ACTA CHEMICA SCANDINAVICA 19 (1965) 2089–2099

A Fluorescence Spectrophotometer Recording Corrected Excitation Spectra between 100°K and Room Temperature

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The present communication describes a fluorescence spectrophotometer which records approximately true excitation spectra between 300°K and about 100°K. A monitoring device is interposed in the beam of exciting light between the first monochromator and the sample cell. The signal from the monitor instructs a servomotor to adjust the slits of the monochromator for the exciting light. In this way the quantum intensity of the exciting light is kept at a preselected level. The frequency selected for excitation, and variations in the output of the Xenon lamp, are both almost without influence on the number of quanta which hits the fluorescence cell per unit time. The function of the monitor has been checked by means of ferrioxalate actinometry. Corrected excitation spectra as displayed by the instrument are presented for fluorescein, quinine, tryptophan and 5-hydroxytryptamine. The spectral sensitivity data necessary for the correction of recorded emission spectra are given. The emission spectra of quinine and β-naphthol have been corrected and are compared to data from the literature.

It is generally recognized that the fluorescence excitation spectra and emission spectra which can be recorded on most commercial instruments, are greatly distorted versions of the true spectra. The apparent excitation spectra deviate strongly from the true ones because the number of exciting quanta hitting the fluorescent sample per unit time decreases rapidly with increasing frequency in the ultraviolet region. The quantum intensity of the exciting light at different frequencies is determined by the output of the light source (usually a Xenon lamp) as well as by the dispersion, transmission, and slit widths of the excitation monochromator.

The shapes of the emission spectra are strongly influenced by the spectral dependence of the sensitivity of the multiplier phototube in combination with the emission monochromator.

These instrumental factors vary from instrument to instrument, even of the same type. The characteristics of the exciting light may also vary from time

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to time in a given instrument because the lamp tends to be unstable and susceptible to ageing phenomena, and because mirrors and silica windows may deteriorate.

Some commercial fluorometers are equipped for recording of luminescence at the temperatures of liquid nitrogen (77°K) or liquid air (93°K). Some instruments can be connected to a liquid thermostat for measurements above about 250°K. The interval of temperature between about 100°K and 250°K is not readily accessible with the equipment commercially available.

The few instruments which have been designed to overcome the difficulties connected with instrumental instability and spectral distortions, have been based either on monitoring of the energy output from the light source,[1] or on measurement of the quantum intensity of the exciting light.[2] The present paper describes a fluorescence spectrophotometer which allows the recording of almost true excitation spectra within a wide range of temperatures. It is largely based on the work of Parker.[2,3] The present communication is intended to serve as a methodological reference for several investigations to be published.

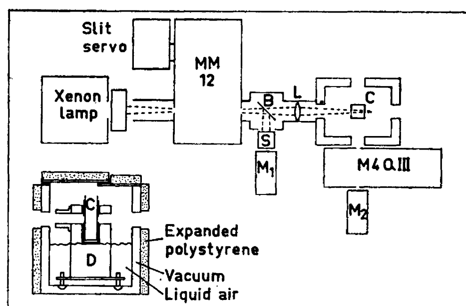
DESCRIPTION OF THE FLUORESCENCE SPECTROPHOTOMETER

The light from a Xenon arc lamp (Osram XBO 450 W) is dispersed by the two silica prisms in a Zeiss MM 12 double monochromator (Fig. 1). Of the exciting light emerging from this monochromator a small fraction is reflected onto the fluorescent screen S by the beam splitter B which is made from a clear silica plate. All photons which hit the screen should be absorbed near the surface of the screen material. S is either a "fluorescein screen" (4.4×10^{-3} M fluorescein in a mixture of equal volumes of 0.2 M sodium carbonate and sodium hydrogen carbonate), a "rhodamine screen" (5×10^{-2} M rhodamine B in ethylene glycol), or a piece of fluorescent glass (supplied by Zeiss for use as fluorescence standard). The low frequency limits of these screens are determined by the absorbances of the screen materials. The rhodamine screen can be used down to 17 000 cm^{-1}, the fluorescein screen down to 20 000 cm^{-1}, and the fluorescent glass down to 31 000 cm^{-1}. The fluorescence of the screen is measured by the multiplier phototube M_1. The output of M_1 is proportional to the number of quanta reflected onto S and almost independent of the frequency of the exciting photons. If the reflectivity of B is independent of frequency, M_1 thus functions as a monitor for the quantum intensity of the light transmitted through the MM 12 monochromator.

The salient feature of the instrument is that the signal from M_1 is fed to a servomechanism. The servomotor regulates the widths of the entrance slit and the exit slit of the monochromator MM 12 simultaneously until the signal from M_1 attains a preselected value. The output of M_1 is also displayed on a millivoltmeter. The millivoltmeter secures easy reproducibility of the servo adjustment from time to time. Furthermore, the signal from M_1 is amplified in the millivoltmeter before reaching the servo input. The automatic slit adjustment compensates for instability of the Xenon lamp and keeps the quantum intensity of the exciting light approximately constant during a scan of the excitation monochromator through a range of frequencies.

Fig. 1. General configuration of the fluorescence spectrophotometer. In the upper half of the figure is shown a diagram of the instrument as seen from above. At the lower left is a vertical section of the cell compartment in the plane of the exciting beam.

B	Silica plate beam splitter
C	Optical cell for the fluorescent sample
D	Brass block
L	Lens focusing the exciting light
M_1	Monitoring photomultiplier (BMS 10/14, 20th Century Electronics Ltd.)
M_2	Photomultiplier for measurement of the fluorescence of the sample in C. (EMI 6256 S)
MM 12	Excitation monochromator
M 4 Q III	Emission monochromator
S	Fluorescent monitor screen



The major fraction of the monochromatic exciting light is transmitted through the beam splitter *B*. The lens *L* brings the beam to focus immediately behind the fluorescence cell, *C*. The fluorescence light emitted by the contents of *C* is dispersed by a Zeiss M 4 Q III monochromator with a single silica prism. The light is measured by a photomultiplier M_2 (EMI 6256 S operated at 2000 V). The voltage produced by the anode current across a resistance (0.01 to 0.2 megohms) is fed without amplification to the Y-axis of a Varian F 80 X—Y recorder.

The monochromators are scanned from higher towards lower frequencies by electric motors. The frequency setting of either monochromator can be made to determine the position of the recorder pen along the *X*-axis. For this purpose the wavelength drums of the monochromators have been linked to ten-turn potentiometers. The *X*-input of the recorder receives the variable D. C. voltages delivered by either potentiometer in connection with batteries.

The sample cell *C* is housed in a metal Dewar vessel externally isolated with expanded polystyrene. The brass block *D* carrying the cell is cooled by liquid air or nitrogen (Fig. 1, lower left). The cooling of the fluorescent sample can easily be made sufficiently slow to counteract the development of cracks in the solidifying solvent. The temperature is measured by a thermocouple in the fluorescent solution immediately above the path of exciting light. The voltage of the thermocouple is either measured by a millivoltmeter or fed to the *X*-axis of the recorder. The latter arrangement permits the fluorescence intensity at selected frequencies to be plotted directly as a function of temperature. After complete evaporation of the cooling liquid, the temperature of the fluorescent sample increases fairly slowly. During a spectral recording at intermediate temperatures, the temperature is thus not entirely constant. The ensuing errors are only moderate, however, unless the spectrum is ex-

tremely sensitive to the temperature, because the change of temperature during a spectral scan rarely exceeds 10 degrees. No disturbing deposition of moisture on the cell C has been noticed.

CALIBRATION OF THE FLUORESCENCE SPECTROPHOTOMETER

Frequency scales. The frequency scales of the monochromators were checked by means of 6 of the lines emitted from a low pressure mercury lamp. Between the lines at 2301.1 Å and 4358.4 Å the scales were never more than 50 cm^{-1} off the correct wavenumber. At 5460.7 Å and 5769.6 Å the error did not exceed 120 cm^{-1}.

Excitation spectra. Fluorescence intensity as a function of the frequency of exciting light evidently represents the true excitation spectrum only insofar as the quantum intensity of the exciting light is independent of the frequency. The exciting light cannot be kept truly constant by the slit servo unless the frequency is without influence on the ratio between the number of photons irradiating the sample per unit time and the signal from M_1. This condition can be fulfilled only in an approximate sense because the frequency exerts some influence on the transmittance and reflectivity of the silica material in the beam splitter and in the fluorescence cells, as well as on the fluorescence quantum yield of the screen and on the optical properties of the condensing lens L.

It was therefore necessary to measure the number of quanta which hits the contents of the cell C at different frequencies with the slits adjusted by the servo to obtain a given signal from M_1. Such measurements were carried out by means of ferrioxalate actinometry. Photochemical quantum yields for the ferrioxalate system have been reported by Hatchard and Parker.[4] The results have been expressed in Table 1 as "spectral distribution of the efficiency of the monitor" in relative units. The monitor efficiency represents the ratio between the signal from M_1 and the number of quanta received per unit time by the contents of C. Table 1 shows that the monitor efficiencies decrease almost monotonously with increasing frequency. This was found both with the fluorescein screen and with the rhodamine B screen. In our instrument the fluorescent sample consequently receives a steadily decreasing quantum intensity of exciting light during a scan of the excitation monochromator from higher towards lower frequencies with the slit servo in operation. Hence the recorded excitation spectra will be relatively too high towards the high frequency end of the ranges scanned.

Emission spectra. The recorded emission spectra are seriously distorted because the sensitivity of the photomultiplier M_2 in combination with the emission monochromator is strongly dependent on the frequency. The spectral sensitivity distribution (Table 2) has been determined as recommended by Parker [3] for fluorometers equipped with a monitor for the exciting light.

To calibrate the emission unit, a magnesium oxide screen was inserted in the place normally occupied by the sample cell C. The screen was made by allowing MgO from burning magnesium to settle to a thick layer on a sheet of aluminium. The layer of MgO was irradiated with monochromatic exciting light of several frequencies. The signal from the monitoring photomultiplier

Table 1. Spectral distributions of the monitor efficiencies.

Wavenumber cm^{-1}	Efficiency of monitor (relative units)	
	Fluorescent screen: Rhodamine B, 5×10^{-2} M in ethylene glycol	Fluorescent screen: Fluorescein, 4.4×10^{-3} M in a mixture of equal volumes of 0.2 M sodium carbonate and sodium hydrogen carbonate
22 500	1.000	1.000
23 000	1.000	0.995
24 000	0.993	0.990
25 000	0.990	0.972
26 000	0.979	0.960
27 000	0.960	0.951
28 000	0.942	0.953
29 000	0.928	0.950
30 000	0.912	0.954
31 000	0.900	0.956
32 000	0.889	0.957
33 000	0.878	0.949
34 000	0.870	0.940
35 000		0.925
36 000		0.902
37 000		0.886
38 000		0.859
39 000		0.834
40 000		0.811
41 000		0.796
42 000		0.778
43 000		0.757

M_1 was the same at all frequencies due to the function of the slit servo. Rhodamine B, 5×10^{-2} M in ethylene glycol, was used as fluorescent screen S. This screen solution can be used down to about 17 000 cm^{-1}. The exciting light was diffusely reflected by the layer of magnesium oxide. The small fraction of the reflected light which entered the emission monochromator was measured by the photomultiplier M_2. The relative intensities of the light reflected from the magnesium oxide at the different frequencies, were calculated taking into account the efficiencies of the monitor (Table 1) and the reflectivities of MgO reported in the literature.[5,6] The irradiated spot on the magnesium oxide screen thus constitutes a light source with known spectral intensity distribution. The size of this luminous area varies slightly with frequency due to changing focal length of the lens L. To minimize any ensuing spectral variation of the fraction of reflected light which reaches the multiplier phototube M_2, the distance between the MgO screen and the entrance slit of the emission monochromator was increased from 5 cm to 10 cm. Furthermore, the slits of the emission monochromator were made considerably wider than the slits of the excitation monochromator. This ensures the passage through

the emission monochromator of the entire band of frequencies contained in the reflected exciting light.

The recorded signals from M_2 at each frequency were now corrected to what they would have been if the quantum intensity of the light reflected from the MgO screen had been the same at all frequencies. These corrected readings were multiplied with the effective bandwidths (in wavenumber units) of the emission monochromator. The figures thus obtained were the sensitivities pertaining to the measurements of fluorescence spectra (Table 2).

Table 2. Spectral sensitivity distribution of the EMI 6256 S multiplier phototube in combination with a Zeiss M 4 Q III quartz monochromator. The sensitivity is given in relative units of: "Signal from the photomultiplier per unit number of quanta per unit time per unit wavenumber interval".

Wavenumber cm^{-1}	Sensitivity
17 000	0.147
18 000	0.339
19 000	0.555
20 000	0.728
21 000	0.871
22 000	0.975
23 000	1.000
24 000	0.985
25 000	0.944
26 000	0.880
27 000	0.805
28 000	0.716
29 000	0.633
30 000	0.556
31 000	0.489
32 000	0.423
33 000	0.362
34 000	0.305

The corrected emission spectra are obtained from the recorded spectra by division with the appropriate values from Table 2. Since the bandwidths of the emission monochromator were given in units of wavenumbers, the ordinates of the corrected emission spectra are: "Relative number of quanta emitted per unit time per unit wavenumber interval".

PERFORMANCE OF THE INSTRUMENT

Excitation spectra. The fluorescence excitation spectra reproduced in Figs. 2, 3, and 4 are intended to demonstrate the general performance of our instrument and to facilitate comparison of our results with those of others. The spectra are given as recorded, *i.e.* without correction by multiplication with the appropriate factors from Table 1.

In the left half of Fig. 2 our excitation spectrum for 5-hydroxytryptamine is compared to the spectrum reported by Parker.[2] The discrepancy between the two excitation spectra seen above 40 000 cm^{-1} can in part be explained

as due to different monitor efficiencies in the two instruments. The efficiency of Parker's monitor increases by 8 % on going from 36 000 cm^{-1} to 43 000 cm^{-1}, while the efficiency of the monitor on our instrument decreases by 16 % in the same interval of frequencies. If the records at 43 000 cm^{-1} are corrected accordingly, the remaining difference is only 1/3 of that shown in the figure.

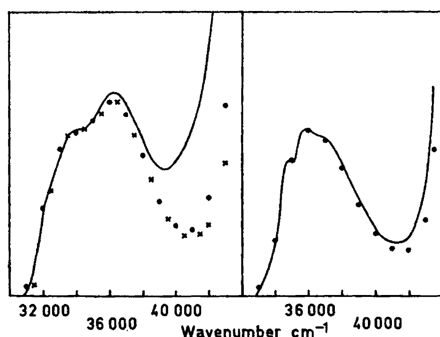


Fig. 2. Left: 5-hydroxytryptamine creatine sulphate in water. ———— Absorption spectrum. ● Fluorescence excitation spectrum as recorded at a concentration of 5×10^{-7} M. × Excitation spectrum of a 10^{-7} M solution published by Parker.[2] Right: L-tryptophan 7×10^{-6} M in water. Same meaning of symbols as in the left half of the figure. Parker used a solution of fluorescein (see Table 1) as monitor screen. The other two excitation spectra are recorded with a fluorescent glass as monitor screen.

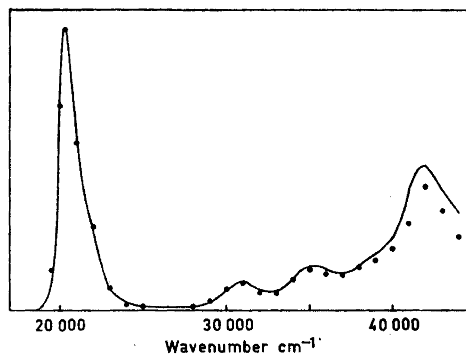


Fig. 3. Fluorescein 8.8×10^{-7} M in 0.1 N sodium hydroxyde. ———— Absorption spectrum. ● Fluorescence excitation spectrum as recorded on the instrument using the concentrated fluorescein solution as monitor screen (see Table 1).

The excitation spectra for tryptophan (Fig. 2, right) and for fluorescein (Fig. 3) suggest that the quantum yields of fluorescence decrease slowly with increasing frequency. Application of the correction factors from Table 1 would exaggerate the divergence between excitation spectra and absorption spectra, and hence emphasize the impression of decreasing quantum yields at higher frequencies. Weber and Teale [7] explicitly claim, however, constant quantum yields for these compounds.

It appears from Fig. 4 that quinine in 0.5 N sulfuric acid fluoresces with a considerably higher yield when excited at 29 000 cm^{-1} than on excitation at 40 000 cm^{-1}. The correction factors in the right column of Table 1 raise the ratio between these apparent quantum yields to 1.47. Moss [8] reported this ratio to be between 1.6 and 1.9. Moss also corrected his spectra by means of ferrioxalate actinometry. Fig. 4 further demonstrates that the fluorescent glass, supplied by Zeiss for use as fluorescent standard, can be used as monitor screen. Below 31 000 cm^{-1} the fluorescent glass cannot be used, however, because its absorbance is too low.

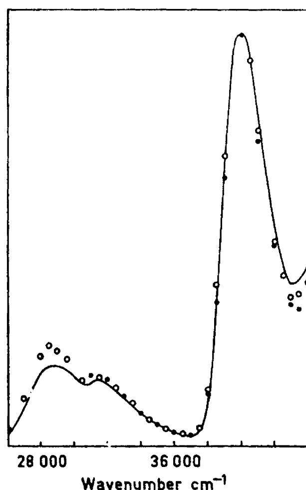


Fig. 4. Quinine in 0.5 N sulphuric acid. Absorbance 0.05 per cm at 40 000 cm^{-1}. ——— Absorption spectrum. ○ Fluorescence excitation spectrum with a fluorescein solution as monitor screen (see Table 1). ● Excitation spectrum recorded while a fluorescent glass served as monitor screen. The interchange of these two screens is seen to be without effect on excitation spectra recorded between 31 000 cm^{-1} and 43 000 cm^{-1}.

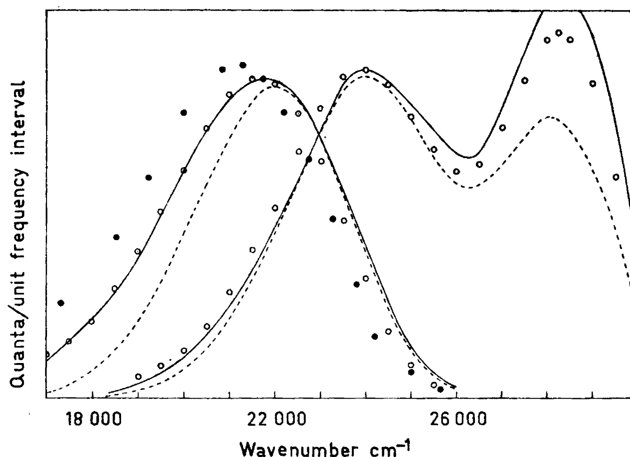


Fig. 5. Fluorescence emission spectra of quinine (left) and β-naphthol (right). – – – Spectra as displayed on the recorder. ——— Spectra corrected according to the sensitivity distribution of Table 2. ○ Spectra published by Lippert *et al.*[9] ● Spectrum of Melhuish [10] adapted to the ordinate of Fig. 5 by multiplication with the squared wavelengths.

Experimental conditions. In our experiment quinine was dissolved in 0.5 N sulphuric acid. The fluorescence was excited at 30 000 cm^{-1} where the absorbance was 0.09 per cm. Lippert *et al.* and Melhuish used more concentrated solutions of quinine and different normalities of sulphuric acid. The differences in question were shown, however, not to influence the shape of the quinine fluorescence emission spectrum.[10]

Our solution of β-naphthol was 10^{-4} M. The absorbance was 0.135 per cm at the wavenumber of the exciting light, 32 000 cm^{-1}. The acetate/acetic acid buffer was 0.02 M with pH 4.67. Temperature 22.7°C. The spectrum of Lippert *et al.* was obtained at 23°C in the same buffer (pH not given) and with exciting light of the same wavenumber as in our experiment, but the concentration of β-naphthol was twice as large.

Emission spectra. The emission spectra to the left in Fig. 5 are of quinine in aqueous solutions of sulphuric acid. Our corrected spectrum agrees fairly well with the spectrum reported by Lippert *et al.*[9] The spectrum given by Melhuish [10] is, however, significantly different from ours.

The spectra to the right in Fig. 5 represent the fluorescence of β-naphthol in a 0.02 M acetate/acetic acid buffer with pH 4.67. Again the agreement between Lippert's spectrum and our corrected spectrum is fairly good.

DISCUSSION

Monitor efficiencies. The data in Table 1 are reliable only to the extent that the photochemical quantum yields of the ferrioxalate solution, as given by Hatchard and Parker,[4] are correct. The yields were stated to be "within a small percentage of the true values". Hatchard and Parker measured quantum yields only in the region between 23 000 cm^{-1} and 39 400 cm^{-1}. We have arbitrarily supposed that the quantum yields of the ferrioxalate actinometer are constant from 39 400 cm^{-1} to 43 000 cm^{-1} and from 23 000 cm^{-1} to 22 500 cm^{-1}. Below 22 500 cm^{-1} the absorbance of the actinometer solution is inadequate for convenient use in determinations of monitor efficiencies. Whenever the solutions of rhodamine B or fluorescein are used as fluorescent screens for exciting light with wavenumber below 22 500 cm^{-1}, the corresponding monitor efficiencies are supposed to remain constant down to the low frequency limits of the screen solutions.

A source of error in the actinometric measurements which had to be investigated, was the possible presence of stray light in the beam of exciting radiation. Stray light would lead to decomposition of the actinometric solution in excess of that due to the monochromatic light. This effect of stray light would be especially noticeable at the higher frequencies where wide slits and long irradiation times (5.5 h at 43 000 cm^{-1}) had to be used. Too high estimates of the intensity of the monochromatic component irradiating C would be the result. To test for stray light a glass filter, which absorbed the monochromatic component while most of the stray light would be transmitted, was placed in front of the entrance slit of the excitation monochromator. This was done at several wavenumber settings of the excitation monochromator in the interval between 35 000 cm^{-1} and 43 000 cm^{-1}. In all these experiments it was found that the glass filter completely abolished the photochemical reduction of the actinometric solution. We conclude that the excitation monochromator does not transmit significant amounts of stray light.

Excitation spectra. The differences between the excitation spectra and absorption spectra shown in Figs. 2, 3, and 4 all suggest falling fluorescence quantum yields towards higher frequencies. Application of the correction factors from Table 1 would amplify this trend. It must be emphasized, however, that some sources of error do remain. Spectral variations in the cross section of the exciting beam within the cell C, as well as polarization of the fluorescence light, could be responsible for minor discrepancies between absorption spectra and excitation spectra. Such effects must be eliminated before reliable conclusions can be drawn concerning small variations of the fluorescence quantum efficiency with the frequency of exciting light.

Nevertheless, the excitation spectra are sufficiently accurate for most practical purposes. Thus the spectra are good enough to identify a fluorescent compound. Discrepancies between excitation spectra and absorption spectra are readily discovered. Usually the excitation spectra can be used directly as recorded, since nothing is gained from corrections for the small spectral variations of the monitor efficiencies.

Spectral sensitivity distribution and emission spectra. In one respect our determination of the spectral sensitivity distribution differed from the procedure adopted by Parker. The MgO screen in Parker's setup was intentionally situated far away from the focus of the lens L, to avoid errors due to spectral dependence of the non-uniformity of the area of illumination on the screen. However, this geometry introduces a systematic error because the aperture of the beam of exciting light increases with the reciprocal focal length of L, and hence varies with frequency. The small fraction of the exciting beam which is interrupted by the MgO screen consequently decreases with increasing frequency. Hence, as the frequency increases, the fraction of the exciting light hitting M_2 decreases, and the sensitivity is accordingly estimated too low. To avoid this error we have placed the MgO screen close to the focus of L, to secure that the cross section of the beam is well within the area of the screen at all frequencies.

The sensitivity data in Table 2 have been calculated from measured signals from M_2 on the basis of monitor efficiencies taken from Table 1. Hence Table 2 is at least as inaccurate as Table 1. The estimates of sensitivity below 22 500 cm^{-1} are reliable only to the extent that the assumed invariance of the efficiency of the rhodamine B monitor between 22 500 cm^{-1} and 17 000 cm^{-1} is correct. We are not in a position to evaluate the limits of possible errors in Table 2.

The spectra in Fig. 5 are intended to demonstrate whether or not our sensitivity distribution yields corrected spectra which are compatible with those obtained by other workers. The discrepancy between Melhuish's spectrum and the two other quinine spectra is difficult to explain. Our corrected spectra for quinine and β-naphthol tend to rise above the spectra reported by Lippert *et al.* towards the higher frequencies. The difference between the two emission spectra of β-naphthol must, however, be interpreted with due allowance for experimental errors. Since the emission spectrum and absorption spectrum of β-naphthol overlap above 29 000 cm^{-1}, the high frequency end of the emission spectrum tends to be suppressed due to reabsorption of the fluorescence light. This effect must have been more pronounced in the spectrum given by Lippert *et al.* because the absorbance of their solution was 0.2 per cm at 30 000 cm^{-1}, *i.e.* twice the absorbance of our solution. Furthermore, the spectrum of β-naphthol is so sensitive to minor alterations in the buffer concentration [9,11] that inaccuracies in the preparation of the buffer may be responsible for the differences between the two spectra. We conclude that the sensitivity figures in Table 2 may be somewhat too low at the higher frequencies, but that this statement is rather uncertain due to possible experimental errors.

If the figures in Table 2 are too low at the higher frequencies, corrected emission spectra will be disproportionately amplified toward the higher

frequencies. Consequently, if the fluorescence quantum yield of two compounds are compared, a too high relative yield will be assigned to the substance which fluoresces in the higher frequency range.

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Received July 29, 1965.