 or less. Under that condition:

$$\frac{I_F}{I_{ex}} \alpha \text{ Absorbance}$$

if the fluorescence quantum yield is independent of the exciting wavelength. If this latter condition is not satisfied, one observed a disagreement between the absorption and the fluorescence excitation spectra. Indeed, that was the case for all the measurements obtained in the present study. The excitation bandwidth was 16.5 nm. The fluorescence quantum yield profiles as a function of excitation wavelength were obtained by dividing the excitation spectra by the absorption spectra.

Polarization Measurements

For polarization measurements a 3 M 105-UV-WRMR polarizer was placed in the excitation light path and a HNP'B polarizer in the emission path. All measurements were corrected for the buffer contribution.

Methylated-DNA Polarization Measurements

In Buffer: A sample of methylated-DNA having an absorbance of 0.5 at 260 nm was prepared in buffer. The polarization was measured at two emission wavelengths (365 and 420 nm) while changing the excitation wavelengths (250, 265, 280, 295 and 300 nm). The emission and the excitation bandwidths were 16.5 nm. The degree of polarization P was calculated from

$$P = \frac{I_{VV} - I_{VH}\,(I_{HV}/I_{HH})}{I_{VV} + I_{VH}\,(I_{HV}/I_{HH})}$$

where V and H stand for vertical and horizontal orientations, respectively, and the first subscript refers to the excitation polarizer whereas the second refers to the emission polarizer.

The voltage was changed from 1200V to 1500V depending on the signal strength and the excitation wavelength.

In 80% Glycerol and 20% Buffer: The conditions for these measurements were identical with those above.

Polarization Measurements of 7-Me GMP

In Glycerol: A sample of 7-Me GMP having an absorbance of 0.1 at 260 nm was prepared in glycerol. Excitation was at 250, 265, 280, 295 and 300 nm. Polarization was measured at two emission wavelengths 350 and 440 nm. The emission bandwidth was 9.9 nm, whereas the excitation bandwidth was 13.2 nm.

In Buffer: The same experiment was carried out using a 7-Me GMP sample with an absorbance of 0.3 at 258 nm in buffer (at pH 6.6 and pH 5.0).

Measurements of Fluorescence Decay Times of Methylated DNA and 7-Me GMP

The fluorescence decay times were measured by exciting with a Pomfret interference filter that had a transmission maximum at 290 nm and a FWHM of 7 nm. The fluorescence was observed through Corning filters 7-60 and 5-59 for methylated DNA and 7-Me GMP, respectively.

CHAPTER III

RESULTS

Native DNA is a very weakly fluorescent macromolecule (fluorescence quantum yield $q \cong 2 \times 10^{-5}$, Daniels, 1973). The fluorescence yield is enhanced very much by methylating some of the guanine bases at position N_7; methylation of other bases occurs to a much lower extent (Ramstein et al., 1971, see Discussion). It has been found that methylation of DNA does not change greatly its properties: it does not prevent the intercalation of dyes into the DNA (Ramstein et al., 1972), it does not change the molecular weight nor does it cause any double strand breakage (Ramstein et al., 1971). It does, however, increase the flexibility of DNA to some extent. Still, Me-DNA is a very useful model of native DNA. In the present work we have carried out spectroscopic studies by using the guanine of Me-DNA as an intrinsic fluorescent probe.

Figure III.1 shows the fluorescence spectra of Me-DNA in buffer. The fluorescence emission was observed from 320 nm to 500 nm while exiting at different excitation wavelengths (250, 260, 270, 280 and 295 nm). It is seen that the spectra exhibit a strong dependence on the excitation wavelength, shifting to the blue with increasing excitation wavelength.

We compared these results with those obtained by employing the model nucleotide 7-Me GMP as the emitter. Figures III.2 and III.3 for pH 6.6 and 5.0 show that the dependence on the exciting wavelength is reduced to a great extent in that case. This comparison implies that

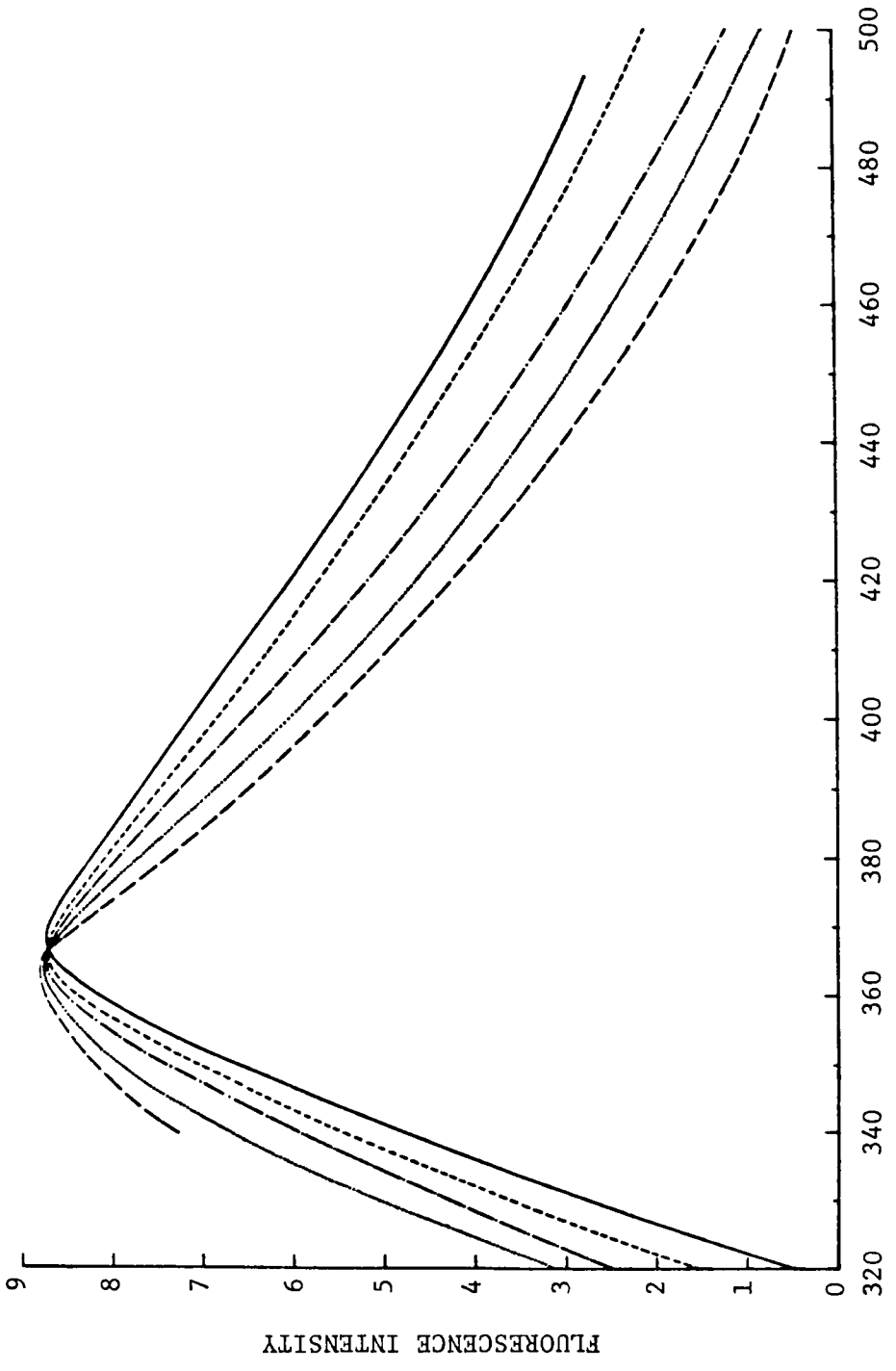

Fig. III.1. Fluorescence spectra of Me-DNA in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 6.6) for the following excitation wavelengths: (——) 250 nm, (- - - -) 260 nm, (—·—) 270 nm, (------) 280 nm, (— —) 295 nm. The spectra are normalized at the peak.

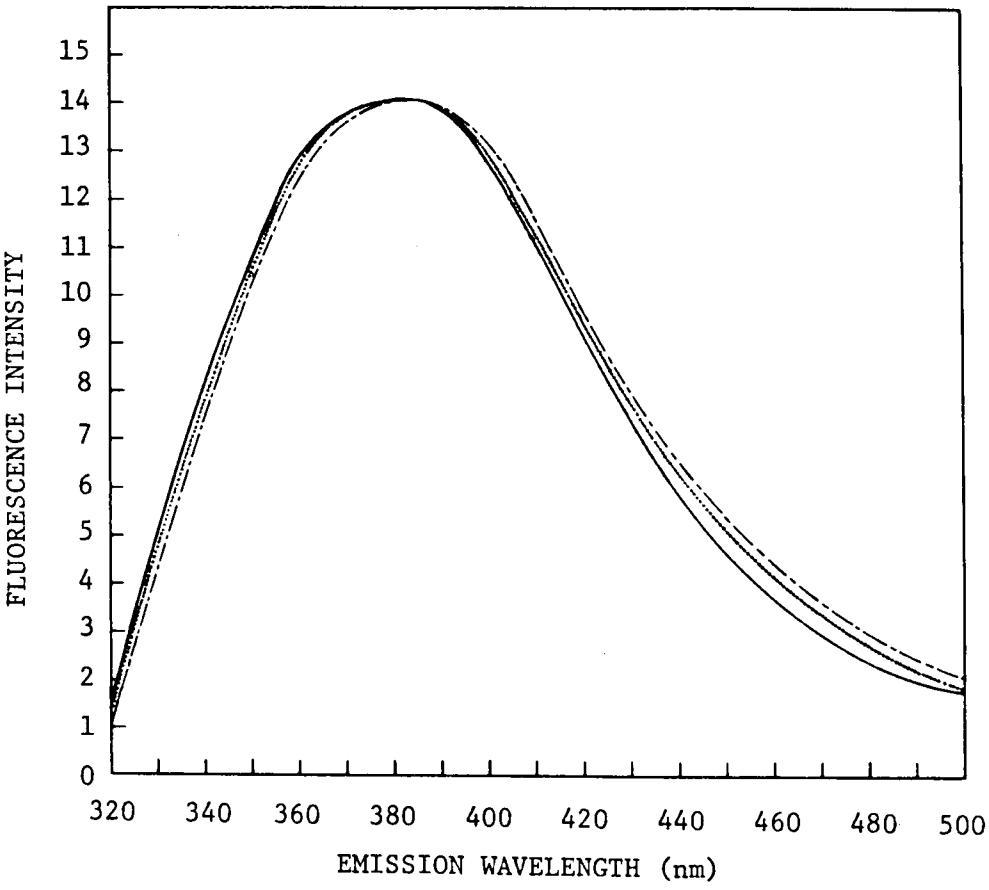

Fig. III.2. Fluorescence spectra of 7-Me GMP in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 6.6) for the following excitation wavelengths: (—·—) 260 nm, (·····) 280 nm, (——) 290 nm. The spectra are normalized a at the peak.

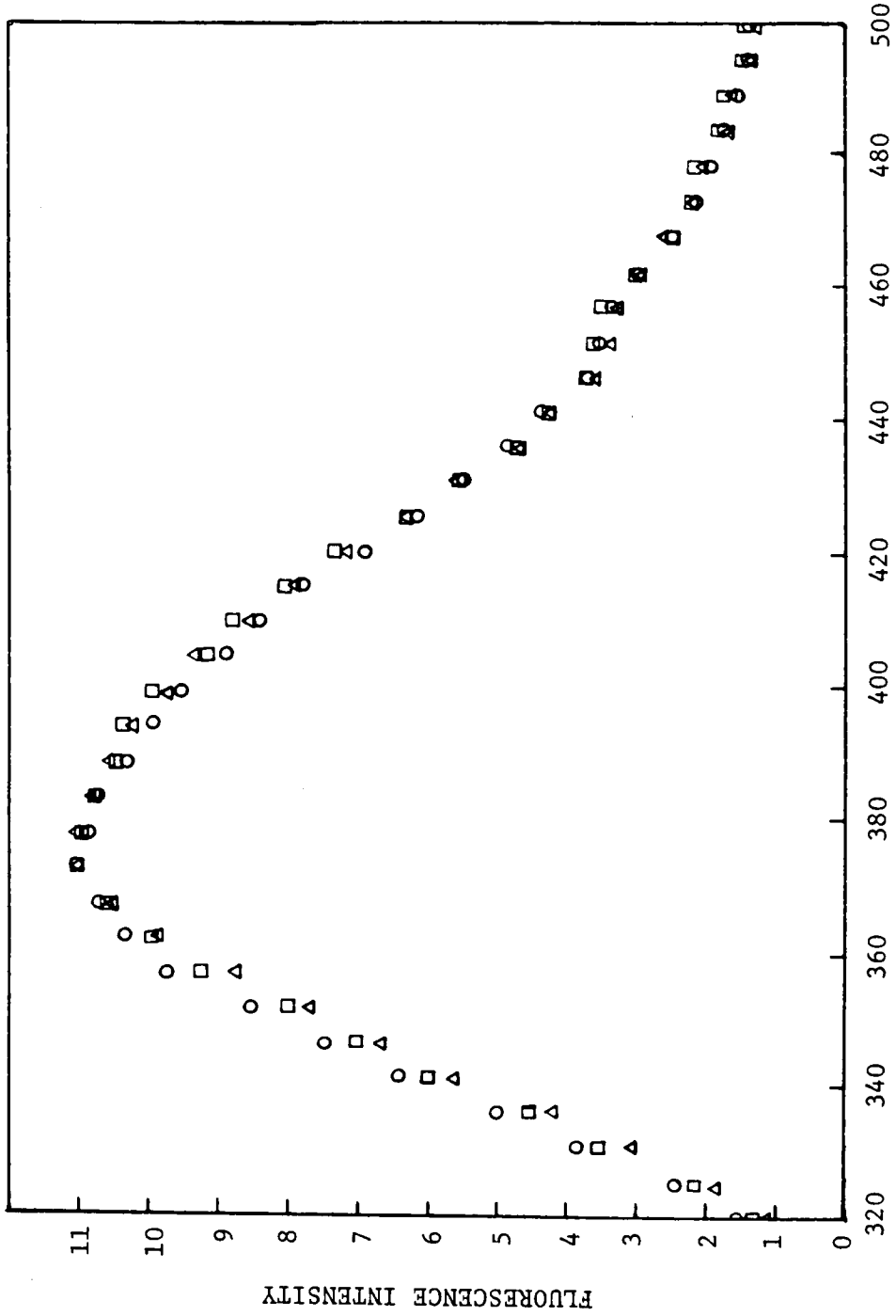

Fig. III.3. Fluorescence spectra of 7-Me GMP in buffer (5×10^{-5} M sodium cacodylate, 0.01 M NaCl, pH: 5.0) for the following excitation wavelengths: 260 nm (Δ), 280 nm (□), 290 nm (○). The spectra was normalized at the peak.

the heterogeneity in the emission properties of Me-DNA is not due wholly to the intrinsic properties of Me-guanine, but it also has its origin in the configuration of the emitter when it is a building block of the genetic material. The possible origin of this effect is further commented upon in the Discussion. It should be noted here that Leng et al. (1968) reported the fluorescence spectrum of 7-Me GMP which is in agreement with our data. It is of interest to note that the fluorescence spectrum of Me-DNA that matches rather closely that of 7-Me GMP (pH 5.0) is for the excitation wavelength of 280 nm (Fig. III.4).

Excitation spectra of Me-DNA were obtained at the emission wavelengths of 350, 390, and 440 nm and are shown in Fig. III.5. It should be noted that Leng et al. (1968) reported an excitation spectrum of 7-Me GMP (ph 5.0 at 0°C and 60°C) which is very narrow and is centered around 292 nm. In contrast, our excitation spectra are broad and show only a moderate difference from the absorption spectra (Figs. III.6 and III.7). In the cases of both Me-DNA and 7-Me GMP the excitation spectra are seen to differ from the absorption spectra and to exhibit a dependence on the emission wavelength. That dependence is much stronger in the former case than in the latter. This observation is in line with the heterogeneity of the fluorescence spectra which we reported above. Absorption spectra of Me-DNA, 7-Me GMP in glycerol and in buffer (ph 6.6 and 5.0) are shown in Figs. III.8, III.9, III.10 and III.11, respectively.

A fluorescence depolarization study was also undertaken for both Me-DNA and 7-Me GMP in buffer as well as in the presence of glycerol

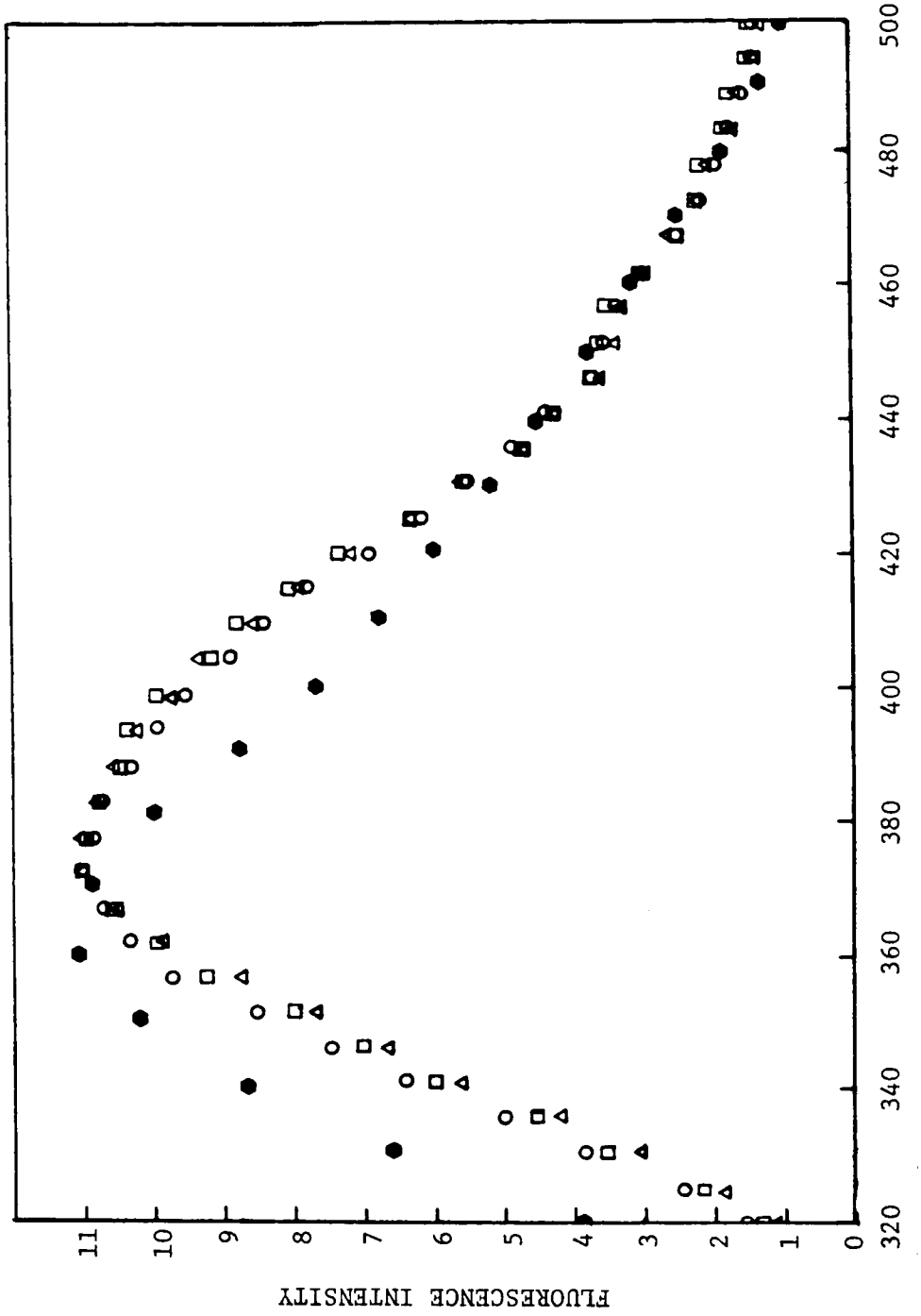

Fig. III.4. Fluorescence spectrum of Me-DNA for the excitation wavelength of 280 nm (●) in comparison to the fluorescence spectra of 7-Me GMP at the following excitation wavelengths: 260 nm (Δ), 280 nm (□), and 290 nm (○).

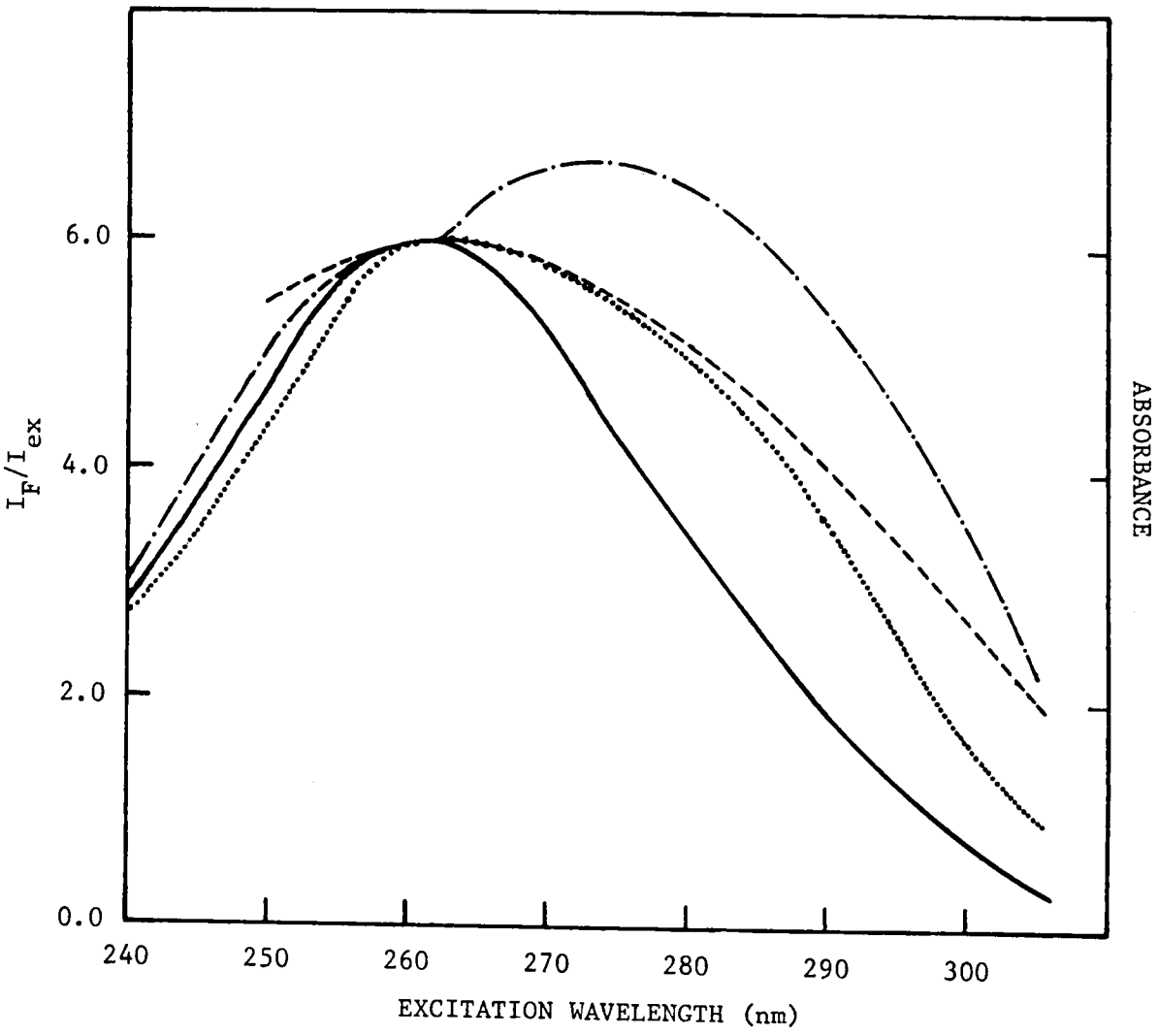

Fig. III.5. Excitation and absorption spectra of Me-DNA in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 6.6). I_F and I_{ex} are the fluorescence and exciting light intensities, respectively. Emission wavelength: 350 nm (·····), 390 (—·—), 440 nm (----). The excitation spectra were normalized at 260 nm. (——) absorption spectrum.

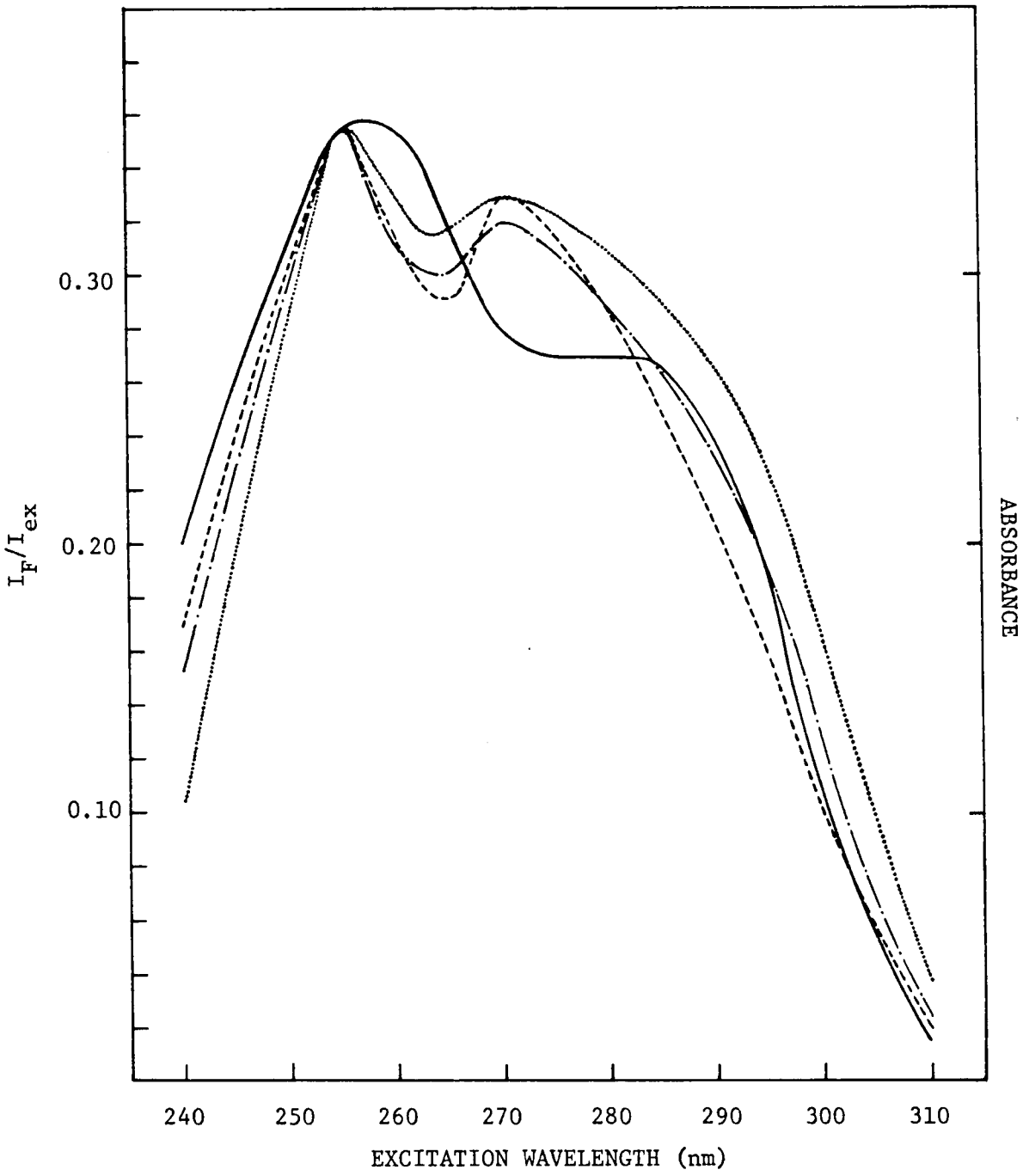

Fig. III.6. Excitation and absorption spectra of 7-Me GMP in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 6.6). I_F and I_{ex} are the fluorescence and exciting light intensities, respectively. Emission wavelengths: (·····) 350 nm, (—·—) 400 nm, (- - -) 450 nm. The excitation spectra were normalized at 255 nm where there is a maximum (——) absorption spectrum.

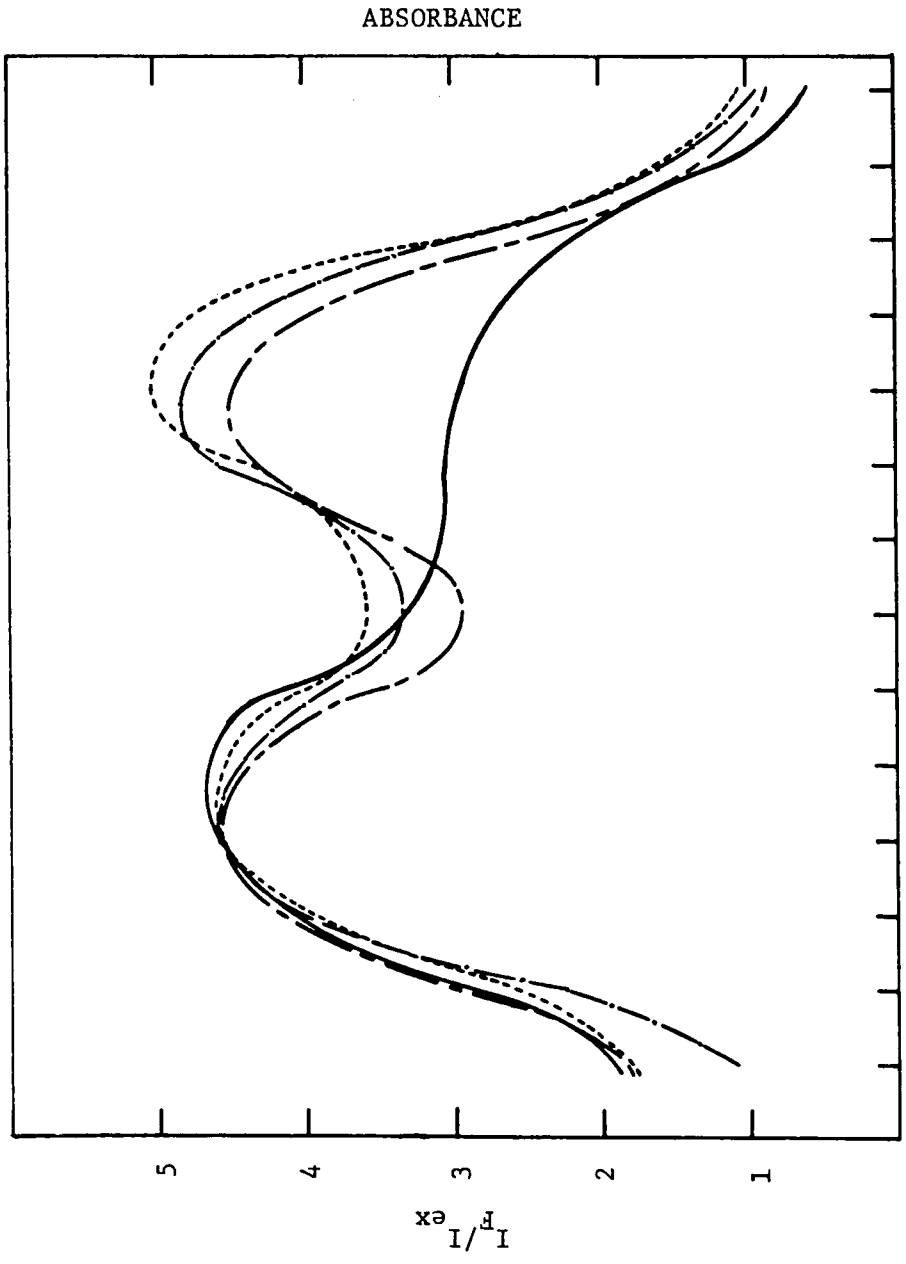

Fig. III.7. Excitation and absorption spectra of 7-Me GMP in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 5.0). I_F and I_{ex} are the fluorescence and exciting light intensities, respectively. Emission wavelengths: (- - -) 350 nm, (—·—) 400 nm, (·—·—) 450 nm. The excitation spectra were normalized at 255 nm where there is a maximum (——) absorption spectrum.

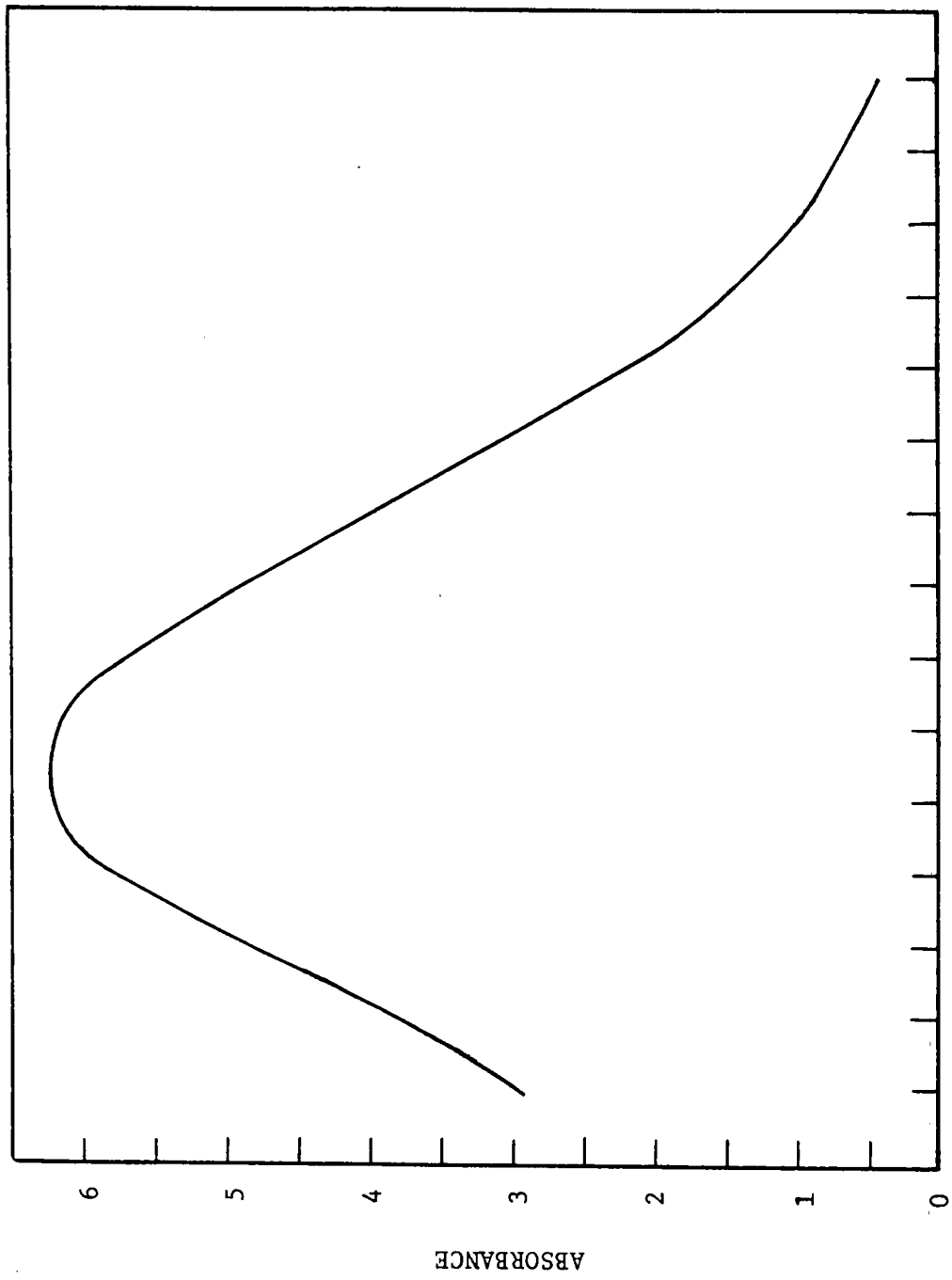

Fig. III.8. Absorption spectrum of methylated DNA in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 6.6).

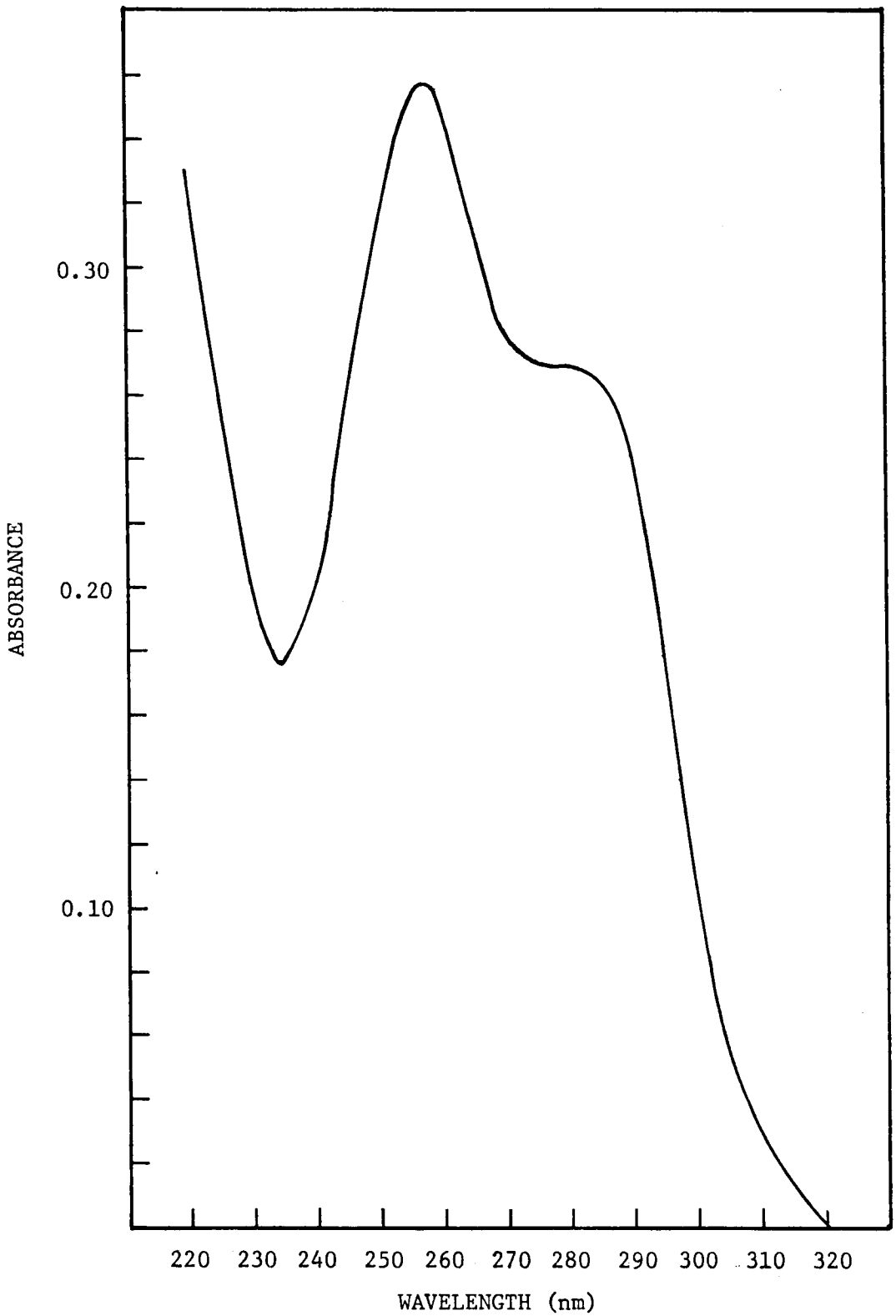

Fig. III.9. Absorption spectrum of 7-Me GMP in glycerol.

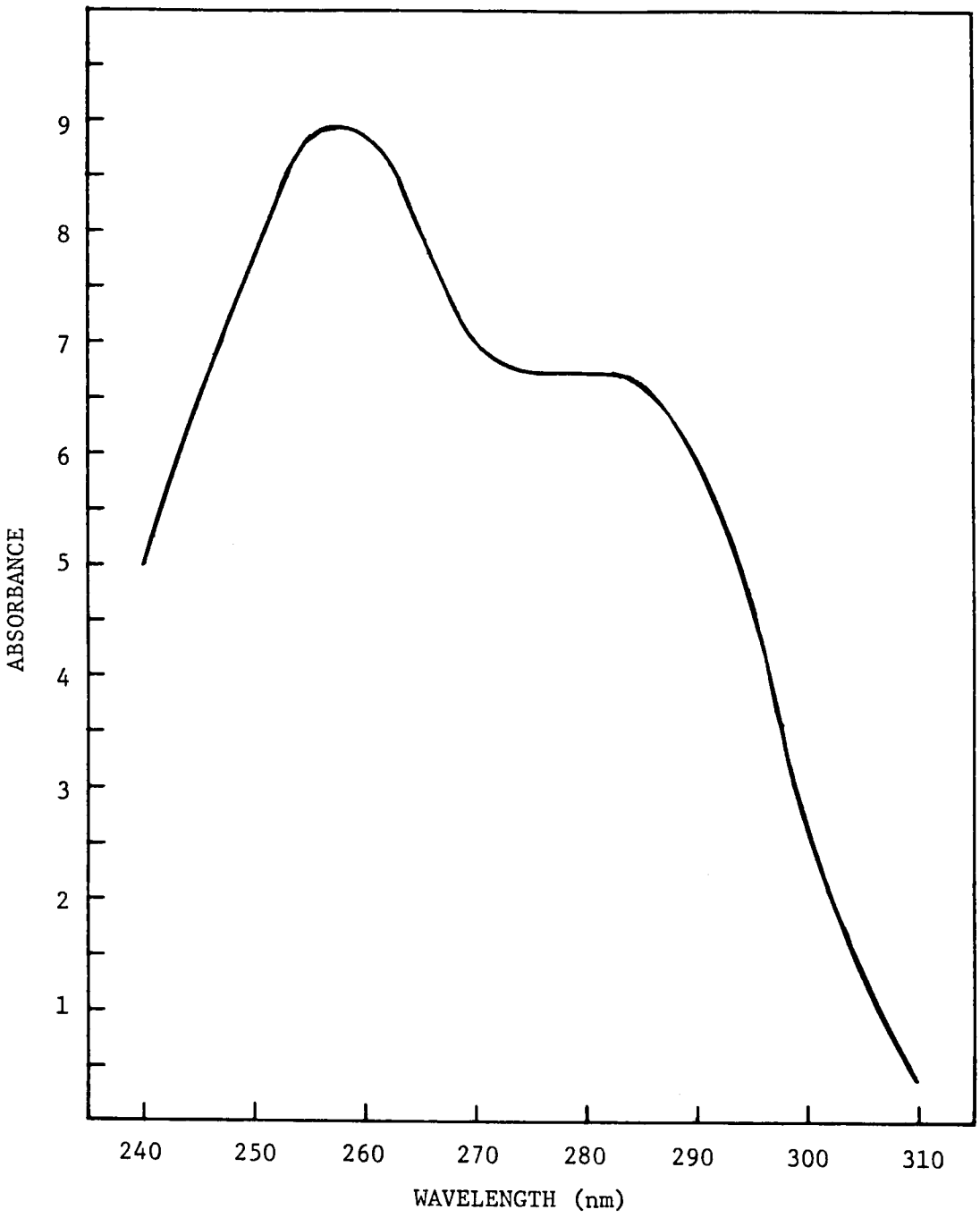

Fig. III.10. Absorption spectrum of 7-Me GMP in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 6.6).

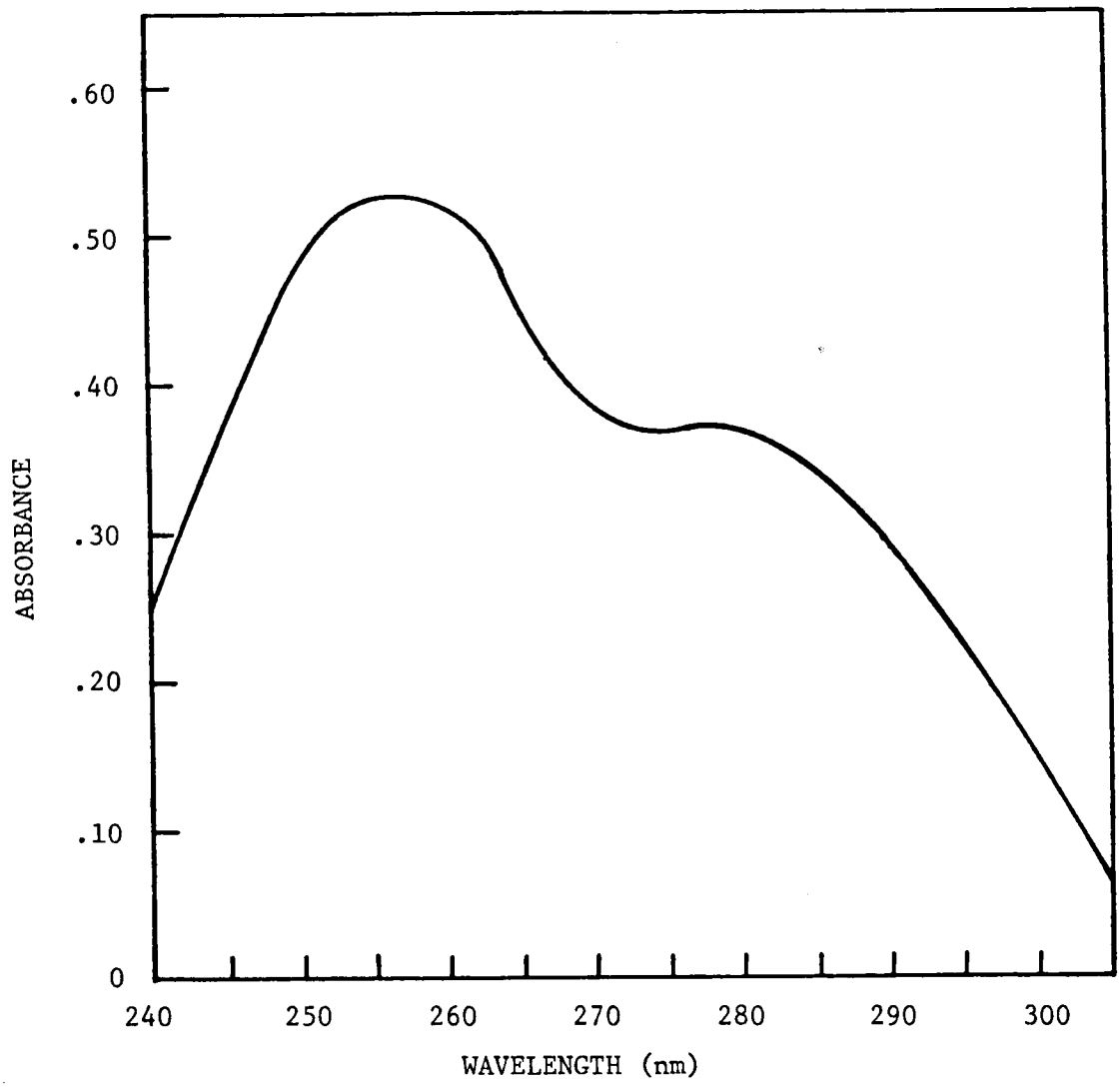

Fig. III.11. Absorption spectrum of 7-Me GMP in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 5.0).

(Tables III.1, III.2, and III.3). The fluorescence decay time was measured for 7-Me GMP in buffer (pH 6.6) and was found to be 0.18 ± 0.05 ns. (Fluorescence decay profile is shown in Fig. III.12.)

We then used the dependence of the fluorescence quantum yield (q) on excitation wavelength, depicted in Fig. III.13, to convert this value to that for excitation wavelength of 290 nm. The average correction factor, obtained from the three emission wavelengths, was 2.06. Thus, the value of (q) at 290 nm is ≅0.0008. It should be noted that this is the value of (q) uncorrected for the contribution to the absorption spectrum by A, C, and T.

The formula used to calculate the fraction of light (A) absorbed by Me-GMP at each excitation wavelength is:

$$A = \frac{\varepsilon_G \times 0.21}{(\varepsilon_G + \varepsilon_C)\ 0.21 + (\varepsilon_A + \varepsilon_T)\ 0.29} \quad .$$

The factors 0.21 and 0.29 that enter this equation arise from the percentage of GC and AT base pairs of calf thymus DNA. The extinction coefficient pertaining to the particular excitation wavelength is ε for each nucleotide. The results are shown in Table III.4. We used the absorption spectra of A, C, and T from UV Absorption Spectra, P-L Biochemicals, Milwaukee, Wisconsin, 1973, and Handbook of Biochemistry and Molecular Biology, third edition, edited by G. D. Fasman, 1975. Also shown in Table III.4 are the values of (q) for the emission wavelength of 350 nm that were corrected for the contribution to absorption by A, C, and T. Those values were also plotted in Fig. III.14 for comparing them with the values of (q) for 7-Me GMP.

Table III.1

Polarization measurements of methylated DNA in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 6.6) and in 80% glycerol

Excitation wavelength	Emission wavelength 365 nm		Emission wavelength 420 nm	
	Degree of Polarization, P			
	In buffer	In 80% glycerol	In buffer	In 80% glycerol
*250 nm	0.20	0.26	0.11*	0.22
265 nm	0.21	0.25	0.21	0.23
280 nm	0.25	0.24	0.18	0.23
295 nm	0.28	0.32	0.27	0.27
300 nm	0.25	0.34	0.25	0.27

*The standard deviation in the values of the degree of polarization is about ±0.01, except for Me-DNA in buffer excited at 250 nm and for the emission monitored at 420 nm; due to relatively poor signal-to-noise ratio in that case, the standard deviation is ±0.02.

Table III.2

Polarization measurements of 7-Me GMP in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 6.6) and in glycerol*

Excitation wavelength	Emission wavelength 350 nm		Emission wavelength 440 nm	
	Degree of Polarization, P			
	In buffer	In glycerol	In buffer	In glycerol
250 nm	0.081	0.172	0.107	0.218
265 nm	0.055	0.109	0.073	0.119
280 nm	0.102	0.225	0.096	0.198
295 nm	0.150	0.299	0.110	0.228
300 nm	0.158	0.344	0.119	0.261

*The standard deviation in the values of the degree of polarization is about ±0.005.

Table III.3

Polarization measurements of 7-Me GMP in buffer
(5×10^{-2} M sodium cacodylate, 0.01 M NaCl,
pH: 5.0)

Excitation wavelength	Emission wavelength 350 nm	Emission wavelength 440 nm
	Degree of Polarization, P	
250 nm	0.119	0.100
265 nm	0.067	0.033
280 nm	0.158	0.062
295 nm	0.165	0.069
300 nm	0.176	0.114

The standard deviation in the values of the degree of polarization is about ±0.005.

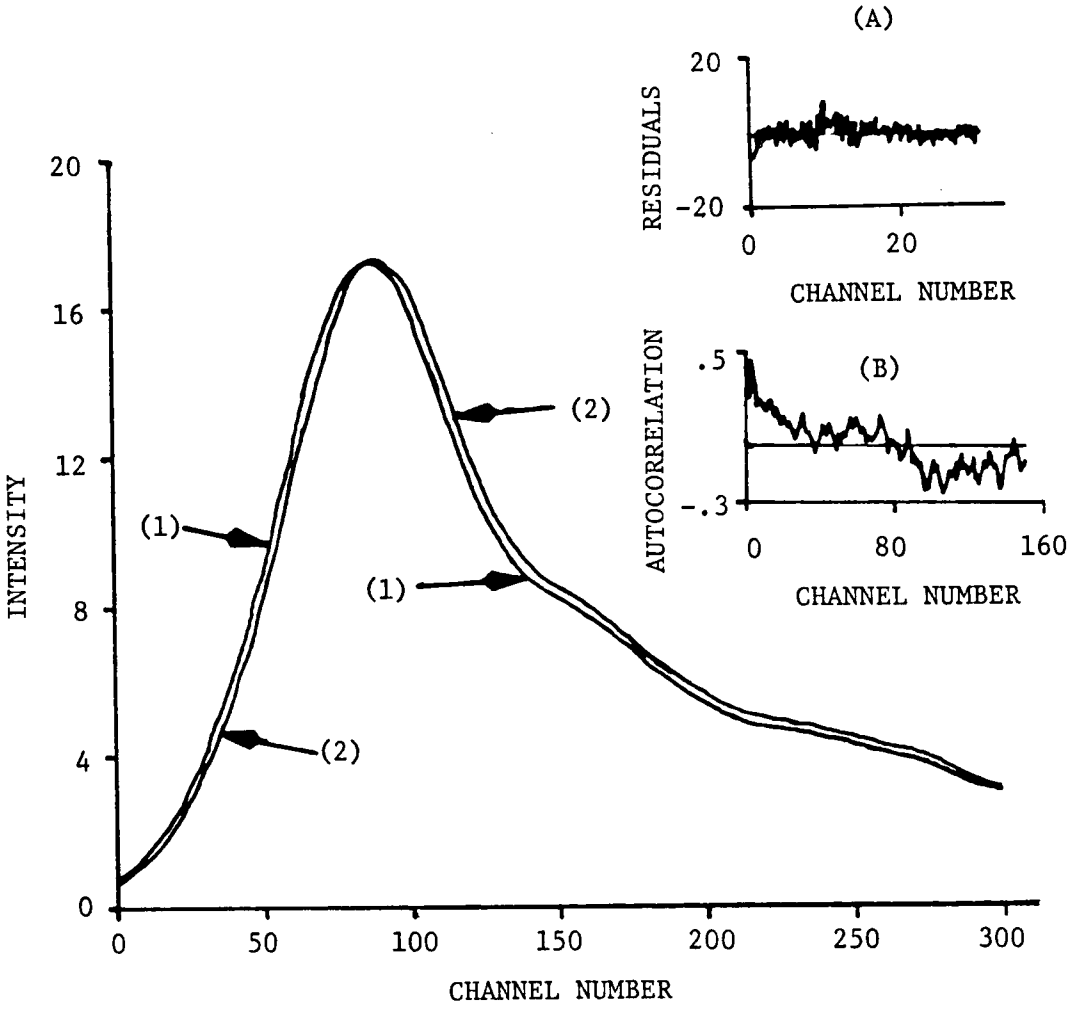

Fig. III.12. Nonlinear regression analysis of the fluorescence decay data for 7-Me GMP in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 6.6). Curve (1) represents the exciting light pulse profile. Curve (2) represents the plot of the fluorescence experimental decay profile which coincides with the convoluted profile. Insert A: Deviations between experimental and convoluted data at each channel. Insert B: Autocorrelation function of the residuals. The plot B shows a trend which implies that there may be more than one component in the decay. The decay profile, however, is too fast to allow a double exponential analysis. Channel width = 0.067 ns.

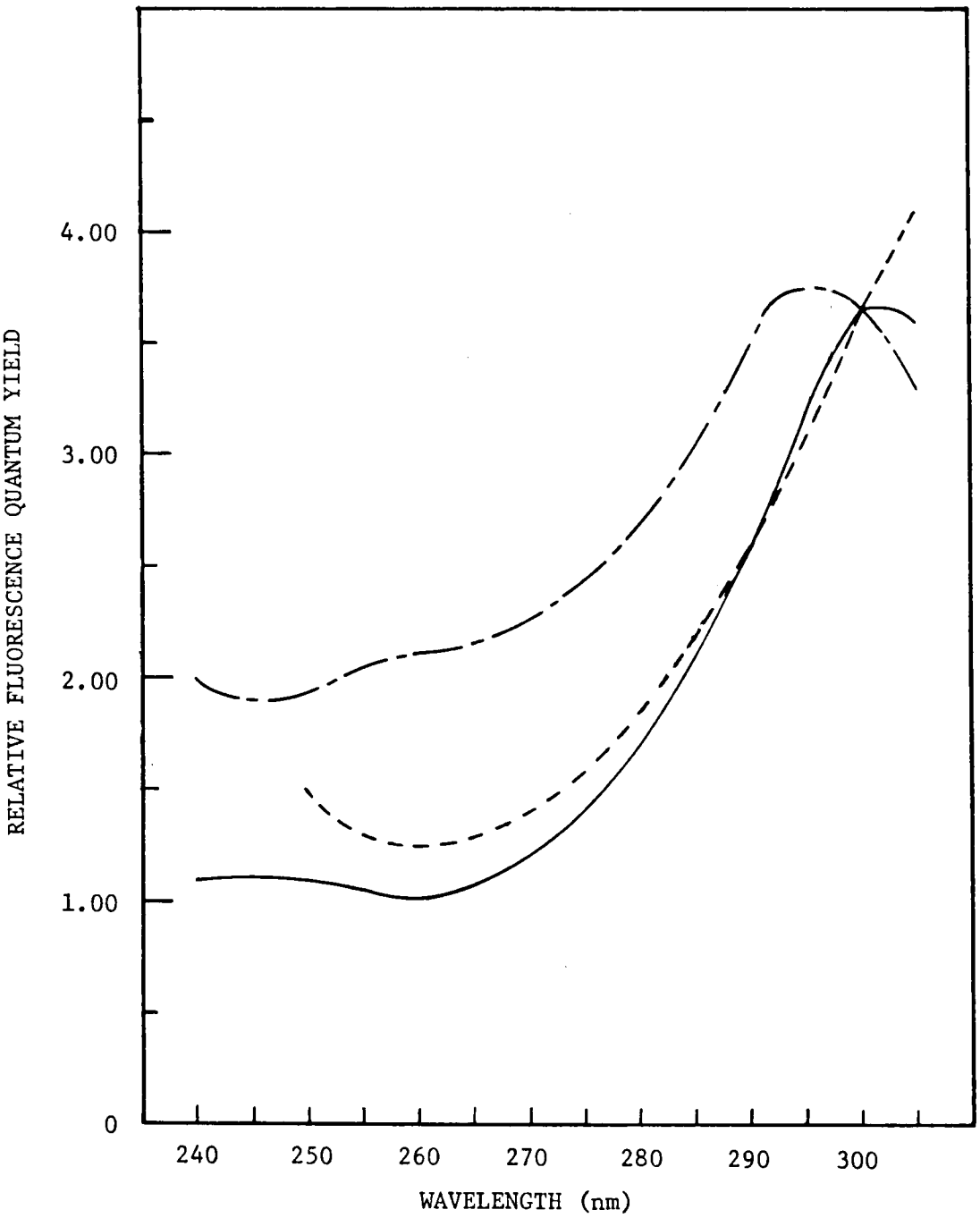

Fig. III.13. Relative fluorescence quantum yield of Me-DNA at the following emission wavelengths: 350 nm (—·—), 390 nm (———), and 440 nm (- - - -). The curves are normalized at λ_{ex} 300 nm.

Table III.4

Calculation of percentage of light absorbed by the G-residue and of the corrected fluorescence quantum yield

	$\varepsilon(10^3\ M^{-1}\ cm^{-1})$							
λ_{ex} (nm)	Me-GMP	CMP	AMP	TMP	A*	C^+	q_{350}	(q_{350}) corrected
240	4.90	6.97	6.15	2.50	0.21	4.80	2.00	9.60
245	6.90	6.32	9.00	3.46	0.23	4.30	1.90	8.20
250	9.40	6.20	12.20	4.85	0.24	4.20	1.92	8.06
255	10.00	6.45	14.60	6.93	0.22	4.50	2.03	9.14
260	10.00	7.31	15.40	8.75	0.20	5.00	2.08	10.40
265	8.90	8.39	14.00	9.63	0.18	5.60	2.11	11.80
270	7.40	9.00	10.30	9.42	0.17	5.90	2.22	13.10
275	7.00	8.70	5.84	8.10	0.20	5.00	2.43	12.15
280	6.90	7.30	2.60	6.93	0.25	4.00	2.72	10.90
285	6.50	5.11	0.76	4.85	0.34	2.90	3.10	9.00
290	5.70	2.49	0.08	2.63	0.47	2.13	3.52	7.50
295	4.30	0.72	0.00	0.55	0.74	1.35	3.72	5.00
300	2.70	0.13	0.00	0.24	0.85	1.18	3.65	4.30
305	1.30	0.00	0.00	0.00	1.00	1.00	3.30	3.30

*Fraction of light absorbed by the Me-GMP residues.

$C^+ = 1/A$; is the correction factor used to multiply the (q) values for correcting for the contribution of A, C and T to absorption.

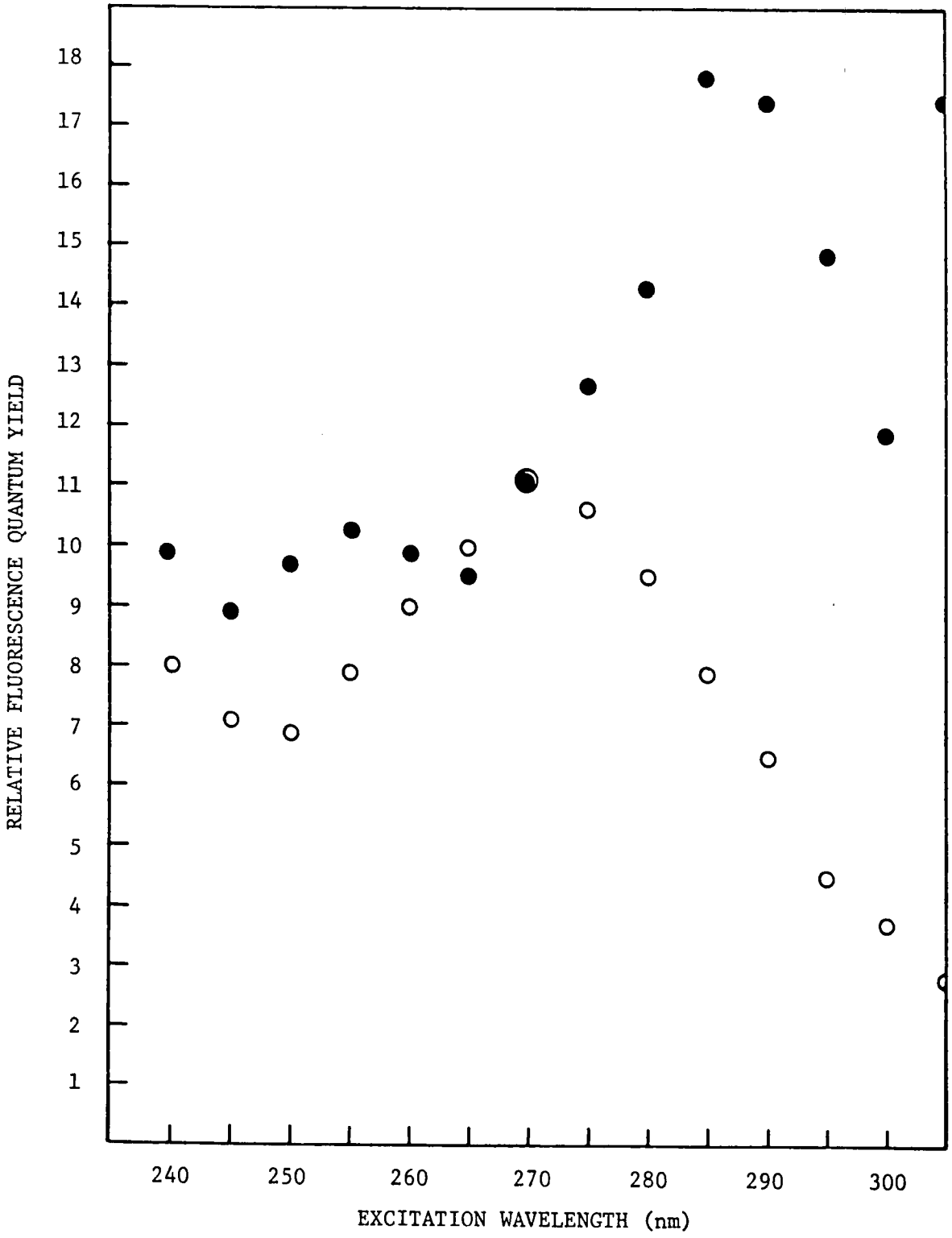

Fig. III.14. Relative fluorescence quantum yield of 7-Me GMP in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 5.0) (●) and that of Me-DNA corrected for absorption by A, C, T (○). The curves were normalized at λ_{ex} 270 nm.

Thus, at the excitation wavelength of 290 nm only 47% of light is absorbed by the Me-G residues. Therefore, q = 0.0008/0.47 ≅ 0.0017. The fluorescence decay time was measured and found to be 0.11 ± 0.06 ns. (The fluorescence decay profiles are shown in Fig. III.15). We note that:

$$q = \frac{K_f}{K_f + \Sigma K_i}$$

$$\tau = \frac{1}{K_f + \Sigma K_i}$$

where K_f is the radiative rate constant and K_i is the sum of the rate constants of radiotionless transitions. From these equations and from the values of (q) and (τ) we have

$$K_f = \frac{q}{\tau} = \frac{0.0017}{0.1 \times 10^{-9}} \cong 1.7 \times 10^7 \ S^{-1}$$

$$\Sigma K_i = \frac{1}{\tau} - K_f = \frac{1}{0.1 \times 10^{-9}} - 1.7 \times 10^7 \cong 10^{10} \ S^{-1} \ .$$

We also measured the fluorescence quantum yield of 7-Me GMP at ph 6.6 and 5.0 for the excitation wavelength of 265 nm. The values are 0.011 and 0.014, respectively. We then corrected these values in order to convert them to the excitation wavelength of 290 nm. The correction factors, obtained in a manner similar to that used for Me-DNA, were 1.07 and 1.70 for pH 6.6 and 5.0, respectively. Thus the values of (q) are 0.012 and 0.024, respectively. The fluorescence decay times were measured and found to be 0.18 ± 0.05 and 0.20 ± 0.05 ns, respectively. (The fluorescence decay profiles are shown in Figs. III.12 and III.16,

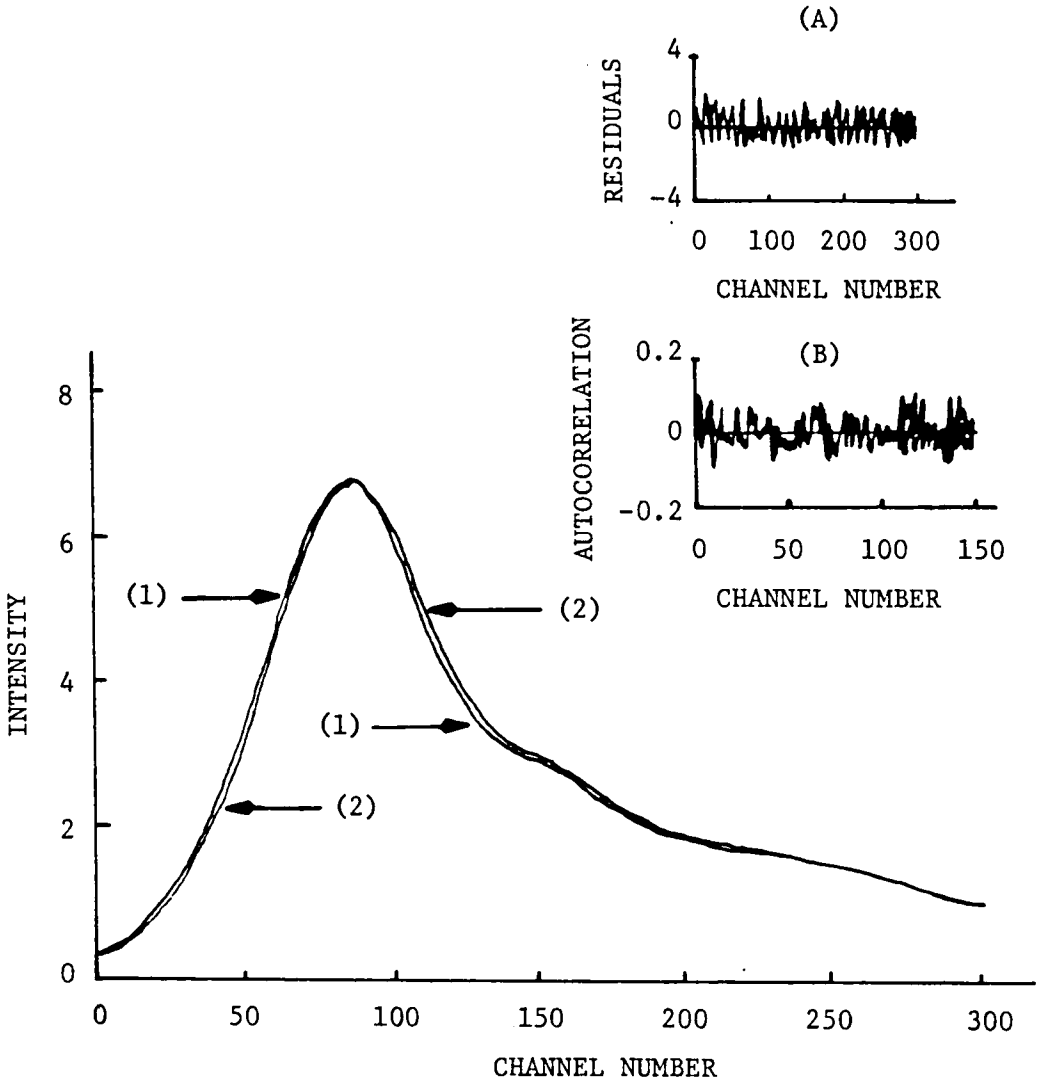

Fig. III.15. Nonlinear regression analysis of the fluorescence decay data for Me-DNA in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 6.6). Curve (1) represents the exciting light pulse profile. Curve (2) represents the plot of the fluorescence experimental decay profile which coincides with the convoluted profile. Insert A: Deviations between experimental and convoluted data at each channel. Insert B: Autocorrelation function of the residuals. The lack of any specific trend in A and B implies a satisfactory fit of the experimental data. Chanel width = 0.067 ns.

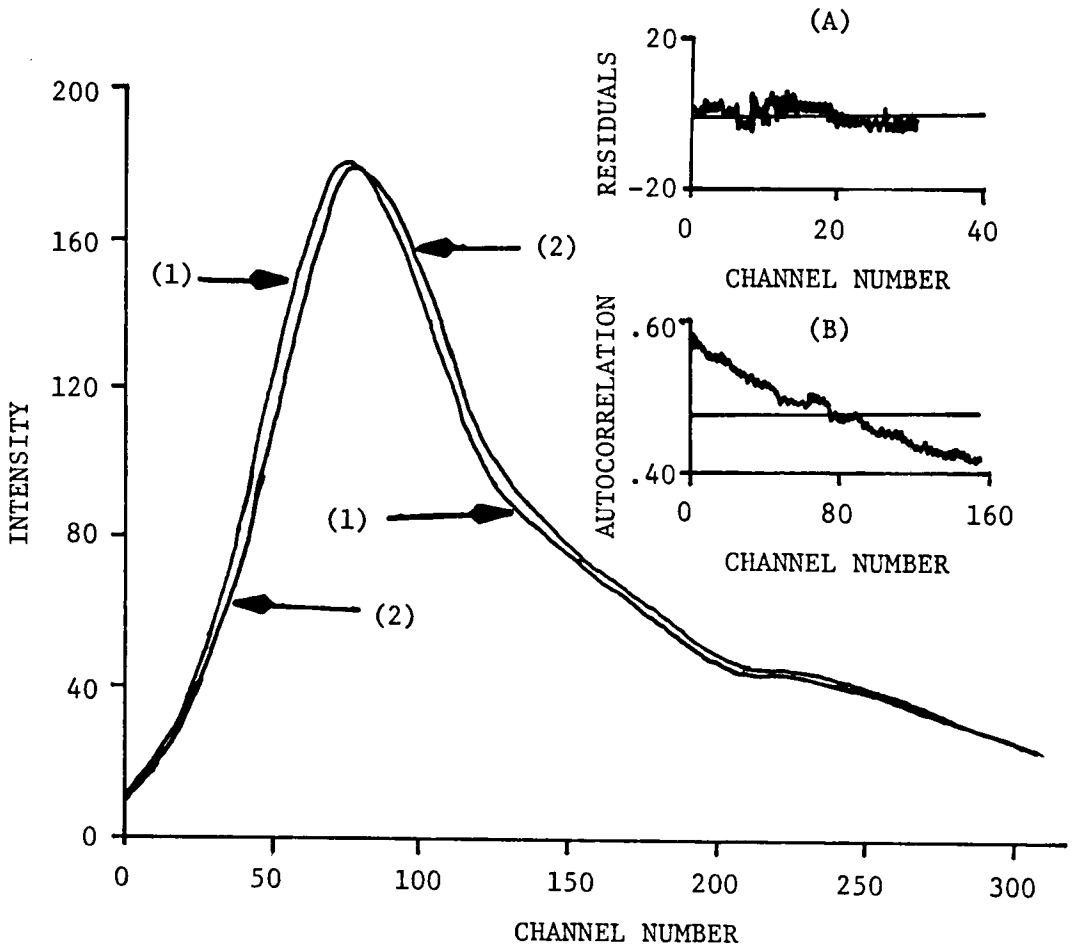

Fig. III.16. Nonlinear regression analysis of the fluorescence decay data for 7-Me GMP in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 5). Curve (1) represents the exciting light pulse profile. Curve (2) represents the plot of the fluorescence experimental decay profile which coincides with the confoluted profile. Insert A: Deviations between experimental and convoluted data at each channel. Insert B: Autocorrelation function of the residuals. The plot B shows a trend which implies that there may be more than one component in the decay. The decay profile, however, is too fast to allow a double exponential analysis. Channel width = 0.061 ns.

$$K_f = \frac{q}{\tau} = \frac{0.012}{0.18 \times 10^{-9}} = 6.67 \times 10^7 \ s^{-1}$$

$$\Sigma K_i = \frac{1}{\tau} = K_f = \frac{1}{0.18 \times 10^{-9}} = 6.67 \times 10^7 = 5.6 \times 10^9 \ s^{-1} \ .$$

For pH 5.0,

$$K_f = \frac{0.024}{0.2 \times 10^{-9}} = 1.2 \times 10^8 \ s^{-1}$$

$$\Sigma K_i = \frac{1}{\tau} = K_f = \frac{1}{0.2 \times 10^{-9}} = 1.2 \times 10^8 = 4.9 \times 10^9 \ s^{-1} \ .$$

The relative fluorescence quantum yields of 7-Me GMP in buffer (pH 6.6 and 5.0) are shown in Figs. III.17 and III.18, respectively.

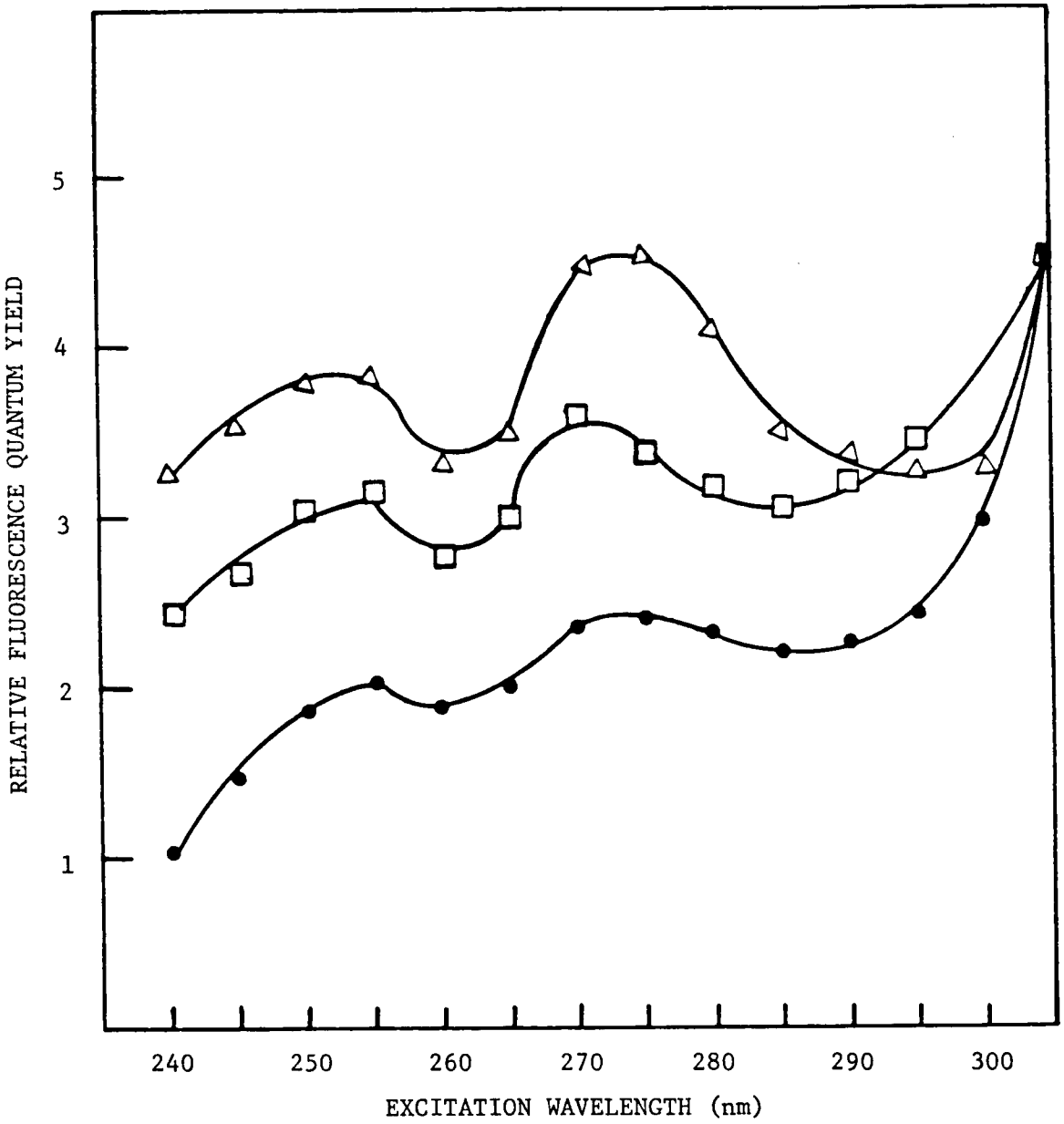

Fig. III.17. Relative fluorescence quantum yield of 7-Me GMP in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 6.6) for the following emission wavelengths: 350 nm (●), 400 nm (□), and 450 nm (△). The curves are normalized at λ_{ex} 305 nm.

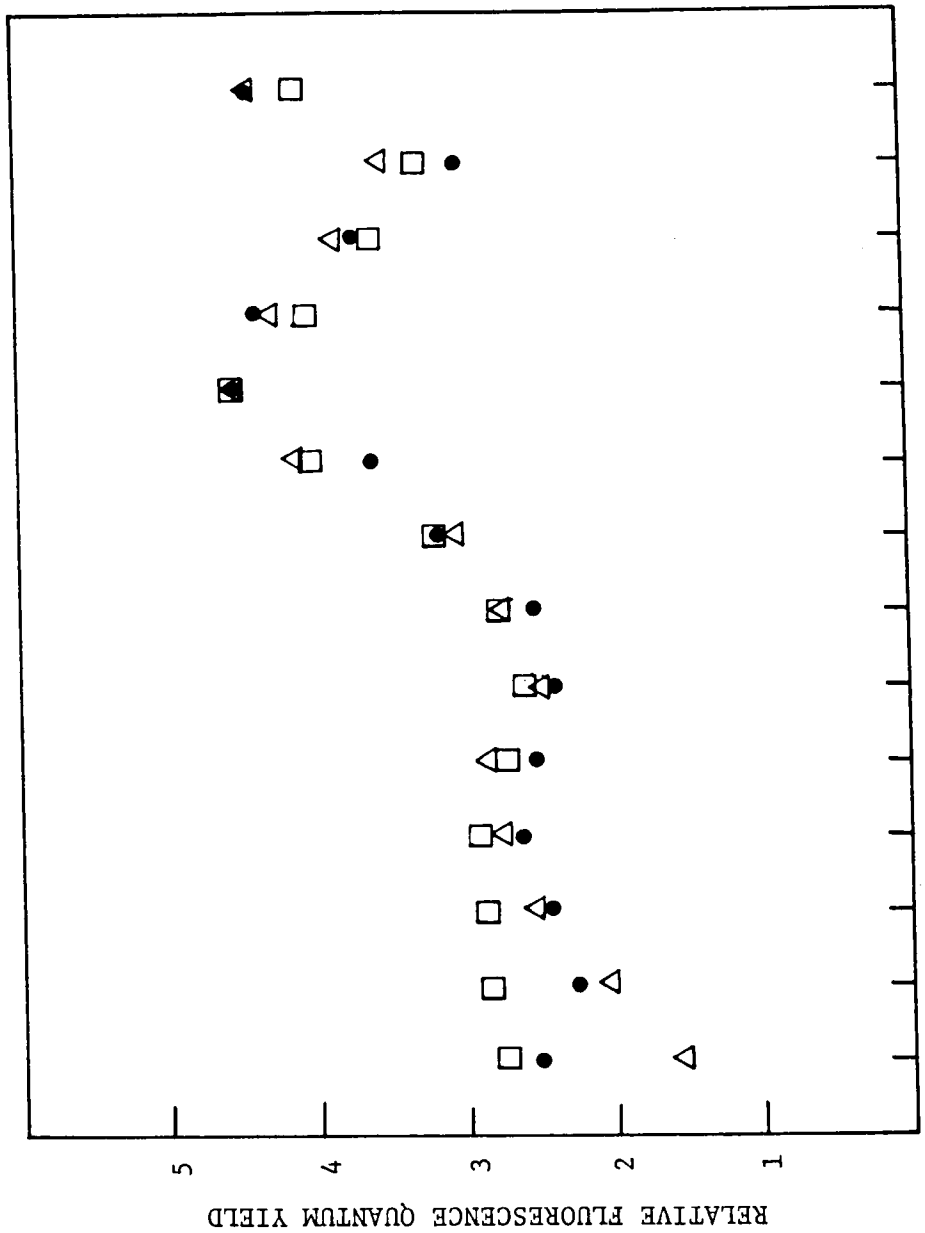

Fig. III.18. Relative fluorescence quantum yield of 7-Me GMP in buffer (5×10^{-2} M sodium cacodylate, 0.01 M NaCl, pH: 5.0) for the following emission wavelengths: 350 nm (●), 400 nm (□), and 450 nm (△). The curves were normalized at λ_{ex} 285 nm.

CHAPTER IV

DISCUSSION

The present study reports on the excited state properties of Me-DNA. It has been found that by treating DNA with dimethylsulfate, guanine and adenine are preferentially methylated. Methylation occurs mainly on the N-7 of guanine residues and to a lesser extent on the N-3 of adenine residues (Ramstein et al., 1971; Lowley and Brookes, 1963). Ramstein and his group found that methylated calf thymus DNA contains at least 3 times as many 7-Me G residues than 3-Me A residues (Ramstein et al., 1971). Figure IV.1 of the present study shows clearly that the fluorescence of 3-Me A is very weak as compared to that of 7-Me G. We compared the fluorescence quantum yield of 3-Me A with that of 7-Me G and found the ratio to be 0.163. Thus, the relative contribution of 3-Me adenine to the fluorescence of Me-DNA is $0.30 \times 0.163 = 0.0489 \cong 5\%$. So, the fluorescence emission of Me-DNA is practically due to 7-Me GMP only, i.e., we have only one emitter.

Before discussing the results for Me-DNA, it is constructive to look at the results for 7-Me GMP which is the fluorophore of Me-DNA.

As seen from Fig. III.3, p. 20, the fluorescence spectrum of 7-Me GMP at ph 5.0 is virtually independent of the excitation wavelength. At that pH the proton at N_1 is not dissociated, as it has a $pK_a = 7.1$ (Hendler et al., 1970), and therefore there is only one emitting species. At ph 6.6, however, there is some contribution from the anionic species and the fluorescence spectrum as seen (Fig. III.2,

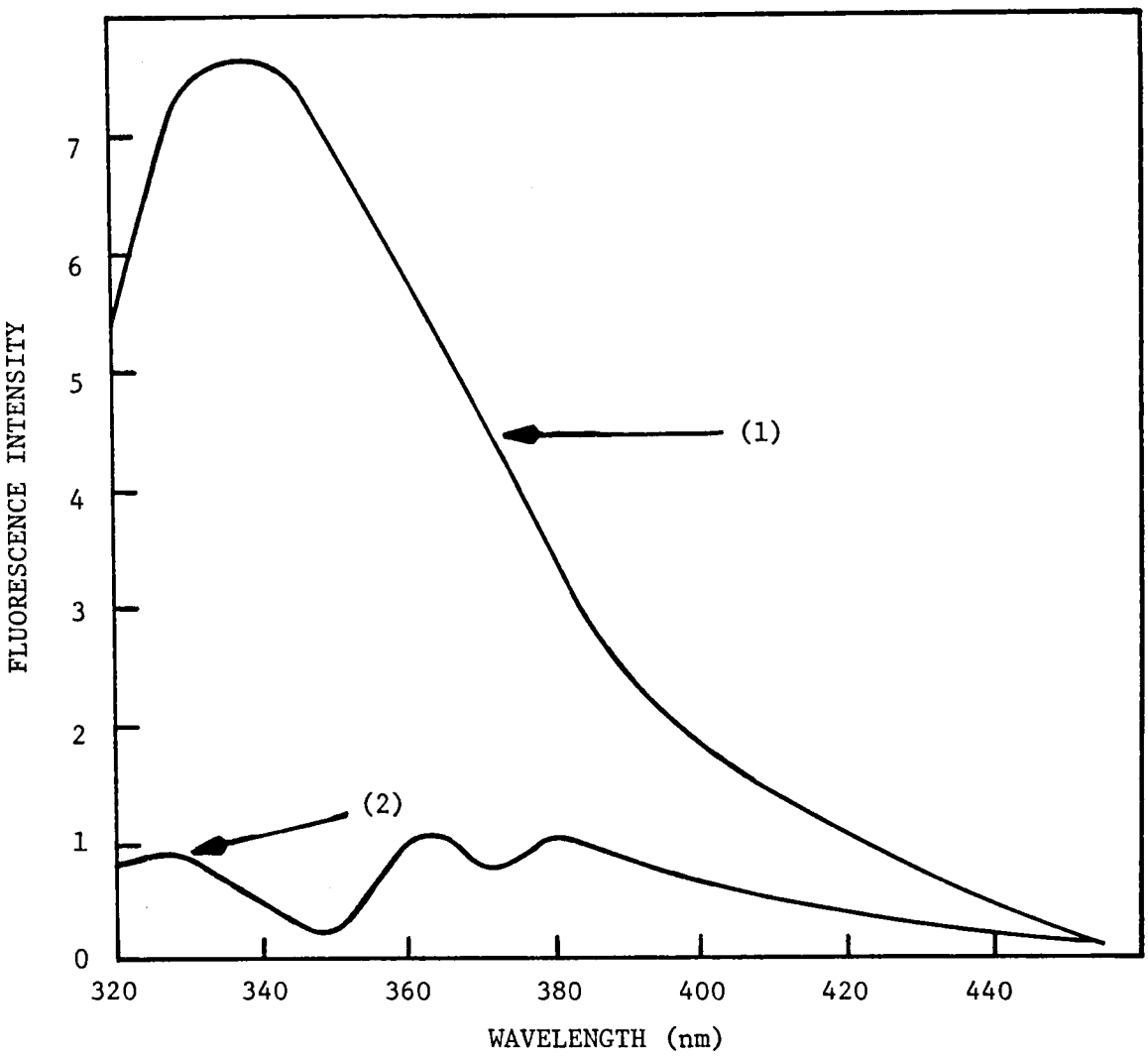

Fig. IV.1. Fluorescence spectra of 7-Me guanine (1) and 3-Me adenine (2). The excitation wavelength was 280 nm.

p. 19) depends slightly on the excitation wavelength. The excitation spectra in both pH values show some dependence on the emission wavelength (Figs. III.6, p. 24 and III.7, p. 25). Moreover, as can be seen from Figs. III.17, p. 42 and III.18, p. 43, the fluorescence quantum yield varies with excitation wavelength. The values of the degree of polarization were measured both in buffer (ph 5.0 and 6.6) and in glycerol solutions. Glycerol was used to get higher polarization values since polarization is higher in a rigid medium. From Tables III.2, p. 32 and III.3, p. 33 we can see the following:

1. In Buffer:
   (a) Emission wavelength (350 nm): polarization values are low for short excitation wavelengths as compared to those for the excitation wavelength of 300 nm.
   (b) Emission wavelength (440 nm): polarization values are not too different at different excitation wavelengths.
2. In Glycerol:
   (a) Emission wavelength (350 nm): the same trend is observed as in buffer. Here, however, there is a clearly defined dip at the excitation wavelength of 265 nm which is not so well defined in buffer as a result of the higher uncertainty associated with those measurements.
   (b) Emission wavelength (440 nm): again here the same trend is observed as in buffer and the dip at the excitation wavelength of 265 nm is observed.

We can conclude then from Tables III.2 and III.3 that the values of the degree of polarization of 7-Me GMP (in buffer and in glycerol) show

a strong dependence on excitation wavelength when the emission is monitored at the blue end of the fluorescence spectrum. When the emission is monitored at the long wavelength region of the spectrum, however, the degree of polarization shows only a weak dependence on excitation wavelength. In all cases the polarization values at the excitation wavelength of 265 nm were the lowest.

Wilson and Callis (1976) studied the degree of polarization of GMP and GPG as a function of excitation and emission wavelengths at -120°C. They found that P shows a strong dependence on excitation wavelength, increasing as the wavelength increases. They also found a weak dependence of P on the emission wavelength. These results are in qualitative agreement with our findings for the P values of 7-Me GMP (Tables III.2, p. 32 and III.3, p. 33). Similarly, Callis (1979) reported that the degree of polarization of guanine depends strongly on the excitation wavelength while it shows only very weak dependence on the emission wavelength both at room temperature and at -125°C. In both cases, the dip at the excitation wavelength of 265 nm, reported in the present work, was not observed.

It should be noted in this connection that Callis found that the degree of polarization exhibits a similar behavior in the case of A, although the variation with excitation and emission wavelengths is smaller. For C and T, however, the polarization was virtually independent of excitation and emission wavelengths. The relative excitation spectra of all DNA bases has been studied by Daniels (1971 and 1973), and Morgan and Callis (1979) and were found to be

significantly different from the corresponding absorption spectra. As it was mentioned earlier, we also found that the excitation spectra of 7-Me GMP are significantly different from the absorption spectra (Figs. III.6, p. 24 and III.7, p. 25).

Several possibilities were proposed for explaining the dependence of the fluorescence quantum yields of the DNA bases on excitation wavelength. Daniels (1973) suggested that the dependence related to the formation of tautomers. It is also possible that there are more than one electronic transition involved in the absorption and fluorescence processes. Daniels (1973) assigned the ^1L_a and ^1L_b states for the two maxima in the absorption spectrum of guanine based on the correlation between the absorption spectra of purines and pyrimidines and those for benzene. Indeed, the strong dependence of the degree of polarization on excitation wavelength and its weak dependence on emission wavelength, observed in the present study, may imply the presence of more than one absorbing transition, but only one emitting transition. Another explanation was offered by Becker (1980). He proposed that the dependence of the fluorescence quantum yield of nucleic acid components on excitation wavelength is due to the equilibrium coexistence of hydrogen-bonded and free forms each having different photophysical properties. But, when the medium is water, as in our case, then it would be expected that the hydrogen-bonded species will predominate. This explanation, therefore, appears to be less attractive. It is of interest to note here that the radiative rate constant, K_f, was calculated from the Strickler and Berg equation (Strickler and Berg,

1962) for 7-Me GMP, pH 5.0, and found to be 1.4×10^8 S^{-1}. This value is in good agreement with the value of 1.2×10^8 S^{-1} obtained experimentally (see Results). This implies that no significant change in the molecular geometries of the ground and the emitting electronic states takes place.

A comparison of the optical properties of Me-DNA with those of 7-Me GMP shows that there are important differences in the two cases. The fluorescence spectra of Me-DNA (Fig. III.1, p. 18) show a strong dependence on the excitation wavelength, shifting to the blue as the excitation wavelength increases, contrary to that of 7-Me GMP at pH 5.0 which is virtually independent of excitation wavelength (Fig. III.3, p. 20). The fluorescence quantum yield of Me-DNA, corrected for absorption by A, C, and T, as a function of excitation wavelength exhibits differences from the behavior of that parameter of 7-Me GMP, pH 5.0 (Fig. III.14, p. 37). It is seen that at short excitation wavelengths the two curves agree to a relatively good extent but at long excitation wavelengths the curve for Me-DNA is much lower than that for 7-Me GMP. Also, the fluorescence quantum yield (q) of Me-DNA is about 14 times lower than that of 7-Me GMP (see Results). These differences imply that the optical properties of Me-DNA cannot be explained on the basis of emission from monomeric methylated guanine. These properties can be rather readily explained on the basis of emission from both monomeric G residues as well as from G residues that have an excimer-like conformation in their ground electronic state. That would explain the heterogeneity in the emission spectral properties, the low value of

q (as aggregates tend to be weakly fluorescent), and the dependence of (q) on excitation wavelength. It would also explain the weak dependence of the value of the degree of polarization, P, on the excitation wavelength and particularly the absence of a minimum at 265 nm, as the presence of more than one absorbing species would tend to smear out such dependence. In connection with this discussion, the absence of a very pronounced effect of 80% glycerol on P (Table III.1, p. 31) implies that flexibility of the G residues is not the source of the low values of P in buffer. The use of DNA with higher GC content would enhance the contribution from excimer-like structures and provide further evidence for the proposed mechanism of emission from Me-DNA. Such studies are proposed for further work. The evidence obtained in the present work regarding the ability of the G residues to approach each other and form excimer-like structures is an interesting finding which is of relevance in the photochemistry of the genetic material.

The question of energy transfer from the A, C, and T residues is now examined. G is the residue that absorbs at the longest wavelength region and, therefore, can serve as an acceptor of excitation energy from the other nucleotides. Figure III.14, p. 37, shows the q of Me-DNA (corrected for absorption by A, C, and T) vs. the excitation wavelength. The graph drops rather strongly above 285 nm. It turns out that in that spectral region the contribution of A, C, and T to absorption is small with G being the most absorbing species (Table III.4, p. 36). This observation tends to support a mechanism involving the transfer of excitation energy from A, C, and T to G at the DNA level.

The main conclusions of the present study are:

1. The data for the fluorophore 7-Me GMP support the notion that more than one species or electronic states are involved in the absorption and emission processes. The dramatic decrease in the value of the degree of polarization when exciting at 265 nm provides a clear indication of the heterogeneous nature of the light-absorbing molecular population.
2. After taking into account the contributions of A, C, and T residues, the optical properties of Me-DNA cannot be accounted for by considering the monomeric G residue as the only emitter. Apparently, neighboring G residues are able to approach each other and form excimer-like structures in the ground electronic state.
3. Evidence has been obtained for the occurrence of transfer of excitation energy from the A, C, and T residues to the G residue.

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