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The Fluorescence of Guanine and Guanosine

Effects of Temperature and Viscosity on Fluorescence Polarization and Quenching

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The fluorescence of guanine and guanosine dissolved in different acidified mixtures of water and methanol has been investigated by the use of polarized light. The fluorescence polarization spectra at low temperatures gave evidence for a third absorptive electronic transition within the envelope of the two major $\pi \rightarrow \pi^*$ transitions in the range between 35 000 cm^{-1} and 42 000 cm^{-1}. This additional transition may be of $n \rightarrow \pi^*$ or $\pi \rightarrow \pi^*$ type. The influence of viscosity and temperature on the previously described displacement of emission maxima towards higher frequencies, which accompanies cooling, has been studied further by varying the proportional amounts of water and methanol in the solvent. Rotational depolarization of the fluorescence has been measured and related to the temperature and composition of the solvent. Discrepancies between the measured macroscopic viscosity and the resistance against molecular movements have been pointed out. Approximate figures for the oxygen quenching are given. Cooling and addition of water to the solvent were both found to counteract oxygen quenching. Evidence for increased basicity of guanine and guanosine at low temperature is described. Adverse effects of high concentration of sulfuric acid, especially at low temperatures, are shown. The roles of temperature and viscosity in solvent quenching have been examined, without evidence for any definite set of mechanisms having been found. Revised fluorescence quantum yields are given. The highest yields were obtained near 150°K, *viz.* about 0.5 for guanine and 0.8 for guanosine. Our determinations of fluorescence quantum yields have been checked against data obtained by others, using similar and different methods. There was satisfactory agreement between the yields of Melhuish and Parker and our data. Substitution of anthracene for quinine as fluorescence standard did not alter our yields. The method of Weber and Teale appears, however, to give considerably higher yields for anthracene, phenol, tryptophan, and tyrosine than our method. To explain these discrepancies, possible errors in the procedure of Weber and Teale due to asymmetric scattering by large glycogen molecules, are discussed.

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Cooling of acid solutions of guanine derivatives in water:methanol (1:9 v/v) has previously been reported to greatly increase the fluorescence quantum yields and to move the emission maxima towards higher frequencies.[1a] Recently, the luminescence of some guanine derivatives has been studied at 77°K as functions of pH in ethylene glycol:water (1:1).[1b] In the present work polarized light has been used in further studies of the influence of cooling on the fluorescence of protonated guanine derivatives, and to obtain fluorescence polarization spectra. Rotational depolarization of the fluorescence has been investigated. The mechanisms of quenching have been analyzed. Solvent mixtures of different viscosities have been used in an attempt to separate the effects of temperature and viscosity. A preliminary account of our polarization data has been published.[2]

EXPERIMENTAL

Materials. Water was distilled twice in all-quartz equipment. Sulfuric acid, A.R. grade and methanol, optical grade ("Uvasole") were obtained from E. Merck, AG., Darmstadt, Germany. This methanol was unsatisfactory for high sensitivity work. In our experiments, however, further purification was unnecessary because the concentrations of fluorescent solutes were relatively high, and because the fluorescence cell emitted more light than the methanol. Phenol, A.R. grade, was also from Merck. Guanine (free base), guanosine, L-tryptophan and DL-tyrosine were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. The purity of guanine and guanosine was considered adequate in view of previous investigations.[1a,3] L-Tryptophane and L-tyrosine obtained from Dr. Theodor Schuchardt & Co., München, Germany, were used before and after recrystallization. Quinine hydrogen sulfate and anthracene (blue fluorescence), laboratory reagent grade, were products of The British Drug Houses, Ltd., Poole, England.

Fluorescence spectrophotometer. The basic instrument has been described previously.[4] It has since been equipped with Glan-Thompson polarizers for the exciting and the emitted light. The polarizer prisms are heated electrically to avoid condensation of moisture

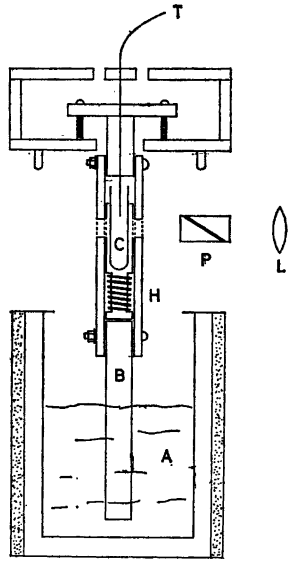

Fig. 1. Semi-schematic drawing of the redesigned cell holder.

A: Liquid coolant (air or nitrogen); B: Copper bar; C: Sample cell; H: Electric heater coil; L: Condensing lens for the exciting light; P: Glan-Thompson polarizer prism; T: Thermocouple (chromel-alumel).

when the sample is cooled. The cell holder has been redesigned to facilitate cooling and heating of the sample cell (Fig. 1). In most experiments a cylindrical silica cell with internal diameter 0.6 cm was used. Oxygen effects were investigated in square silica cells by bubbling nitrogen or oxygen through the fluorescent sample. The spectral sensitivity distribution of the photometer [4] had to be redetermined with the polarizer in the path of the emitted light.

Procedure. Since the polarizers reduce the sensitivity of the fluorescence spectrophotometer, relatively high concentrations of guanine and guanosine were used, *viz.* about 5×10^{-5} M. The corresponding absorbances were between 0.2 and 0.3 cm^{-1} at 35 000 cm^{-1}.

Fluorescence quantum yields were calculated from integrated, corrected emission spectra. Quinine in 0.5 N sulfuric acid was employed as standard. The apparent yield of this standard solution was found to decrease from 0.546 (Melhuish's value [29]) to 0.44 when the exciting light was changed from 366 nm to 35 000 cm^{-1} (*i.e.* 286 nm).

Apart from the quantum yield, several factors influence the measured fluorescence intensities. Corrections have to be applied to compensate for these. Thus the contraction of the solvent accompanying cooling leads to an increased concentration and absorbance of the fluorescent solute. This was compensated by multiplication of the intensity readings with the relative solvent volume at the temperature in question. The volume-temperature relationships of the three solvent mixtures used were measured in separate experiments. The increase of index of refraction due to the increased density at low temperatures [5] tends to reduce the fraction of the emitted light which enters the emission monochromator.[6] The corresponding correction will be discussed below.

Polarization leads to uneven spatial distribution of the emitted light.[7] In our determinations of quantum yields both polarizers were oriented to transmit vertically polarized light, *i.e.* light with the electric vector perpendicular to the plane defined by the exciting beam and the direction of observation of the fluorescence. If the emitted light is polarized positively, the intensity of the light emitted in any direction in this plane is above the average taken over the whole sphere. Hence a correction factor smaller than unity has to be used. On the basis of Ref. 7 this factor can be shown to be:

$$(3 - P)/3(1 + P)$$

P is the degree of polarization, defined by:

$$P = (I_z - I_x)/(I_z + I_x)$$

I_z and I_x, respectively, are the intensities of emitted light with electric vector parallel to and perpendicular to the electric vector of the exciting light.

The degree of polarization was measured at the emission maxima, the exciting light being vertically polarized. The readings obtained when the exciting light was horizontally polarized were used to correct for instrumental artefacts.[8]

The viscosities of the mixtures of water and methanol were measured with a rotating cylinder viscometer (Haake Viscotester VTL-180) as previously described.[1a]

RESULTS

Viscosities of the water:methanol mixtures. The interpretation of some of the fluorescence data requires knowledge of the solvent viscosities over a large range of temperatures. Fig. 2 shows the viscosities of the three water: methanol mixtures used as solvents (1:3, 1:9, and 1:19 v/v) plotted against reciprocal temperature. Near room temperature the viscosity was not greatly influenced by the proportional amounts of water and methanol. Increasing proportions of water considerably increased the slopes of the lines. Hence, at low temperatures the addition of water strongly increased the viscosity. The slope of each line increased as the temperature was lowered, the transition between regions of different slopes being confined to small temperature ranges.

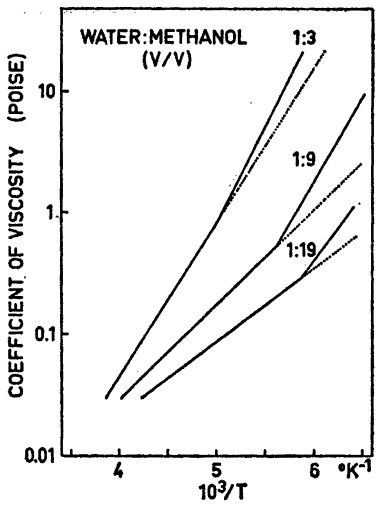

Fig. 2. Coefficients of viscosity measured in three different mixtures of water and methanol plotted against reciprocal temperature. Proportions of water and methanol (v/v) are shown at each curve. Experimental lines are fully drawn. The dotted lines represent the extrapolations used in the discussion.

Fluorescence polarization spectra at low temperatures. Fig. 3 shows the degree of polarization, P, of the fluorescence of guanine and guanosine as functions of the wavenumber of the vertically polarized exciting light. The solvent was water:methanol (1:9 v/v), and the temperatures were sufficiently low for P to have attained its low temperature limits within experimental error. Fig. 3 has been arranged to display the correspondence between the maxima in the absorption and fluorescence excitation spectra (taken from Ref. 1a) and the values of P.

The polarization spectra of guanine and guanosine both show that the maximal values of P near 0.4 were found only near the low frequency limits

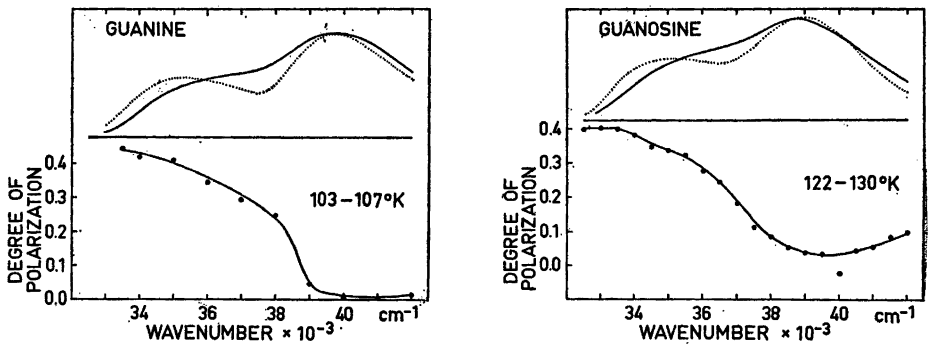

Fig. 3. Fluorescence polarization spectra at low temperatures in acid solutions (lower half of figure). Water:methanol was 1:9 (v/v) and sulfuric acid was 0.1 N. For comparison, the upper half of the figure shows fluorescence excitation spectra at low temperatures (dotted curves) and absorption spectra at room temperature (fully drawn curves) taken from Ref. 1a.

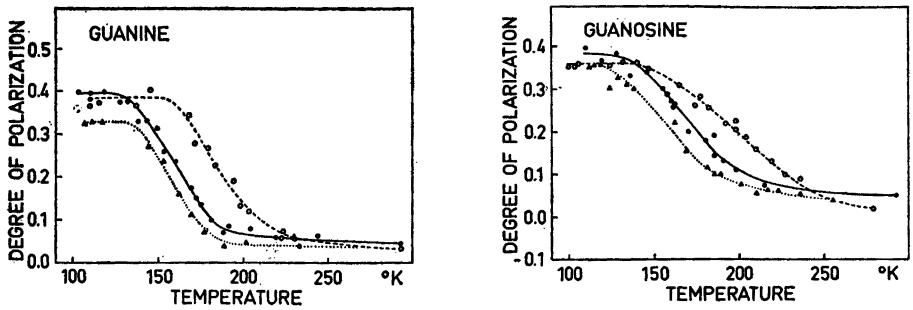

Fig. 4. Fluorescence polarization as function of temperature, measured in three different mixtures of water and methanol. The exciting light was vertically polarized, wavenumber 35 000 cm^{-1}. The degree of polarization was measured at the emission maxima. The symbols refer to solvent composition: Water:methanol 1:3 (v/v) O - - - - - O, 1:9 ● ——— ●, and 1:19 ▲ . . . . . ▲.

of the lowest absorption maxima. The degree of polarization was close to zero in the maxima at 39 000—40 000 cm^{-1}.

Effects of solvent composition and temperature on the degree of polarization. The main purpose of these measurements (Fig. 4) was to obtain correction factors for the determinations of fluorescence quantum yields. Therefore, the wavenumber of the vertically polarized exciting light was 35 000 cm^{-1}, which was also used when the yields were measured, although a slightly lower wavenumber would have been required to obtain maximal values of P at low temperatures (Fig. 3).

As expected, cooling increased the degree of polarization. At room temperature and at very low temperatures P was independent of solvent composition within experimental error. At intermediate temperatures, however, P was enhanced when the viscosity was increased by the addition of water.

Table 1. Solvent viscosity and degree of polarization, P. An intermediate value of P, *viz.* 0.22, has been chosen. The coefficients of viscosity in three different water:methanol mixtures corresponding to $P = 0.22$ have been tabulated. The coefficients of viscosity given in parentheses have been taken from the dotted, extrapolated lines in Fig. 2, while the other values have been obtained from the experimental, fully drawn lines in Fig. 2. Relative fluorescence quantum yields, F/F_0, have been taken from Fig. 7.

	Guanine			Guanosine		
Water:methanol (v/v)	1:19	1:9	1:3	1:19	1:9	1:3
Temperature, T °K	153	161	185	156	171	193
Coefficient of viscosity, η poise	1.5	3.4	3.6	1.1	1.1	1.6
T/n (based on η in parentheses)	(0.75) 204	(1.5) 107	(2.8) 66	(0.62) 233	(0.80) 214	(1.45) 133
F/F_0	0.91	0.91	0.83	0.90	0.81	0.73

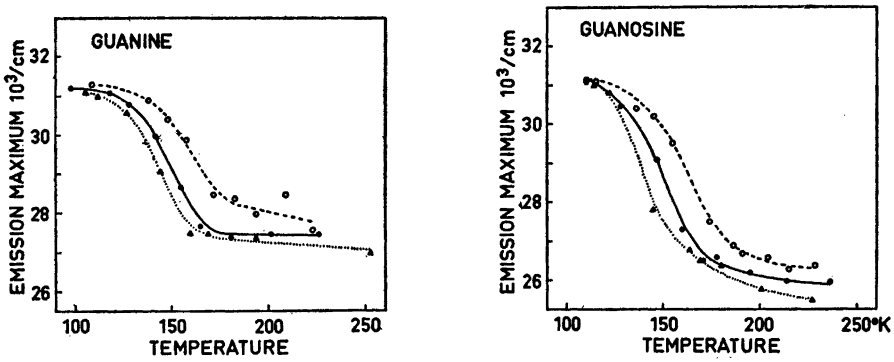

Fig. 5. Wavenumber of the fluorescence emission maximum in three different mixtures of water and methanol plotted as a function of the temperature. The maxima refer to corrected emission spectra, the ordinate of which was: "Quanta emitted per unit wavenumber interval". Same symbols as in Fig. 4.

To test whether the relationship between P and the macroscopic viscosity of the solvent was independent of the composition of the water:methanol mixture, Table 1 has been arranged on the basis of data from Fig. 4. It is evident that only slightly different macroscopic viscosities were needed in the three solvents to bring P to 0.22, *i.e.* a value midway between the limits of P at room temperature and at low temperature.

Wavenumber of emission maximum. Effects of solvent composition and temperature. Fig. 5 confirms the earlier observation that cooling moves the emission maximum towards higher wavenumbers. The location of the emission maximum at very low temperatures was independent of the solvent composition, and was the same for guanine and guanosine. At intermediate temperatures the maximum was shifted towards the low temperature limit when the viscosity was increased by the addition of water. Table 2 shows, however,

Table 2. Solvent viscosity and location of emission maximum. The tabulated viscosities correspond to emission maxima located midway between the limits at high and low temperatures. The wavenumber of this intermediate emission maximum was 29 125 cm^{-1} for guanine and 28 625 cm^{-1} for guanosine. The viscosity coefficients of the three water:methanol mixtures have been obtained from the dotted, extrapolated lines in Fig. 2. At the temperatures and viscosities given in the table the quenching of the fluorescence was insufficient to move the emission wavenumbers to any measurable extent.

	Guanine			Guanosine		
Water:methanol (v/v)	1:19	1:9	1:3	1:19	1:9	1:3
Temperature, T °K	143	150	165	140	150	163
Coefficient of viscosity, η poise	1.4	3.5	20	1.8	3.5	24
T/η	102	43	8.3	78	43	6.8

that the location of the emission maximum is not rigidly determined by the macroscopic viscosity, but is influenced by the solvent composition in other ways as well.

FLUORESCENCE QUANTUM YIELDS

Before the influence of solvent composition and temperature on the fluorescence quantum yields could be determined, it was necessary to investigate whether the concentration of sulfuric acid or the presence of oxygen was of critical importance.

Effects of sulfuric acid. Two peculiarities in the effects of sulfuric acid were found. First, 0.001 N sulfuric acid was sufficient to give maximal fluorescence of guanine at low temperatures, while ten times as much H_2SO_2 was required for the fluorescence to be maximal at room temperature. Guanosine exhibited analogous behaviour at concentrations of sulfuric acid about ten times higher than in the experiments with guanine (Table 3, the five lower rows).

Table 3. Fluorescence quantum yields of guanine and guanosine at high and low temperatures. Effects of variations of the proportional amounts of water, methanol and sulfuric acid. The yields were determined with quinine in 1 N sulfuric acid as standard. Use of anthracene as fluorescence standard, the quantum yield of which has also been determined by Melhuish,[29] would give nearly identical results (Table 4).

Solvent		Guanine			Guanosine		
Water: methanol v/v	H_2SO_4 N	293°K	147°K	111°K	293°K	147°K	111°K
1:3	0.038	0.024	0.40		0.017	0.65	
1:10	0.05	0.030	0.50	0.39	0.026	0.78	0.56
1:20	0.05	0.030	0.54		0.028	0.76	
1:9	0.001	0.017	0.51	0.43			
1:9	0.01	0.024	0.50	0.39	0.020	0.93	0.65
1:9	0.1	0.031	0.50	0.33	0.033	0.78	0.54
1:9	1.18	0.020	0.32	0.09	0.033	0.78	0.54

Secondly, Table 3 shows that the fluorescence of guanine was considerably decreased by high concentrations of sulfuric acid. The fluorescence was reduced to about 2/3 of its maximal value at room temperature and at 147°K by 1.18 N H_2SO_4. At still lower temperatures this effect of sulfuric acid was greatly enhanced (Table 3). This second effect of sulfuric acid was, however, not observed with guanosine.

Oxygen quenching. Saturation of the solutions with oxygen under atmospheric pressure at 293°K reduced the fluorescence of guanine and guanosine only slightly (10 % or less). Partial removal of oxygen had more profound effects. Thus the fluorescence increased about 15 % in the solutions rich in water (water:methanol 1:3 v/v). This increase was amplified to between 50 and 100 % in different experiments in the solutions with 1/10 or less water.

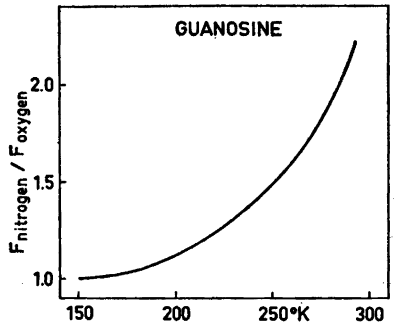

Fig. 6. Oxygen quenching of the fluorescence of guanosine as a function of temperature. The solvent was water:methanol 1:19 (v/v) with 0.05 N sulfuric acid. Ordinate: Fluorescence yield after equilibration with bubbling nitrogen in 15 minutes/ fluorescence yield after 5 minutes subsequent equilibration with oxygen at atmospheric pressure.

Fig. 6 describes a typical experiment (guanosine in water:methanol 1:19 v/v) and shows that the oxygen quenching is counteracted by cooling and abolished near 170°K.

Effects of temperature and solvent composition on fluorescence quantum yields. Fig. 7 illustrates the considerable increase of the fluorescence which accompanied cooling in the three different air saturated mixtures of water and methanol. The fluorescence yields, F, have been expressed as fractions of theoretical maximal yields, F_0, which were equated to 1.1 times the highest measured yield for each curve. Absolute fluorescence quantum yields referred to quinine as standard are given at selected temperatures in Table 3.

Increasing proportions of water decreased the fluorescence at room temperature and possibly at 147°K (Table 3). At intermediate temperatures, however, the fluorescence was augmented when the viscosity was increased by the addition of water (Fig. 7). These effects of solvent composition were hardly discernible from a comparison of the curves obtained in water:methanol 1:19 and 1:9 v/v, but the use of water:methanol 1:3 demonstrated the effects clearly.

The apparent decrease of fluorescence yield previously reported[1a] to accompany cooling below 155°K were observed again in the present work (Fig. 7). The phenomenon was, however, less pronounced in the latter series

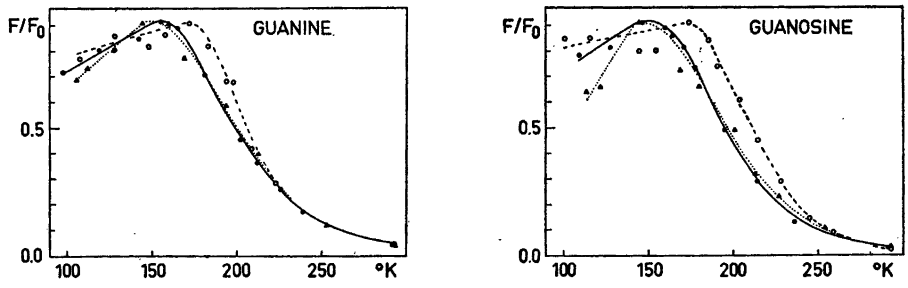

Fig. 7. Relative fluorescence quantum yields as functions of the temperature in three different mixtures of water and methanol. The yields have been expressed as fractions of theoretical maximum yields, which have been set to 1.1 times the highest observed yields (see discussion). Same meaning of symbols as in Fig. 4. Sulfuric acid was 0.01 N in water:methanol 1:9 (v/v), but was increased to 0.05 N in the other two mixtures.

of experiments where polarized light and the appropriate corrections were utilized. Furthermore, Fig. 7 and Table 3 show that high concentrations of sulfuric acid and decreasing proportions of water in the solvent tend to enhance this adverse effect of extreme cooling on the fluorescence yield.

Accuracy of fluorescence quantum yields. In order to detect systematic errors in our determinations of quantum yields, the fluorescence quantum yields of anthracene, phenol, tryptophan, tyrosine and salicylate were determined and compared to values from the literature (Table 4). The figures for anthracene and phenol indicate that our results are in satisfactory agreement with the data obtained by Melhuish [29] and Parker.[9,10] The yields for anthracene and phenol given by Weber and Teale [11] have been corrected to their corresponding values in air free ethanol to allow comparison with the other data. These corrections have been based on effects of deaeration and interchange of solvents measured in the present investigation. It is readily apparent that the method of Weber and Teale tends to produce considerably higher quantum yields than the other methods. This tendency is even more pronounced with tryptophan and tyrosine. The yields of salicylate are not, however, subject to the same discrepancy.

DISCUSSION

Viscosities of the water:methanol mixtures. The viscosities were measured in order to allow interpretations of the effects of viscosity on various molecular relaxation phenomena involved in fluorescence processes. The uncertain relationship between the measured "macroscopic viscosity" and the "microscopic viscosity" which determines the resistance offered against movements of individual solute molecules, does, however, seriously limit the reliability of attempted interpretations. It is known, for instance, that the resistance against the diffusion of oxygen [14] and against rotational Brownian movements of luminescent molecules [15,16] may be considerably lower than the bulk viscosity indicates.

Several sources of error are inherent in the measurements of macroscopic viscosity. First, the temperature was probably not entirely uniform throughout the liquid in the viscometer. This error was likely to be at a minimum in the period of slow, spontaneous reheating of the viscometer. Fig. 2 is based on measurements in this phase. Secondly, the shearing movements to which the liquid was subjected in the viscometer altered the state of this liquid, as evidenced by visible crystallization. The unstirred liquids in the fluorescence cell remained clear down to much lower temperatures. It may well be that stirring altered the viscosity. This error is probably of significance only in the low-temperature portions of the curves in Fig. 2. Thus, it must be admitted that the latter source of error may contribute to the fairly sudden increase of the slopes of the curves in Fig. 2 at certain stages of cooling, although visible crystallization did not occur at the temperatures shown. Analogous changes of slopes of curves like those in Fig. 2 are known to occur in hydrogen bonded liquids where the degree of association tends to increase on cooling.[17] The suddenness of the change of slope may be connected with restrictions imposed on molecular rotations by cooling.[18]

Fluorescence polarization spectra at low temperatures. It is appropriate to compare our spectra for the protonated guanine and guanosine to polarization data for neutral guanine and guanosine in the literature.[19] Protonation appears to alter the polarization spectra in two ways. First, the degree of polarization, P, at 39 000—40 000 cm^{-1} is changed from —0.2 to zero (Fig. 3). This indicates that the angle between the absorptive transition at 39 000—40 000 cm^{-1} and the transition associated with fluorescence decreases from 70° to about 55° upon protonation.

Secondly, P fails to maintain the maximum value of about 0.4 all the way across the lowest absorption band (Fig. 3). This cannot easily be explained by any plausible shape of the overlapping tail of the next major absorption band. The explanation may be that a third absorption band is intercalated between the two main bands. Whether this third absorption band represents another $\pi \rightarrow \pi^*$ transition [27,31] or whether an $n \rightarrow \pi^*$ transition is responsible, is uncertain. The contribution of an $n \rightarrow \pi^*$ transition to the absorption at 35 000—36 000 cm^{-1} would explain the fall in the value of P because the transition moment of the $n \rightarrow \pi^*$ transition is perpendicular to the molecular plane, while the $\pi \leftarrow \pi^*$ transition associated with fluorescence is polarized in the molecular plane.[20,21] Similarly, the alternative hypothetical third $\pi \rightarrow \pi^*$ transition must be polarized at an angle to the fluorescence transition.

P as a function of temperature and solvent. The degree of polarization, P, is decreased by the rotational Brownian movements which occur within the lifetime of the fluorescent excited state. This is expressed quantitatively in the Perrin-Levshin formula.[22]

$$1/P - 1/P_0 = (1/P_0 - 1/3)(RT/V\eta)t \qquad (1)$$

P_0 is the limit of P at infinite viscosity or 0°K.
V is the molar volume of the assumed spherical body performing rotational Brownian movements.
η is coefficient of viscosity in poise.
t is the lifetime of the fluorescent state.

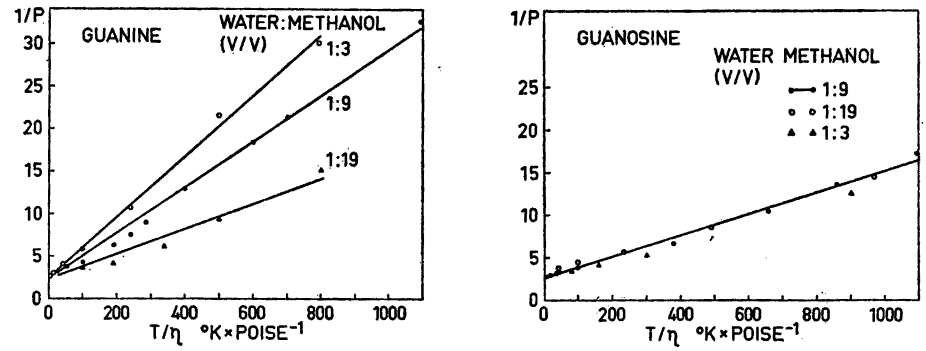

Fig. 8. Reciprocal degree of polarization ($1/P$) plotted against T/η. (T = absolute temperature, η = coefficient of viscosity). This plot is based on the Perrin-Levshin formula (eqn. 1). The values of P were taken from Fig. 4. The coefficients of viscosity have been taken from the low-slope portion of each curve in Fig. 2 and from the dotted, extrapolated lines.

The curves in Fig. 4 confirm eqn. 1 qualitatively in the sense that an increase in the viscosity by the addition of water increases the degree of polarization.

To examine whether eqn. 1 is obeyed quantitatively, the values of $1/P$ have been plotted against T/η in Fig. 8. Two corrections had to be applied to the measured data to obtain straight lines as predicted by eqn. 1. First, P had to be corrected to the smaller value it would have had in the absence of quenching. This correction is based on the assumption that quenching decreases the lifetime of the excited state,[22] *i.e.* that the quenching processes are competitive with fluorescence.

Secondly, it was found that the viscosities taken from the dotted, extrapolated lines in Fig. 2 yielded straight lines in Fig. 7, while the higher viscosities obtained from the fully drawn, experimental lines (Fig. 2) did not. We conclude that the resistance towards molecular rotations appears to follow the simple Arrhenius equation for a rate process with activation energy independent of temperature, even if the macroscopic viscosity shows considerable departure from this simple behaviour as evidenced by the increasing slopes of the lines in Fig. 2.

Fig. 8 shows that the Perrin-Levshin formula is obeyed in our system only when the somewhat uncertain assumption as to the simple Arrhenius behaviour of the "microscopic viscosity" is introduced. The figure illustrates further difficulties inherent in the concept of "microscopic viscosity": The curves for guanine in Fig. 8 indicate that the addition of water to the solvent increases the measured macroscopic viscosities more than the resistance towards molecular rotations. The data for guanosine, however, indicate that the attachment of ribose to N-9 perturbs this relationship, since all points are scattered around the same straight line. We conclude that the "microscopic viscosity" depends on specific interactions between solute and solvent molecules.

Table 1 further illustrates the discrepancies between macroscopic and microscopic viscosity. Thus the macroscopic viscosity which corresponds to a given resistance towards rotation of the fluorescent solute, as evidenced by a given intermediate value of P, tends to increase as the proportion of water increases. This tendency is clear irrespective of whether measured or extrapolated values for the viscosity are used. It is also shown in Table 1 that the viscosity needed to hinder the rotation of guanine to a specified extent is somewhat larger than the viscosity which affects the rotation of guanosine equally. This observation is in accordance with eqn. 1 since the attachment of ribose to the guanine nucleus is likely to increase the molar volume of the rotating unit.

Wavenumber of emission maximum. Effects of solvent composition and temperature. At high viscosities the fluorescence occurs from intermolecular "Franck-Condon excited states".[23] Low viscosities, however, permit the equilibrium excited state of lower energy to be attained through relaxation of intermolecular distances before most of the fluorescence photons are emitted.[1a,23] Hence the average wavenumber of the emitted photons tends to decrease as the viscosity decreases.

Comparison of Figs. 4 and 5 demonstrates that considerably lower temperatures and higher viscosities are required to arrest the solvated fluorescent

molecules in the intermolecular Franck-Condon state than to inhibit completely rotations of the excited solute. This probably shows that the force driving the relaxation of the intermolecular distances towards the equilibrium values is particularly strong near the Franck-Condon state, *i.e.* that this force is elastic.

Table 2 shows that the resistance towards the relaxation of intermolecular distances increases much more slowly than the macroscopic viscosity as the proportion of water is increased. This is more pronounced than the analogous phenomenon in Table 1. The attachment of ribose to N-9 does not influence perceptibly the relaxation from the intermolecular Franck-Condon state.

Summary of discrepancies between macroscopic and microscopic viscosity. The results show that the resistance to molecular movements bears no simple relation to the measured macroscopic viscosity. The microscopic viscosity is even different for different types of molecular movements. The solvation sphere of the excited solute may preferentially contain one of the solvent components. Thus it is possible that the immediate surroundings of the fluorescent molecule vary less than the bulk of the solution when the relative proportions of water and methanol are altered. This may explain why the addition of water tends to increase the microscopic viscosity by a smaller amount than the effect on the measured macroscopic viscosity. If the molar volume of the rotating unit varies with the solvent composition, and if the force driving the relaxation of intermolecular distances also varies with the solvent, the interpretation of our results in terms of microscopic viscosity is of limited value. We conclude that the measurements of polarization and emission maxima do not give entirely reliable indications as to the viscosity which could be used in a meaningful interpretation of the role of viscosity in solvent quenching.

FLUORESCENCE QUANTUM YIELDS AND QUENCHING MECHANISMS

Effects of sulfuric acid. Protonation of the guanine ring, probably at the N-7 position, is a prerequisite for the appearance of strong fluorescence.[1a,3] A reasonable interpretation of our results (Table 3) is that the basicity of N-7 increases as the temperature is lowered, since a smaller amount of sulfuric acid was required to accomplish the protonation at low temperature than at room temperature. The pK_a appears to be at least one unit higher at 147°K than at 293°K. This conclusion is applicable to guanosine as well as to guanine.

The other peculiarity in the effects of sulfuric acid was observed with guanine only, *viz.* a pronounced decrease of fluorescence in very acidic solutions. The observation that this effect was amplified by very low temperatures and high viscosities rules out collisional quenching as the cause. Whether another proton is attached to guanine in the ground state or in the excited state, or whether the guanine cation forms a non-fluorescent complex with the sulfate anion at high concentrations of sulfuric acid, remains to be settled.

Oxygen quenching. The data on the oxygen effect are only approximate since oxygen cannot be completely removed with the equipment presently at our disposal. Furthermore, the evaporation of the solvent during equilibra-

tion with the gas which bubbles through the solution, leads to an increase in the absorbance. The corresponding increase in the fluorescence intensity tends to mask the oxygen quenching in our experiments, because equilibration with oxygen was performed after the measurement of "oxygen free" fluorescence. The extinction of oxygen quenching at low temperature is reasonable in view of the diffusion process involved. It must be admitted, however, that the equilibration of the fluorescent solution with the ambient atmosphere during the cooling procedure may contribute to this result. Since oxygen is about 5.5 times more soluble in methanol than in water, it is not surprising that the addition of water was found to counteract the oxygen quenching.[24,25]

Solvent quenching. Effects of temperature and solvent composition. Since oxygen and possibly sulfate ions account for only a small fraction of the serious quenching observed at higher temperatures in our system, we assume that the main quenching mechanism depends on interactions between the excited solute and the ambient solvent molecules, *i.e.* solvent quenching. The simplest possible set of reactions which can account for our observations is:

Process	Reaction	Rate
Fluorescence	$A^* \rightarrow A + h\nu$	$k_1 A^*$
Quenching without activation energy	$A^* \rightarrow A$	$k_2 A^*$
Solvent quenching with thermal activation energy, E	$A^* \rightarrow A$	$k_3 A^* e^{-E/RT}$

The fluorescence quantum yield, F, is then

$$F = k_1/(k_1 + k_2 + k_3\ e^{-E/RT})$$

It follows that

$$1/F - 1/F_0 = (k_3/k_1)\ e^{-E/RT}$$

where

$$1/F_0 = 1 + k_2/k_1$$

F_0 evidently becomes the low temperature limit of F.
Rearrangement and logarithmic transformation yields

$$\log_{10}(1/(F/F_0)-1) = -0.4343\ E/RT + \text{constant} \qquad (2)$$

To test whether our results fit eqn. 2, $\log_{10}(1/(F/F_0)-1)$ has been plotted in Fig. 9 against $1/T$. The quantum yields have been corrected to the "oxygen free" values by means of the curve in Fig. 6, except for the data obtained in water:methanol 1:3 v/v where the oxygen effect was small. By trial and error F_0 has been chosen to be higher than the highest measured value of F for each curve by a factor of 1.1. The error due to a discrepancy of a few per cent between the chosen F_0 and the true F_0 affects significantly only the extreme lower right portions of the plots in Fig. 9.

Fig. 9 documents that eqn. 2 describes our results satisfactorily. Addition of water to the solvent appears to enhance the activation energy of the solvent quenching. This is illustrated in Table 5 which also permits comparison of the activation energies of quenching and the activation energies of viscous flow which have been derived from the lower slope of each curve in Fig. 2. Since the two sets of activation energies show similar dependence on the

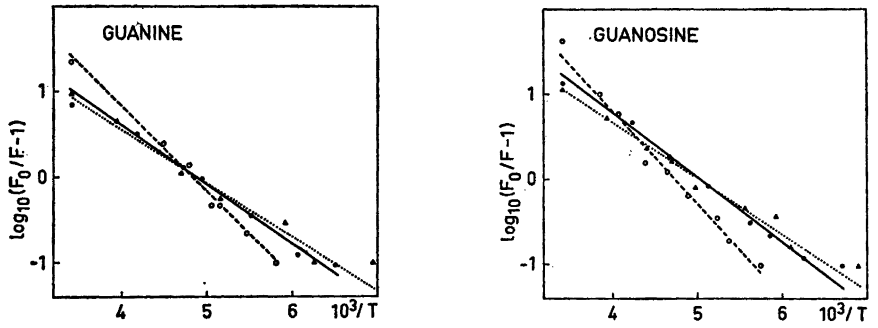

Fig. 9. The influence of temperature on the fluorescence quantum yields, F, examined with reference to eqn. 2. Same meaning of symbols as in Fig. 4. The values of F_0 have been set to 1.1 times the highest measured F for each curve (see discussion).

solvent composition, a reasonable interpretation is that solvent quenching operates through molecular movements similar to those involved in diffusion and viscous flow. An alternative expression for the rate of the solvent quenching would then be:

$$\text{rate} = k_4(T/\eta)$$

If the viscosity is supposed to follow the simple Arrhenius equation (*i.e.* straight lines in Fig. 2), it can be shown that the rate expression assumes a form very similar to the one used above. Hence our results do not show clearly whether molecular movements against viscosity are sufficient to produce solvent quenching, or whether such quenching involves other processes with activation energy somehow dependent on the solvent. Bowen's proposal [26] that thermal activation produces quenching only if subsequent "diffusion type movements, less frequent in viscous solvents" can be performed, did not fit well with our results. Drobnik and Augenstein [27] pointed out that the fluorescent solute should be more susceptible to solvent quenching in the equilibrium excited state than in the intermolecular Franck-Condon state where the positions of the ambient solvent molecules are unfavourable to strong solute-solvent interactions. The corresponding role of viscosity would be to control the rate with which the easily quenched equilibrium excited state is attained. A comparison of Figs. 5 and 7 shows that our results are compatible with this idea, since appreciable solvent quenching does not occur until most of the fluorescence is emitted from the equilibrium excited state.

Sources of error in our determinations of fluorescence quantum yields. The quantum yields in Table 3 can be correct only if the effective, apparent quantum yield of our quinine standard solution is 0.44. Repeated measurements have shown that the apparent yield falls from 0.546 at 366 nm (Melhuish's value [29]) to 0.44 when excited at 35 000 cm^{-1}. If this decrease in quantum yield is due to the presence of absorbing impurities at 35 000 cm^{-1}, it does not represent the behaviour of pure quinine, but it does not introduce errors in the quantum yields based on this standard solution. The close agreement between the fluorescence yield of anthracene determined against our quinine

standard (0.26) and the value given by Melhuish (0.27) indicates the absence of significant errors (Table 4).

Some corrections required in very accurate work to relate measured fluorescence intensities to the true quantum yields of fluorescent solutions,[28] have not been applied in the present paper. The most important of these is needed because an increase, dn, of the index of refraction, n, of the solution, reduces the fraction of the fluorescence light which is emitted within a specified small space angle outside the cell, perpendicular to the cell surface.[6] In the present investigation the index of refraction has been altered by the change of solvent composition and by the increase of the density of the solvents due to cooling. For rectangular cells the corresponding correction factor is $(n + dn)^2/n^2$.[6] For cylindrical cells, which were used in the present work, the factor can be shown to be approximately $(n + dn)/n$. It is estimated that the fluorescence yields at 147°K (Table 3) are about 5 % too low due to the omission of this correction.

Accuracy of the quantum yields. Comparison with literature data. The uncertainties inherent in our determinations of quantum yields made it desirable to check our method against data obtained by others using similar and different methods. Our fluorescence yields for anthracene and phenol are in good agreement with the values reported by Melhuish [29] and Parker [9,10] (Table 4). In general, this indicates that our quantum yields are not subject to large errors. Furthermore, the agreement shows that anthracene can be substituted for quinine as fluorescence standard, provided the quantum yield of quinine is assumed to drop from 0.546 to 0.44 when excitation is changed from 366 nm to 35 000 cm^{-1} (286 nm), as shown in a previous study.[4]

Table 4. Fluorescence quantum yields reported by different authors. Anthracene and phenol were dissolved in ethanol. This was deaerated by bubbling nitrogen through the solution. The other compounds were dissolved in water saturated with air. No buffers were added.

Compound	Børresen (present work)	Melhuish [29]	Parker [9,10] (based on Melhuish's value for anthracene)	Shore and Pardee [13]	Weber and Teale [11,12]
Anthracene	0.26	0.27			0.30 (0.38) [a]
Phenol	0.19		0.16		0.32 [b]
Tryptophan	0.106 0.108 [c]			0.06	
Tryptophan, recrystallized	0.119				0.20
Tyrosine	0.087 0.088 [c]			0.04	
Tyrosine, recrystallized	0.088				0.21
Salicylate (sodium salt)	0.27				0.28

[a] Value in parenthesis has been corrected for oxygen quenching.

[b] The yield has been corrected from 0.22 found in water by Weber and Teale to the corresponding value in oxygen-free ethanol.

[c] Commercial samples from different sources.

If our results are approximately true, there must have been a large systematic error in the method of Weber and Teale,[11,12] since they reported considerably higher yields for tryptophan and tyrosine than we have found (Table 4). Shore and Pardee [13] published even lower yields for tryptophan and tyrosine than those obtained in the present work. This supports the suggestion that the values of Weber and Teale may be too high. The latter authors assumed that the scattering of light from a glycogen solution was equivalent to the fluorescence of a substance with quantum yield equal to unity, after proper corrections for the effects of polarization on the spatial distribution of the scattered and fluorescence light had been applied. To explain the high yields of Weber and Teale, we propose a source of error inherent in this assumption which appears not to have been discussed properly in their original paper.[11] Very large glycogen molecules, with molecular weights up to several hundred millions, scatter light preferentially under small angles with respect to the direction of the incident light.[30] This is due to interference between light scat-

Table 5. Activation energies of viscous flow and solvent quenching. The activation energies of viscous flow have been derived from Fig. 2. The lower left (high temperature) portion of each curve has been used in conjunction with the equation

$$\text{Viscosity coefficient} = k\ e^{E/RT}$$

The activation energies of solvent quenching have been derived from the slopes of the lines in Fig. 9 by the use of eqn. 2. Activation energies are given in kcal/mole.

Water:methanol, v/v	Activation energy of viscous flow	Activation energy of solvent quenching	
		Guanine	Guanosine
1:3	5.8	4.6	5.0
1:9	3.6	3.2	3.5
1:19	2.8	2.9	3.0

tered from different areas on the same particle. The light scattered from such particles may be negligible in the perpendicular direction, in which Weber and Teale observed the scattering, compared with the average intensity taken over the whole sphere. This phenomenon was observed with solutions of glycogen from some, but not all, sources by Harrap and Manners.[32] This effect increases as the wavelength of the light decreases.

If a significant fraction of the glycogen in some of the standards used by Weber and Teale were present as such large molecules, the intensity of the light scattered in the perpendicular direction must have been below the average over all directions, and more so than the correction for the effect of polarization compensates for. Thus a disproportionately low perpendicular light intensity represents a quantum yield equal to unity, and a too high quantum yield will consequently be assigned to a measured fluorescence intensity.

This error probably varies from one glycogen preparation to another due to different ranges of molecular weights, even if the average molecular

weights do not differ. It is even conceivable that sedimentation of large molecules during storage might alter the properties of a glycogen standard. Such variations may explain why the data on salicylate (Table 4) did not reveal the same discrepancy as the figures for tryptophan and tyrosine.

This error in the work of Weber and Teale, if present, has been transferred to the quantum yields of the luminescence of adenine and guanine derivatives given by Longworth, Rahn and Schulman,[1b] since these authors used tryptophan as standard with the fluorescence yield given by Weber and Teale.

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