ACTA CHEMICA SCANDINAVICA 19 (1965) 2100–2112

Effects of Cooling on the Fluorescence of Biological Guanine Derivatives

HANS CHR. BØRRESEN*

Institute of Clinical Biochemistry, University of Oslo, Rikshospitalet, Oslo, Norway

The fluorescence of guanine, guanosine, GMP, GDP, and GTP in acid solutions of methanol:water (9:1) increased about twentyfold as the solutions were cooled from 300°K to 165°K. Maximal quantum yields near 0.5 were attained. Further cooling decreased the fluorescence as measured perpendicularly to the exciting beam.

Between 180°K and 130°K the emission maximum of guanine in acid solution moved from 27 500 cm^{-1} to 30 600 cm^{-1}. The derivatives of guanine at acid pH underwent a slightly greater blue shift from 26 000 cm^{-1} to 30 500 cm^{-1}. At alkaline pH considerably smaller blue shifts were observed (1500 cm^{-1}), but they occurred in the same temperature interval as at low pH. The blue shifts have been related to viscosity measurements, and are discussed in terms of long-lived intermolecular Franck-Condon states in rigid media at low temperatures.

In the alkaline range with an apparent pH of about 11.5 in the methanol:water mixture, only guanine itself and GMP emitted observable fluorescence at room temperature. This fluorescence also increased on cooling. The corrected excitation spectra were not, however, congruent with the absorption spectra. Reasons are given for the belief that this incongruity shows the existence of two tautomeric forms of the guanine ring with one net negative charge. According to this interpretation one tautomer is fluorescent at all temperatures, while the other tautomer fluoresces only at temperatures below 180°K or 160°K.

Fluorescence methods are potentially useful in the investigation of the role played by purine bases in biological structures and processes. The sensitivity and specificity of fluorescence spectrophotometry often supersede that of absorption spectrophotometry. Luminescence phenomena add important information to the absorption data as far as the electronic structure of molecules is concerned. Fluorescence can profitably be used in the study of localization and function of fluorescent molecules in intact cells or in exposed animal organs.

* Fellow of *The Norwegian Cancer Society.*

Several factors do, however, limit the usefulness of fluorescence in the study of biological purine derivatives. Thus the weakness of the fluorescence found at room temperature from the derivatives of adenine and guanine [1-3] detracts from the usual attractiveness of fluorescence spectrophotometry as a quantitative or qualitative analytical method. A limited number of studies of the luminescence of purine derivatives at 77°K [4-6] has indicated that the fluorescence quantum yield in some cases increase towards 0.1. Further data are needed, however, to provide a basis for analytical use of low temperature luminescence measurements.

It would be of considerable interest to employ fluorescence methods to investigate the electronic structures of biological purines and to study molecular complexes where purines participate. Unfortunately, however, the purine derivatives are nonfluorescent in their functional state at physiological pH and temperature. Artificial means as protonation at low pH or in some cases deprotonation at high pH are required to create room temperature fluorescence. These peculiarities of purine fluorescence also constitute serious obstacles to the observations of purine bases in the living cell by means of fluorescence microscopy.

In a short communication [7] we have reported evidence which indicates that the possibilities for study of purines using fluorescence methods in conjunction with cooling are by no means exhausted. Thus it was shown that cooling permits analysis of guanine derivatives in the presence of adenine derivatives, since the fluorescence of the guanines was amplified by cooling while the fluorescence of the adenines disappeared at low temperatures. Furthermore, the interaction af alkylating pharmacological agents with purine bases was shown to yield fluorescent purine derivatives.

The rational use of fluorescence methods in purine research requires systematic studies to find out how the fluorescence quantum yield, the emission spectrum and the degree of polarization of the emitted light, depend on the temperature, the viscosity, the pH, the polarity and hydrogen bonding strength of the solvent, and on quenching exerted by ions or molecular oxygen. The present paper deals with a small section of these studies, *viz.* the fluorescence of guanine, guanosine, GMP, GDP, and GTP at temperatures between room temperature and about 100°K.

EXPERIMENTAL

Materials. Guanine, guanosine, and the guanosine phosphates were purchased from Nutritional Biochemicals Corporation and used without further purification. Methanol P.A.grade "Merck" was distilled twice in all quartz equipment to attain a sufficiently low level of absorbing and fluorescent impurities.

Procedure. Fluorescence excitation spectra and emission spectra were recorded on an automatically corrected fluorescence spectrophotometer, the design and function of which have been described previously.[8] The compounds were dissolved either in water or in methanol:water (9:1) in concentrations between 2 and 4 μmoles/l. The absorbancies were thus kept below 0.05 per cm in the frequency range to be investigated. All solutions were saturated with air. The pH was adjusted by means of 10 N sulphuric acid or 4 N KOH. The ratio between methanol and water was not significantly disturbed by the small amounts of acid or base required for this adjustment of pH. A Radiometer capillary glass electrode was used for measurements of pH. The pH meter was calibrated with aqueous standards. This calibration is, however, not valid when pH is to be measured in

solutions with a high proportion of methanol. The liquid junction potential between the aqueous KCl bridge and the methanolic solution must be subtracted from the total voltage measured by the pH meter to find the potential across the glass membrane.[9] We have chosen to omit this correction because the data reported in the present paper can be discussed and reproduced without reference to the true activities of hydrogen ions in the solutions.

The silica fluorescence cell was cylindrical with internal diameter 0.7 cm. Cooling was effected by liquid air in contact with the brass block carrying the sample cell. Temperatures below 100°K had to be avoided because of the high tendency for cracks to develop in the solidifying solvent on further cooling. The temperature was measured with a thermocouple immersed in the fluorescent solution immediately above the path of exciting light. Fluorescence intensity as a function of temperature was displayed directly on the X-Y recorder, the thermoelectric potential being fed to the X-axis and the fluorescence intensity to the Y-axis. The emission monochromator had to be continuously readjusted to follow the displacements of the emission maxima during the cooling process. At intermediate temperatures the fluorescence excitation spectra and emission spectra were recorded while the temperature of the cell was rising spontaneously after completed cooling. This rise of temperature was sufficiently slow to permit each spectrum to be recorded within temperature intervals of 10 degrees. The temperature assigned to each spectrum was the mean of the temperatures measured immediately before and after the recording.

The emission spectra were corrected according to the spectral sensitivity distribution of the instrument.[8] The apparent quantum yields were determined from corrected emission spectra by comparison with the fluorescence of quinine in 0.5 N sulphuric acid. Quinine has been assumed to fluoresce with a quantum efficiency of 0.52 when excited in the peak near 29 000 cm^{-1}, while the yield drops to 0.36 when the wavenumber of the exciting light is 36 000 cm^{-1}.[8,10]

The viscosities of the methanol:water mixture at different temperatures were measured with a rotating cylinder viscometer (Haake Viscotester VTL-180). The viscometer was cooled with liquid air. Measurements of viscosity were performed during the slow rise of temperature after completed cooling. The temperature was measured with a small thermocouple between the rotating inner cylinder and the stationary outer cylinder, except above 190°K where the viscosity was so low that cylinders with very small clearance had to be used. In this case the thermocouple was situated in the liquid immediately above the cleft between the cylinders. Below 136°K the viscosity was too high to be measured with the viscometer at our disposal, while above 250°K the viscosity was too low to be recorded accurately.

RESULTS

Effects of solvent on the fluorescence at room temperature. In Table 1 the fluorescence quantum efficiencies in water and in methanol: water (9:1) are tabulated. At acid pH the use of methanol:water instead of water increased the quantum yields about 3 times. The absorption spectra were only slightly affected by this change of solvent. The emission spectra were the same in both solvents.

At alkaline pH the fluorescence of guanine and GMP was found, respectively, to be 4 and 5 times more intense in methanol:water than in water. Again the absorption spectra were only moderately sensitive to the type of solvent. Emission spectra were not perceptibly influenced by the solvent. Guanosine, GDP, and GTP were all nonfluorescent at high pH in both solvents. Near neutral pH all solutions were devoid of observable fluorescence.

Effects of cooling on fluorescence yields. Cooling of the acid methanol: water solutions increased the fluorescence intensities considerably (Fig. 1). These solutions exhibited unexpected maxima of fluorescence intensity between 170°K and 160°K, where apparent quantum yields of about 0.5 were

Table 1. Fluorescence quantum yields of guanine and some guanine derivatives. The yields were determined relative to the fluorescence of quinine in 0.5 N sulphuric acid. In all cases the wavenumber of the exciting light was 36 000 cm^{-1}. Quinine was assumed to fluoresce with a quantum yield of 0.36 when excited at 36 000 cm^{-1} (see text). The pH was adjusted with sulphuric acid or potassium hydroxide.

Compound	Aqueous solution, 295°K		Methanol:water (9:1), 295°K		Methanol:water (9:1)		
	pH	Fluorescence quantum yield	pH	Fluorescence quantum yield	pH	Temp. °K	Fluorescence quantum yield
Guanine	1.40	0.007	1.30	0.023	1.35	165	0.50
	10.90	0.017	11.69	0.064	11.80	135	0.38
Guanosine	1.20	0.009	1.30	0.026	1.32	165	0.54
GMP	1.22	0.009	1.25	0.021	1.40	165	0.52
	11.47	0.006	11.87	0.029	11.80	135	0.30
GDP	1.74	0.007	1.33	0.021	1.50	165	0.44
GTP	1.77	0.007	1.30	0.021	1.42	165	0.50

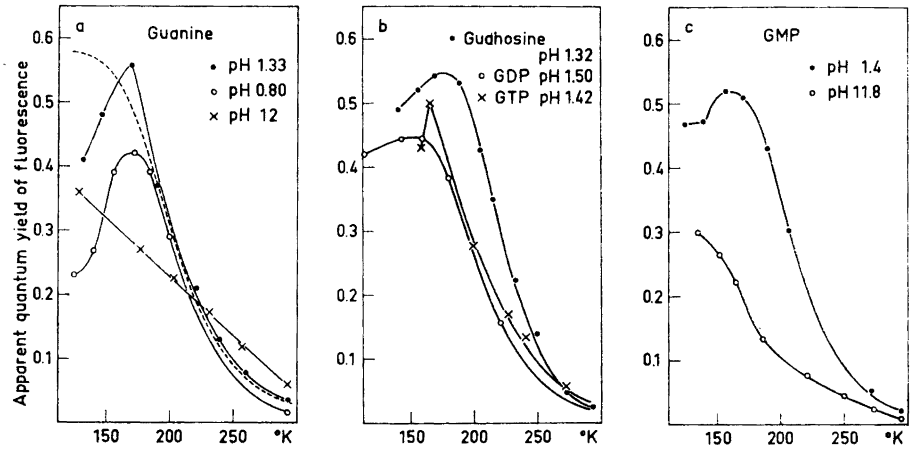

Fig. 1. Apparent fluorescence quantum yields as functions of temperature. The solvent was methanol:water 9:1. The quantum yields were calculated from the areas below the corrected emission spectra, using quinine in 0.5 N sulphuric acid as quantum yield standard. The yield of this standard was assumed to be 0.36 during excitation at 36 000 cm^{-1} (see the text), which was the wavenumber of the exciting light in all experiments shown in Fig. 1. The heights of the emission spectra have not been corrected for the various effects arising from the contraction of the solvent which accompanies the cooling.

Experimental curves are fully drawn. The stippled curve has been calculated from eqn. 1 with the omission of the term containing the viscosity coefficient. The activation energy of quenching (3.2 kcal/mole) was obtained from Fig. 6. The choice of constants in the equation is discussed in the text.

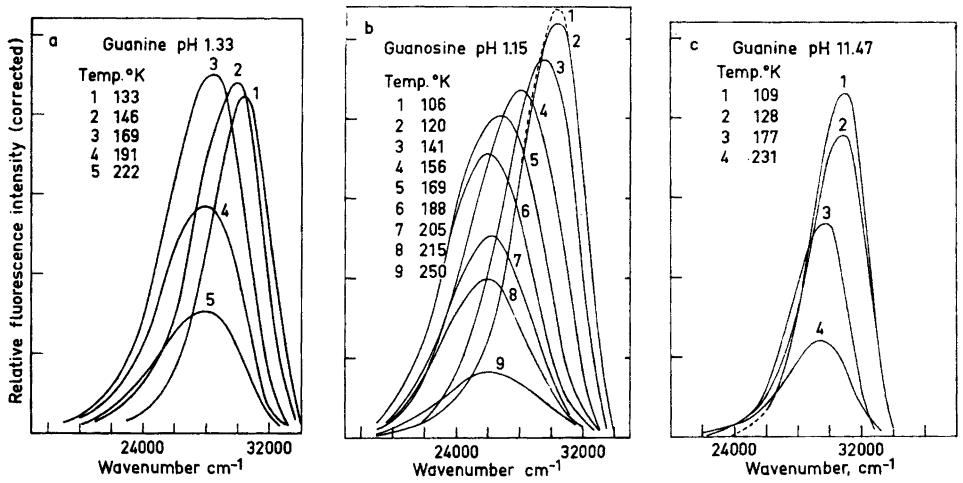

Fig. 2. Fluorescence emission spectra in methanol:water (9:1) at different temperatures. Since the spectra have been corrected according to the spectral sensitivity distribution given in Ref. 8, the unit along the ordinate is quanta per unit interval of wavenumber. The slit width of the emission monochromator (Zeiss M 4 Q III) was 0.2 mm. The pH was adjusted with 10 N H_2SO_4 or 4 N KOH.

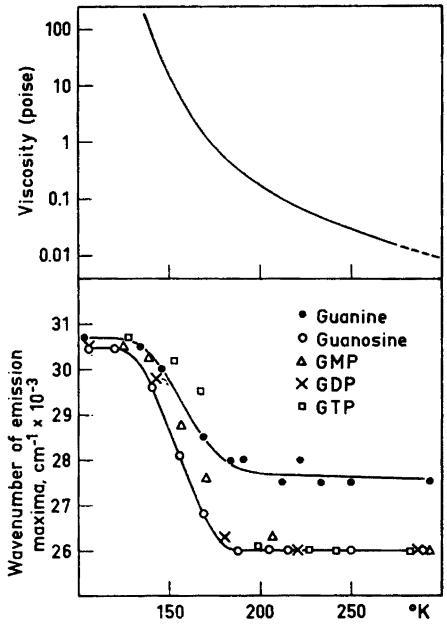

Fig. 3. The upper half shows the viscosity of the methanol:water (9:1) solvent as a function of temperature. In the lower half the wavenumbers of the emission maxima at acid pH in methanol:water (9:1) are plotted as functions of temperature.

attained. Further cooling invariably decreased the fluorescence intensity as measured perpendicularly to the exciting beam.

At alkaline pH the fluorescence of guanine and GMP also increased, but no temperature optimum was seen (Fig. 1). The other guanine derivatives did exhibit some fluorescence at alkaline pH as the temperature was lowered, especially below 190°K, but this fluorescence was too weak to be separated from the luminescence of impurities, and has not been investigated further.

Effects of cooling on the emission spectra. The maxima of the corrected emission spectra in all cases moved towards higher frequencies ("blue shift") as a result of cooling (Fig. 2). The blue shifts began near 180°K and were completed at 130°K. The shifts were half completed near 155°K where the viscosity of the solvent was 6.7 poise (Fig. 3). At the temperature optimum for the fluorescence at acid pH (160°K — 170°K) the spectral shifts were thus only about to appear.

At acid pH the emission maxima of guanosine and its phosphates moved from 26 000 cm^{-1} to 30 500 cm^{-1}. Protonated guanine underwent a slightly smaller shift from 27 500 cm^{-1} to 30 600 cm^{-1}.

At alkaline pH similar, but considerably smaller shifts were observed in the emission spectra of guanine and GMP (Fig. 2). The maxima were located at 29 500 cm^{-1} and 31 000 cm^{-1} at high and low temperatures, respectively.

Excitation spectra at acid pH. The fluorescence excitation spectra at low pH had the same general shape as the absorption spectra (Fig. 4). Both these types of spectra appear to envelop two electronic transitions with corresponding maxima at about 35 000 cm^{-1} and 39 000 cm^{-1} (40 000 cm^{-1} in guanine). The shape of the spectra between 33 000 cm^{-1} and 44 000 cm^{-1} is

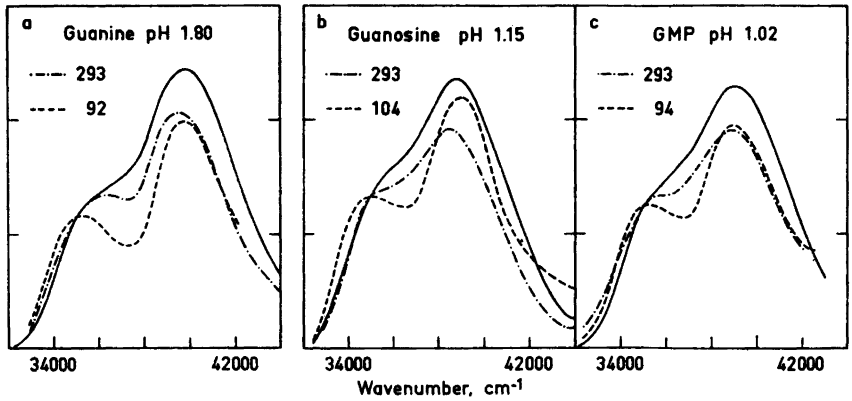

Fig. 4. Fluorescence excitation spectra at acid pH in methanol:water (9:1) at different temperatures. The spectra are reproduced as displayed on the recorder of the fluorescence spectrophotometer, *i.e.* without subsequent corrections for the spectral variation of the monitor efficiency. The effect of the latter correction (Table 1 in Ref. 8) would be to lower the excitation spectra at 40 000 cm^{-1} by multiplication with the factor 0.88, while the ordinates at 35 000 cm^{-1} would be retained. The slits of the excitation monochromator (Zeiss MM 12) were 2 mm at 44 000 cm^{-1}, and were progressively narrowed by the slit servo during the scans towards lower wavenumbers, attaining a width of 0.2 mm at 36 000 cm^{-1}. The absolute temperatures corresponding to the different spectra are shown in the figure. The fully drawn curves are the absorption spectra at room temperature obtained in the same solvent and at the same values of pH as the excitation spectra.

determined by the broadness and relative heights of the bands corresponding to the two electronic transitions.

If the excitation spectra are arbitrarily matched with the corresponding absorption spectra at 35 000 cm^{-1}, the bands with maxima at 39 000 cm^{-1} (or 40 000 cm^{-1}) are found to be about 20 % lower in the excitation spectra than in the absorption spectra. This appears to explain why the inflections at 37 000 cm^{-1} are more pronounced in the excitation spectra at room temperature than in the absorption spectra.

Cooling further deepened the minima in the excitation spectra near 37 000 cm^{-1}. No consistent trend was evident in the effect of cooling on the relative heights of the two bands in the excitation spectra. No vibrational structure appeared.

Excitation spectra at alkaline pH. At room temperature guanine and GMP gave rise to excitation spectra which were very different from the absorption spectra (Fig. 5). Of the two bands in the absorption spectra in the range between 32 000 cm^{-1} and 43 000 cm^{-1} only the band with maximum at about 35 000 cm^{-1} was apparent in the excitation spectra. The excitation spectra at room temperature were the same in methanol:water as in aqueous solutions.

Cooling had somewhat different effects on the two compounds which fluoresced between pH 11 and 12. In the excitation spectrum of guanine a new shoulder appeared on cooling below 180°K. This shoulder evidently corresponds to the band in the absorption spectrum with maximum between 40 000 cm^{-1} and 41 000 cm^{-1}.

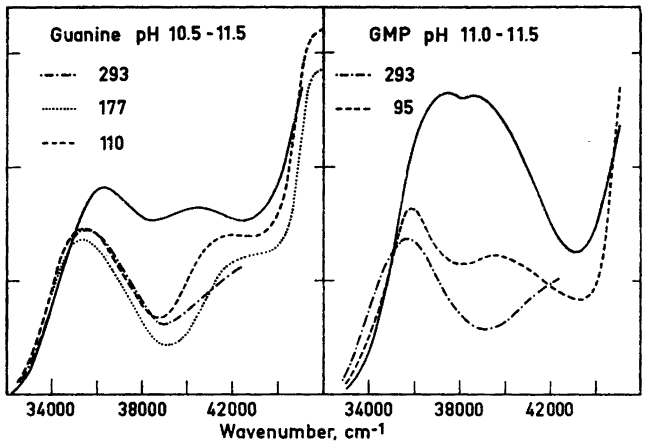

Fig. 5. Excitation spectra and absorption spectra at alkaline pH. Same legend as Fig. 4.

An analogous new maximum also appeared in the excitation spectrum of GMP at low temperatures. Cooling below 160°K was, however, necessary to reveal it. As would be expected from the absorption spectrum of GMP, the new low temperature maximum in the excitation spectrum is located at 39 500 cm^{-1}, *i.e.* at a lower wavenumber than in guanine.

DISCUSSION

Sources of error in the determinations of fluorescence quantum yields. Cooling of a fluorescent solution usually enhances the quantum yield of fluorescence. It is generally recognized, however, that it is difficult to measure accurately this effect of cooling. [11] The polarization of the emitted light which is likely to occur at the lowest temperatures and highest viscosities attained in our experiments, leads to an uneven distribution of the fluorescence light among the different directions in space. Positive polarization reduces the fraction of light emitted perpendicularly to the exciting beam provided the latter is unpolarized as in our experiments. [12] This effect may be responsible for the decrease of apparent fluorescence quantum yields which was observed in the acid solutions on cooling below 160°K. Whether the emitted light really is polarized in the positive sense, remains to be investigated.

The measured fluorescence intensities need corrections for the change of absorbance at the wavenumber of the exciting light (36 000 cm^{-1}) which accompanies the cooling. This absorbance is influenced by the contraction of the solvent (9.5 % per 100°K), by the shape of the absorption bands at low temperatures, and by the increase of refractive index of the solvent.[13]

Another effect of the changing refractive index, which should be taken into account, is to alter the fraction of the emitted light which eventually enters the emission monochromator. The magnitude of this effect depends on the detailed geometry of the instrument.

It is considered unlikely that the combined effects of these sources of error amount to more than 10—20 % at 165°K. Since the required corrections are

difficult to evaluate, and since they are small compared to the true effects of temperature and viscosity, no attempt has been made to compensate for the errors.

Interpretational ambiguities. The other main group of difficulties pertains to the interpretation of the effects of temperature on fluorescence quantum yield. It is usually assumed that the increase of fluorescence, which accompanies cooling of the solution, occurs because at least one of the quenching processes, which competes with the fluorescence, needs thermal activation energy. The picture is obscured, however, because some quenching processes are also inhibited by the increase of viscosity at low temperatures. To separate the effects of temperature and viscosity, a number of solvents with different temperature-viscosity relationships must be employed. This is no simple task, because other solvent characteristics than viscosity and temperature may determine the fluorescence yield.

In our experiments the fluorescence yield is not likely to show a simple dependence on temperature and viscosity, since the possible quenching processes are too numerous. Thus quenching due to interaction between the excited solute and the solvent (solvent quenching) can only in a tentative way be regarded as the main temperature dependent quenching mechanism. Collisional quenching due to the presence of the electrolytes used to adjust pH, or due to molecular oxygen in the solution, are impeded by low temperatures and high viscosities. On the other hand the increasing fluorescence yields, which accompany cooling, imply increasing lifetimes of the excited solute molecules, whereby the occurrence of quenching collisions within the lifetime of the excited singlet states is rendered more probable.

Analysis of the effects of temperature on the fluorescence yields at acid pH. Although our results are unable to confirm unequivocally any definite set of mechanisms leading to quenching of fluorescence, we have chosen to illustrate our empirical curves (Fig. 1) in terms of a scheme similar to the one proposed by Bowen.[11] This scheme implies that collisional quenching is unimportant, and that solvent quenching not only requires thermal activation energy, E, but that thermal activation leads to quenching only if the viscosity is low enough to allow certain diffusion-like movements to occur. Our data necessitated the introduction of a temperature independent quenching process since the quantum yields are smaller than 1 even at very low temperatures. Furthermore the absolute temperature was included in the preexponential factor in the expression for the rate of the thermally activated quenching to render the equation consistent with transition state theory:

$$\log(1/F - k_1) + \log(1 + k_2/(T/\eta)) - \log T = -0.4343\,E/RT + \text{constant.} \quad (1)$$

F = fluorescence quantum yield
η = viscosity coefficient (poise)

In Fig. 6 are shown plots of the left side of this equation against $1/T$, based on the experimental values of F and η. The best linearities of the plots were achieved when a value of 100 was chosen for k_2, and when k_1 was assigned a value which gives theoretical maximal quantum yields at zero degrees between 1 and 5 % higher than the maximal apparent yields actually measured near 165°K. Unless k_2 is considerably larger than 100, the viscosity term is

found to be of quantitative significance only below 200°K where the viscosity coefficient exceeds 0.2 poise.

The stippled curve in Fig. 1 has been drawn to illustrate the assumed effect of viscosity. The curve has been calculated from eqn. 1 with the omission of the term containing the viscosity coefficient. The activation energy of quenching was found from Fig. 6 to be 3.2 kcal/mole, and the values of k_1 and k_2 were the same as was assumed in the preparation of Fig. 6. The remaining constant in eqn. 1 was adjusted to make the theoretical curve fit the experimental value of F at 200°K. The effect of viscosity appears to be to raise the experimental values of F above the theoretical curve which was calculated without the viscosity term. Viscosities in excess of 0.2 poise, which are obtained in the methanol:water solvent (9:1) below 200°K, are necessary for this effect to become apparent. The factors which decrease the fluorescence intensity below 165°K, tend to obscure the enhancement of fluorescence which is due to the high viscosity.

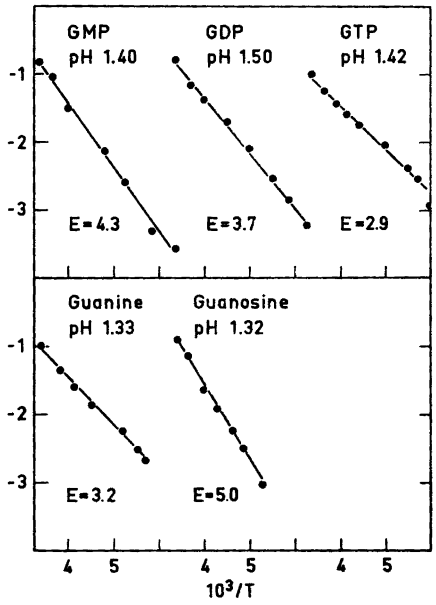

Fig. 6. The experimental curves of Fig. 1 at low pH examined with reference to eqn. 1: See text.

The sums of the terms to the left of the equality sign have been plotted along the ordinate, against the reciprocal temperature along the abscissa. The values assigned to k_1 correspond to theoretical maximal quantum yields between 1 and 5 % above the maximal apparent yields experimentally attained. The curves in Fig. 6 were all rendered fairly linear by letting k_2 assume the value 100. The linearity does not persist at temperatures below 170°K (or $10^3/T$ above 5.9) due to the factors which are responsible for the decrease of fluorescence on cooling below 165°K. Activation energies of quenching, E, derived from the slopes of the lines, are given below each curve in kcal/mole.

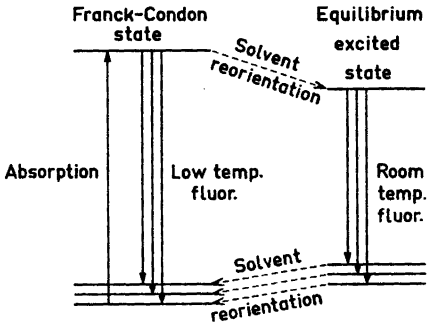

Fig. 7. Fluorescence from the equilibrium excited state at room temperature (right), and from a Franck-Condon state at low temperatures and high viscosities (left). The diagram is modified from Fig. 4 of Ref. 14 by taking into account the solvent reorientation in the ground state.

Frequency shifts of emission spectra. The pronounced frequency shifts which occur in the emission spectra between 180°K and 130°K are presumably due to the longevity of intermolecular Franck-Condon states in rigid media. [14] The argument is based on the assumption that the equilibrium distances between the solute and the surrounding solvent molecules are not the same in the excited state as in the ground state. Following excitation at room temperature some energy is quickly dissipated as the molecules move to new equilibrium distances (Fig. 7). At low viscosities this reorientation is completed before any appreciable fluorescence is emitted. Hence light emission occurs predominantly from the equilibrium excited state. At the end of the radiative transitions another adjustment of intermolecular distances ensues, *viz.* back to the original equilibrium configuration. The latter molecular movements appear to have been overlooked by Hercules and Rogers. [14] Evidently the photon energy of the emitted light has been lowered due to molecular relaxation phenomena both in the excited state and in the ground state.

If the viscosity is very high, the solvent cage surrounding the solute is rigid, and no reorientation of the solvent molecules occurs during the lifetime of the excited singlet state. Consequently radiative transitions follow the downward arrows in the left half of Fig. 7, which clearly correspond to higher photon energies and wavenumbers than the arrows to the right in the figure. Our results indicate (Fig. 3) that a viscosity of 100—200 poise renders the solvent cage stiff in the above sense.

At a viscosity of about 8 poise the blue shifts of emission maxima are half completed (Fig. 3). This implies that the relaxation time of the solvent cage is now of the same order of magnitude as the lifetime of fluorescent states, *i.e.* 10^{-8} sec. This is reasonable in view of what is known about the influence of viscosity on such relaxation times: The rates of relaxation processes, like diffusion, are usually assumed to be proportional to T/η. [15] The value of T/η at 155°K where $\eta = 8$ poise is decreased by a factor of 10^3 compared to its value at room temperature. The relaxation time increases by the same factor, *i.e.* from about 10^{-11} sec in a non viscous solvent at room temperature [16] to 10^{-8} sec at 155°K in our methanol:water mixture, which is just the lifetime quoted above for fluorescent states.

The emission maxima of guanosine and its phosphates at acid pH undergo blue shifts amounting to 4500 cm^{-1}. This is equivalent to 12.8 kcal/mole. No special significance can be assigned to this figure, however, in terms of number and strengths of the bonds between solute and solvents, because electronic transitions do not lead to simple breakage of one or more intermolecular bonds. The large blue shifts rather suggest that the equilibrium distances of the intermolecular bonds are profoundly influenced by the electronic transitions.

The considerably smaller blue shifts (1500 cm^{-1}) experienced with guanine and GMP in the anionic state at high pH, indicate that the electronic transitions in these cases do not disturb the equilibrium distances of the intermolecular bonds nearly as strongly as at acid pH.

Fluorescence excitation spectra at acid pH. If the fluorescence quantum yield is independent of the wavenumber of the exciting light, the fluorescence excitation spectra will be found to follow closely the absorption spectra. Apparent exceptions to this rule occur when the emitted light is polarized, and the

degree of polarization depends on the wavenumber of the exciting light, because of the uneven spatial distribution of the emitted light.

The excitation spectra at room temperature (Fig. 4) indicate that the quantum yields are about 20 % lower when the wavenumber of the exciting light is near 40 000 cm^{-1}, than they are when only the electronic transition with corresponding absorption maximum at 35 000 cm^{-1} is affected by the exciting light. Significant errors due to polarization are unlikely at the small viscosity of the methanol:water mixture at room temperature. The rate of thermally activated quenching processes evidently determines the quantum yields at room temperature in our experiments. We conclude that one or more of these quenching mechanisms appear to be favoured by high frequency excitations.

Cooling deepened the minima in the excitation spectra at 36 000—37000 cm^{-1}, presumably due to analogous changes in the absorption spectra at low temperatures. Such changes in the absorption spectra can be expected for the following reason: A significant fraction of the absorption at 36 000 cm^{-1} is due to the low frequency tail of the electronic transition which corresponds to the maximum near 39 000 cm^{-1}. But the absorption in such low frequency wings of a band is mainly due to molecules which are strongly disturbed by interaction with neighbouring molecules and hence raised energetically. [17] At low temperature the thermal energy needed to create such heavily disturbed molecules is no longer available, and the corresponding light absorption disappears.

Further discussion of the relationships between excitation spectra and absorption spectra at low temperatures must be postponed until absorption data and polarization measurements at low temperatures are available.

Tautomerism and excitation spectra at high pH. The large discrepancies between excitation spectra and absorption spectra shown in Fig. 5 cannot be explained in terms of reasonably small spectral variations of the fluorescence quantum yields, but rather suggest the presence of more than one absorbing species in the solution. This may be due to molecular association or to tautomerism. [18]

In the cases displayed in Fig. 5 a third explanation must be considered because the guanine rings undergo two acidic dissociations between pH 9 and 13. If the pH ranges of these two dissociations overlap significantly, deprotonated guanine rings with one and two negative charges may exist simultaneously. In the methanol:water mixture (9:1) the absorption spectra were not as sensitive to pH, however, as they would have been around pH 11 if the acidic dissociations did overlap. In aqueous solutions the pK_a values are known to be too far apart for the overlap to be of importance. [3]

The formation of molecular complexes at high pH will be opposed by the negative charge of the guanine rings. Experimental evidence against the fluorometric significance of complex formation was provided by the linear relationship which was found between the fluorescence intensity and the concentration of guanine or GMP.

To check the remaining hypothesis, -tautomerism-, the absorption spectra were examined under different conditions. Variations of temperature between 10°C and 70°C altered these spectra only very slightly. In view of this finding

it is not likely that the increase of fluorescence due to cooling is brought about by major displacements of the tautomeric equilibria towards the fluorescent tautomer. The use of methanol-water as solvent instead of water was also almost without effect on the absorption spectra. This shows that the four- or five-fold increase of fluorescence intensity which accompanied this change of solvent at room temperature, was not due to shifts of the tautomeric equilibria.

The tautomers of guanine and GMP, which are fluorescent at room temperature, appear to be of similar structure since their excitation spectra are scarcely distinguishable, and since their emission spectra are identical.

The difference in structure between guanine and GMP does express itself, however, in the absorption spectra of the tautomers which are devoid of fluorescence at high temperatures. The overlap between the absorption bands of the two tautomers of GMP is more pronounced than the corresponding overlap in the absorption spectrum of guanine. The fluorescence quantum yields, which were calculated with reference to the absorbances at 36 000 cm^{-1}, have consequently been estimated erroneously to low for the fluorescent tautomer, especially in the case of GMP.

The characteristic changes which were found in the excitation spectra at low temperatures corroborate the assumption that the tautomers which are nonfluorescent at room temperature are rendered fluorescent by cooling.

We conclude that tautomerism accounts satisfactorily for the peculiarities in the fluorescence properties of guanine and GMP around pH 11.

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Received July 29, 1965.