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Fluorescence Quantum Yield Measurements: Vitamin B_6 Compounds

Abstract. *Quantum yields for pyridoxal, pyridoxamine, and pyridoxamine-5-phosphate at 25°C in neutral aqueous solution were 0.048, 0.11, and 0.14, respectively. The fluorescence efficiencies were highly temperature-dependent, and the compounds were extremely sensitive to light. There were marked discrepancies between the values obtained in this study for quantum yield and fluorescence polarization of pyridoxamine-5-phosphate and the values recently reported by others.*

It is desirable, when studying the fluorescence of solutions, to be able to measure absolute quantum yield (Q) conveniently and accurately. The quantum yield, Q, is the ratio of the number of photons emitted to the number absorbed. Spectrophotofluorometers have been calibrated by a number of methods (*1–3*) which make it possible to obtain corrected emission spectra with such instruments. The Aminco-Bowman spectrophotofluorometer, patterned after an instrument designed by Bowman *et al.* (*4*), has been used to measure the fluorescence quantum yields of a number of organic compounds by comparison of their corrected emission spectra with that of a fluorescent standard, and these values agreed closely with published data (*5*). However, it was surprising that reliable quantum-yield data obtained under well-defined conditions were available for so few compounds. Fluorescence data are incomplete without knowledge of the quantum yields, since this parameter is a measure of the strength of emission and is somewhat analogous to the extinction coefficient in absorption spectroscopy.

The quantum yields of compounds of the vitamin B_6 group, namely, pyridoxal, pyridoxamine, and pyridoxamine-5-phosphate (PMP) were measured. The rationale for undertaking this study is as follows. The binding of coenzymes such as diphosphopyridine nucleotide to proteins has been successfully studied by fluorimetry (*6*), and Undenfriend suggested that fluorescence techniques could also be used to study combination of vitamin B_6 coenzymes with proteins (*7*, p. 213). However, such a proposal is hampered by the fact that the emissions of free vitamin B_6 compounds have been poorly characterized; although a series of papers by Churchich on this subject has recently appeared (*8–10*). Measurements by Churchich of the quantum yield of PMP with an instrument described by Weber and Young (*11*) gave Q equal to 0.55 in neutral aqueous solution at 25°C, and this figure for quantum yield was used in the calculation of the fluorescence-lifetime and "radiationless" energy-transfer properties of PMP (*9–10*). However, there are no reports giving the impression that PMP possesses as high a fluorescence efficiency as quinine sulfate—one of the most fluorescent laboratory compounds—which has been shown to have a quantum yield of 0.55 at 25°C (*12*). Furthermore, the value of Q equal to 0.55 for PMP was inconsistent with the value of 0.11 reported for this compound (*8*).

I have measured fluorescence quantum yields with an Aminco-Bowman spectrophotofluorometer fitted with an Osram XBO-150 xenon arc lamp and an RCA 1P28 multiplier phototube (*13*). Calibration of the exciting light source was carried out with solutions of Rhodamine B (3 g/liter in ethylene glycol) or 1-dimethylaminonaphthalene-5-sulfonate (0.01M in 0.1M $NaHCO_3$) as fluorescence screens by the method of Melhuish (*2*), which requires the use of an adapter (*14*) to allow fluorescence to be observed from the side of the cuvette which faces the light source. Calibration of the detector system was accomplished with a standard tungsten lamp obtained from the U.S. National Bureau of Standards (*1*) or with the aforesaid xenon lamp after it had been calibrated (*2*).

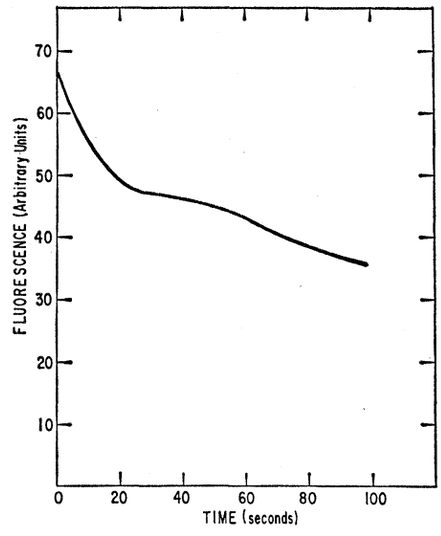

Fig. 1. Photodegradation of pyridoxamine-5-phosphate, $3 \times 10^{-6}M$, in 0.05M potassium phosphate buffer, pH 7.0. Illumination at 326 mμ in a microcuvette in the Aminco-Bowman spectrophotofluorometer. Temperature, 8°C.

Polarization of fluorescence was measured with polarized exciting light; light-stable, ultraviolet, polarizing filters were employed (*13*). Optical densities were determined on a Cary model 11 spectrophotometer. All reagents were obtained from commercial sources (*15*). Pyridoxal, pyridoxamine,

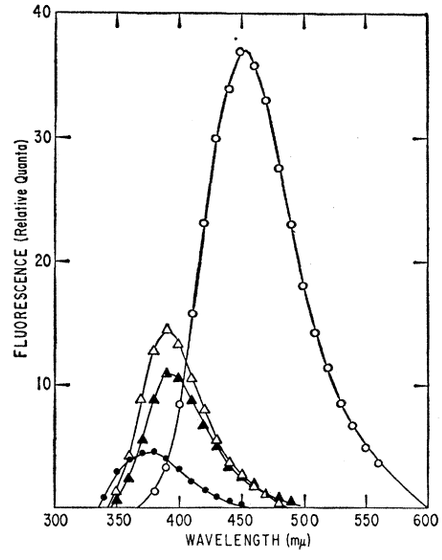

Fig. 2. Corrected emission spectra for quinine in 1N H_2SO_4 excited at 348 mμ (○), pyridoxal excited at 315 mμ (●), pyridoxamine excited at 324 mμ (▲), and PMP excited at 326 mμ (△). Solvent for the vitamin B_6 compounds was 0.05M potassium phosphate, pH 7.0. Areas beneath the curves are proportional to the absolute quantum yield.

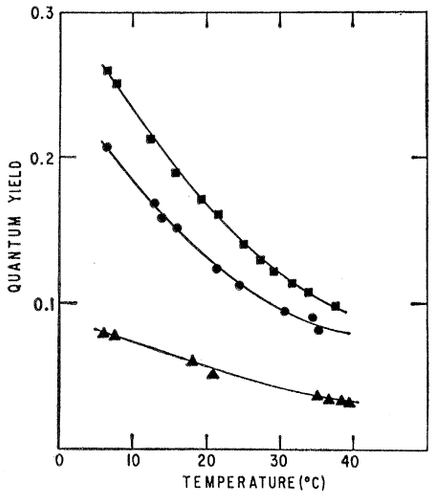

Fig. 3. Temperature dependence of the quantum yields of vitamin B_6 compounds. Curves refer to pyridoxal (▲), pyridoxamine (●), and PMP (■). Buffer and excitation wavelengths as in Fig. 2.

and PMP were examined in 0.05*M* potassium phosphate buffer, *p*H 7.0, and had the extinction coefficients reported by Peterson and Sober (*16*). Quinine had the same extinction at 366 mμ in 1*N* H_2SO_4 as the sample employed by Parker and Rees (*3*).

The method for measuring quantum yield may be summarized as follows. The areas under the corrected emission spectra (expressed as relative quanta per wavelength interval versus wavelength) of a fluorescent standard, quinine in 1*N* H_2SO_4, and of the unknown substance are compared. Light of different wavelengths may be used to excite the solutions, because the relative amounts of light reaching the solutions at different portions of the spectrum are known from the light source calibration. Quinine was excited at 348 mμ and the vitamin B_6 compounds were excited near their respective long wavelength absorption maxima. Quantum yields were calculated from the relation:

$$Q_x = Q_{st} \cdot \frac{F_x}{F_{st}} \cdot \frac{q_{st}}{q_x} \cdot \frac{OD_{st}}{OD_x}$$

in the limit $OD \rightarrow O$, where the subscripts *st* and *x* refer to standard and unknown solutions, Q is the quantum yield, F is the relative fluorescence (determined by cutting out and weighing the area underneath the corrected emission curve), q is the relative photon output of the source at the wavelength employed to excite a given solution, and OD is the optical density at the exciting wavelength. The same slit arrangement is employed in exciting standard and unknown; and under these conditions, the bandwidth of the excitation is constant at different settings of the excitation-grating monochromator (*2*). In order to avoid artifacts due to absorption of the incident light by the fluorescent solution, a microcell is used (*13*), and the effective optical densities are maintained below 0.01. With a calibrated spectrophotofluorometer, one person can obtain and process the spectra needed for determining the quantum yield of an unknown sample in less than an hour.

Initial experiments revealed that the vitamin B_6 compounds were strikingly photosensitive, and it was impossible to obtain reproducible emission spectra under the usual instrumental conditions (Fig. 1). Almost 50 percent of the fluorescence of PMP was lost in 100 seconds. The shapes of curves like that of Fig. 1 depend on the solution volumes, slit widths, temperature, degree of oxygenation, and so forth. Effective avoidance of photodecomposition was achieved by 100-fold attenuation of the light with the insertion of two 30-mesh, blackened, copper screens in front of the second focusing mirror inside the excitation monochromator; this did not alter the shape of the calibration curve for the exciting system. Corrected emission spectra for quinine, pyridoxal, pyridoxamine, and PMP are shown in Fig. 2. Relatively few points are needed to define these curves to the accuracy needed. These curves have been adjusted so that the areas underneath are proportional to the absolute quantum yields of fluorescence. At 25°C, these values are 0.11 ± 0.01 (pyridoxamine), 0.14 ± 0.01 (PMP), and 0.048 ± 0.003 (pyridoxal). The quantum yields of pyridoxamine and PMP approximately doubled in the interval from 26° to 6°C. The quantum yields as a function of temperature are shown in Fig. 3. These data illustrate how important it is that the temperature be specified in reporting quantum yields.

Erroneously low quantum-yield values may be obtained owing to asymmetric distribution of the emission if the fluorescence viewed at right angles to the exciting light is highly polarized; Singleterry and Weinberger (*17*) have given the necessary correction formulas. However, the corrections were negligible in the case of pyridoxal, pyridoxamine, and PMP in 0.05*M* potassium phosphate buffer, *p*H 7.0, at 25°C, since the polarizations were only 0.020 ± 0.010, 0.025 ± 0.010, and 0.035 ± 0.010, respectively. While polarization measurements with our apparatus can frequently be made with a precision as high as ± 0.002 (*13*), the attenuated light and the low quantum yields decreased the signal-to-noise ratio and limited the precision in the case of these compounds.

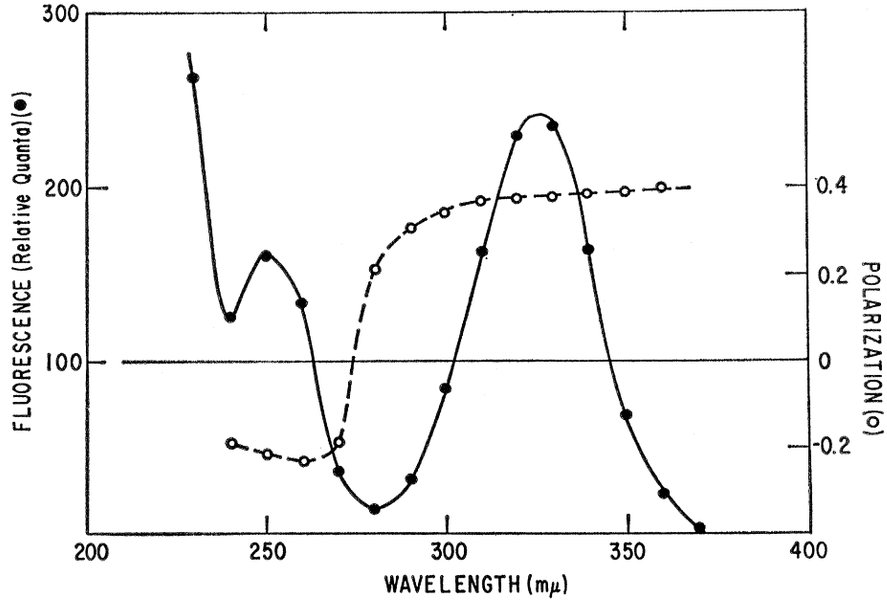

Fig. 4. Excitation spectrum (corrected) and fluorescence polarization spectrum of pyridoxamine-5-phosphate, $3 \times 10^{-5}M$ in a mixture of 90 percent glycerol and 10 percent potassium phosphate (0.05*M*), *p*H 7.0, at 8°C.

Nevertheless, the value of polarization (P) found for PMP appears to differ significantly from the value P equal to 0.06 reported by Churchich (*10*), who employed a double-beam, null-point, polarization photometer also said to measure polarizations to ± 0.002 (*18*).

In addition to data on the emission spectrum and on quantum yield, the fluorescence polarization spectrum has been advocated by Feofilov (*19*) as an independent parameter characteristic of the fluorescence of a given compound. The polarization of PMP fluorescence in 90 percent glycerol was measured as a function of exciting wavelength at 8°C (Fig. 4). The excitation spectrum shows two separated bands having peaks at ~250 mμ and 325 mμ. The polarizations in the regions corresponding to these bands are vastly different; and there is virtually no overlap between the two bands, as indicated by the very sharp transition from a highly positively polarized ($P = +0.39$) to a negatively polarized region ($P = -0.23$) in the excitation spectrum. According to accepted interpretations of such data (*19*), the direction of the absorption oscillators responsible for these two bands are probably mutually perpendicular, and the long wavelength electronic transition for absorption has a dipole moment parallel to that for emission.

It is not clear why there should be such a wide discrepancy between my results for the quantum yield, 0.14, of PMP and that reported by Churchich (*9, 10*), which is about four times the value reported here. Only part of this discrepancy can be accounted for by his use of 1-dimethylaminonaphthalene-5-sulfonate as a fluorescence standard having a quantum yield of 0.53 (*9*). This quantum yield, reported by Weber and Teale (*20*), is much higher than that found in other laboratories (*5, 21*).

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24 August 1965

Disulfide-Bond Cleavage and Formation in Proteins

Abstract. *Disulfide bonds can be cleaved at an alkaline* p*H by treating a protein with excess of a reagent disulfide in the presence of catalytic amounts of thiol. The cleavage products are stable and can be isolated; they contain the mixed disulfide between the reagent and the exposed thiol groups of the protein. The extent of cleavage is readily controlled by the* p*H of the reaction, temperature, and the addition of urea. Disulfide bonds cleaved by the reaction can be re-formed by exposing the mixed disulfide of the protein to catalytic amounts of thiol. Specific side chains can be added on to the thiol groups in native proteins by treatment with a reagent disulfide alone.*

Thiols have been used extensively for the specific cleavage of disulfide bonds in proteins by the disulfide interchange reaction at an alkaline *p*H (*1*). The resulting protein cleavage products have thiol groups which are sensitive to oxidation. When the re-formation of disulfide bonds by air oxidation is under investigation (*2*) this sensitivity is desirable, but it is disadvantageous during physical and chemical studies of the cleavage products. For such studies the exposed protein thiol groups are therefore often protected from undesirable oxidation by alkylation with iodoacetamide or *N*-ethylmaleimide. The alkyl groups cannot, however, be removed subsequently. I have found that under suitable conditions the disulfide interchange reaction can be used for the cleavage of protein disulfide bonds, giving in a single-step process a stable product, insensitive to oxidation, which can be isolated. The product can nevertheless be used in experiments in which disulfide bonds are to be re-formed.

The rationale of the procedure is illustrated by the following equations. Equation 1 shows the first reaction between an ionized thiol ($R\text{-}S^-$) and a protein disulfide bond.

$$\text{Protein-S-S-Protein}' + R\text{-}S^- \rightleftarrows \text{Protein-}S^- + R\text{-S-S-Protein}' \quad (1)$$

In the presence of excess thiol the reaction continues and the protein thiols (Protein-S^- and S^--Protein′) are the chief products.

$$R\text{-}S^- + R\text{-S-S-Protein}' \rightleftarrows S^-\text{-Protein}' + R\text{-S-S-R} \quad (2)$$

In the presence of an excess of the reagent disulfide (R-S-S-R), the reaction in Eq. 2 is replaced by the reaction in Eq. 3.

$$\text{Protein-}S^- + R\text{-S-S-R} \rightleftarrows \text{Protein-S-S-R} + R\text{-}S^- \quad (3)$$

Under these conditions the thiol is not consumed in the overall reaction but serves as a catalyst. The overall reaction, which yields as the products the mixed disulfides between the protein and the reagent (Protein-S-S-R and R-S-S-Protein′), is then more suitably written

$$\text{Protein-S-S-Protein}' + R\text{-S-S-R} \overset{R\text{-}S^-}{\rightleftarrows} \text{Protein-S-S-R} + R\text{-S-S-Protein}' \quad (4)$$

The protein cleavage products are stable, with their thiol groups fully protected from oxidation. Yet, since all the reactions are reversible, the protective groups can at any time be removed or the disulfide bonds may be re-formed by exposing the products to a suitable concentration of the thiol in the absence of the reagent disulfide.

I must emphasize that the disulfide