DETERMINATION OF THE RAMAN SCATTERING CROSS SECTION OF WATER BY COMPARING IT WITH THE FLUORESCENCE OF AQUEOUS DYE SOLUTIONS; QUANTITATIVE DETERMINATION OF "TRACES" BY USING RAMAN SCATTERING AS AN INTERNAL STANDARD

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(Received, April 30, 1983)

The spontaneous Stokes—Raman scattering cross section ($\sigma_{R,t}$) of a band of water between 2900—3700 cm^{-1} was obtained as 1.2×10^{-29} cm^2/molecule·sr, when the Raman scattering was compared with the fluorescence cross section ($\sigma_{F1,t}$) of aqueous fluorescein at an excitation of 480 nm, using a pulsed laser spectrofluorimeter. The fluorescence spectra of very small quantities of dansyl tryptophan (15 fmol) are measured and the amounts are calculated from spectral data ($\sigma_{R,t}$; $\sigma_{F1,t}$) as well.

Introduction

It is very important to know exactly the spontaneous Stokes—Raman scattering of solvents (*e.g.* water, ethanol, methanol, benzene *etc.*) both in spectroscopy [1] and since more recent times, for the quantitative determination of the organic traces of very small quantities (<pmol) dissolved in water or alcohols, where the Raman band of solvents is an internal standard [2, 3]. Several absolute and relative methods for measuring the cross section were applied [4—6], chiefly to determine the intensity of the 992 cm^{-1} Raman line in benzene. But the results of the total differential Raman scattering cross section ($\sigma_{R,t}$) showed a considerable difference (*e.g.* for water $\sigma_{R,t}=0.8\times10^{-29}$ cm^2/molecule.sr [7], $\sigma_{R,t}=4.5\times10^{-29}$ cm^2/molecule.sr [8] at a wavelength of 488 nm in the same plane of polarization for the incident light beam).

In the last few years, the fluorimetric technique made considerable progress *e.g.* the fluorescence spectrum of highly diluted dye solutions ($<10^{-10}$M) [3, 9] can be measured by pulsed laser spectrofluorimeters (PLSF). Since the fluorescence of a dye molecule is twelve orders of magnitude more intensive than the Raman scattering of the solvent molecule, the dye solution — where the ratio of $n_R/n_{F1}\sim10^{12}$ (n_R, n_{F1} is the number of molecules of the solvent and dye per cm^3) — is an excellent medium for the simultaneous investigation of the two molecular processes.

Since by the PLSF technique the fluorescence spectrum of solvents of $\sim10^{-10}$ M can be measured even in a volume less than 1 µl, it means that the detection limit for quantities of several, suitably fluorescent compounds is less than *pg* (fmol). This is

an important result for high-performance liquid cromatography (HPLC) and thin-layer chromatography (TLC) detection [10—12].

In the present study, by using an aqueous fluorescein dye solution, the total differential Raman scattering cross section ($\sigma_{R,t}$) of a band of water between 2900—3700 cm^{-1} compared with the fluorescence cross section of fluorescein ($\sigma_{F1,t}$) could be determined. We measured with a relative method the $\sigma_{R,t}$ data of ethanol and methanol, compared them with $\sigma_{R,t}$ of water; we aimed at calculating the concentration of dansyl tryptophan (Dns-Trp) from a single spectrum of Raman scattering and the fluorescence of Dns-Trp in ethanol, and at comparing the result with that obtained from the dilution of a standard solution.

Materials and methods

In order to determine the Raman scattering cross section of water we decided to use fluorescein dye dissolved in water (pH=12). This dye is one of the organic compounds the most frequently investigated with conventional methods as well as by a pulsed laser fluorimeter [9], and this dye is often used in biological investigations for staining [13]. Fluorescein was purified by chromatography, and the solvent was deionized water. The concentration of the aqueous fluorescein was changed from 10^{-4} to 10^{-11} M.

The tryptophan, a good representative of amino acids, was dissolved in a pH= =10 sodium carbonate buffer and was allowed to react with dansylchloride (dissolved in dimethylformamide) for 3 hours, at room temperature, with intensive stirring. The dansyl tryptophan (Dns-Trp) was separated by TLC (Kiselgel G, ethylacetate-acetic acid-methanol 20:1:1): the spot was eluted by spectral grade ethanol [14]. The initial concentration of the eluted Dns-Trp was measured by a spectrophotometric method [15]. Since the maximum absorption of dansyl derivatives is at 335 nm, the excitation by nitrogen laser suits it well.

The term "detection limit" has the following meaning as used by us: the ratio of SIGNAL to BACKGROUND (or BLANK) (*i.e.* the fluorescence of the solvent over the spectral range to be examined, where the SIGNAL is exhibited) is higher than 2, and the SIGNAL to instrumental NOISE ratio is higher than 10.

The main parts of the pulsed laser spectrofluorimeter are shown in Fig. 1.

The dye laser (DL) pumped by an atmospheric pressure nitrogen laser (TEA NL) contained a 7-diethylamino-4-methyl-coumarin dye. The pulse generator of NL triggers the discharge of NL, makes the stepping motor of the monochromator (M) step through the delaying pulse generator (D) and opens an electric gate (100—500 ns). The multichannel analyzer (MA) (NTA 1024) integrates the photocurrent of the photomultiplier (Ph) (FEU—100) after an amplitude/digital conversion. This complete apparatus is programmed to measure many times the total spectrum (340 nm—640 nm). The position of the sample holder to the incident, polarized dye laser beam is the same as it is in other experiments [7, 8]. It is needed for comparing results.

The pulse energy of the TEA N_2 laser and the dye laser at 480 nm was 75 μJ and 5 μJ, respectively. We chose lenses for L_1, in order that the area of the focused beam in the sample be about 1 nm^2. The duration of pulses was ~1 ns, thus the power density in the sample was 7.5 MW/cm^2 and 0.5 MW/cm^2 with 337 nm and 480 nm, respectively. The saturation photon irradiance (Φ_S) could be calculated by

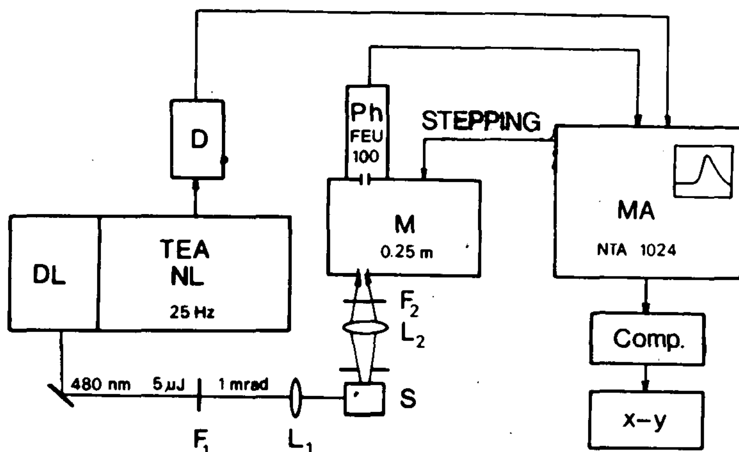

Fig. 1. Block scheme of the pulsed laser spectrofluorimeter. TEA *NL* — atmospheric pressure nitrogen laser, *DL* — dye laser, *D* — delaying pulsed generator, *S* — sample holder, *M* — monochromator, Ph — photomultiplier, MA — multichannel analyzator, Comp. — computer, $x-y$—$x-y$ plotter

Eq. (1):

$$\Phi_S = \frac{1}{\sigma_a} \cdot \frac{1}{\tau}. \tag{1}$$

The former power densities at given wavelengths were about Φ_S, and this was the optimum pulse excitation.

The spectra were recorded after computer correction (comp.) with a transmission function of the monochromator and the photomultiplier on the $x-y$ plotter ($x-y$).

Results

1. *Determination of $\sigma_{R,t}$ of water by comparing the Raman scattering with the fluorescence* Fig. 2 shows the total fluorescence intensity (lg I) as a function of the concentration of aqueous fluorescein (lg c). The linear connection through five orders of magnitude of c showsh the exact dilution of solutions.

In Fig. 3 the dashed line is the corrected total spectrum of the aqueous fluorescein of 10^{-10} M (λ_e=480 nm). The dashed-dotted line is the blank spectrum of the water used. In this case we can see the Raman band of the water of 2900—3700 cm^{-1}, between 560 nm and 580 nm. The continuous line is the fluorescence spectrum of fluorescein, the distribution of which was the same as that of measured at concentrations from 10^{-6} to 10^{-10} M, and these were also identical with the spectrum measured by another conventional spectrofluorimeter at 10^{-5} M.

Having measured the total spectrum at a concentration of 10^{-10} M, we obtained a quantum spectrum, proportional to the photon numbers obtained from fluores-

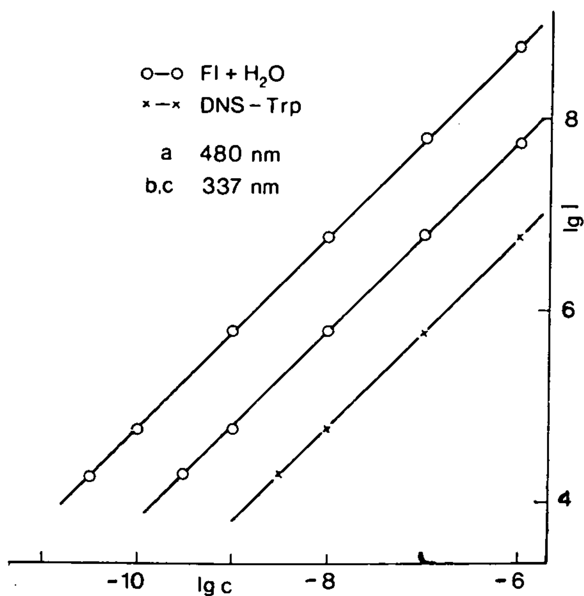

Fig. 2. The total fluorescence intensity (lg *I*) as a function of the concentration (lg *c*) of aqueous fluorescein (a) (λ_e=480 nm), (b) (λ_e=337 nm) and dansyl tryptophan (c) (λ_e=337 nm)

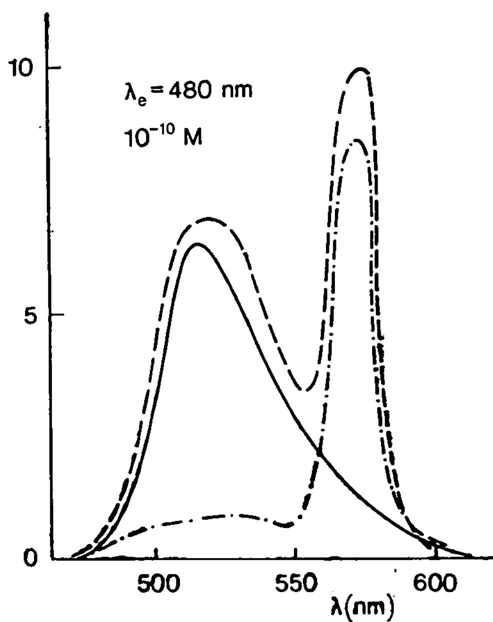

Fig. 3. Fluorescence and Raman spectrum of the aqueous fluorescein at a concentration of 10^{-10} M (dashed line), the blank spectrum of the water (dashed-dotted line) and the fluorescence spectrum of fluorescein (continuous line)

cence (N_{f1}) and Raman scattering (N_R) (N_{f1}/N_R equaled the ratio of the areas under the fluorescence and the Raman band), which was proportionate to the following ratio:

$$\frac{N_{f1}}{N_R} = \frac{n_{f1}}{n_{H_2O}} \cdot \frac{\sigma_{f1,t}}{\sigma_{R,t}}; \quad \text{where} \quad \sigma_{f1,t} = \frac{\sigma_a \cdot \eta}{4\pi}, \tag{2a-b}$$

where σ_a was the absorption cross section at the excitating wavelength (λ_e) and η was the fluorescence quantum yield.

We summarized the experimental and calculated data in Table I. We got for a total differential Raman scattering cross seetion of the band of water (corresponding to the OH bond of 2900—3700 cm^{-1}) $\sigma_{R,t}=1.2\times10^{-29}$ cm^2/molecule · sr.

2. *Raman scattering cross section of other solvents.* We can see in Fig. 4 the corrected Raman scattering spectra of water, ethanol and methanol used, when the excitation wavelength was 480 nm. The total differential Raman scattering cross section of bands between 2800—3600 cm^{-1} were 2.4×10^{-29} cm^2/molecule · sr and 2.0×10^{-29} cm^2/molecule · sr, respectively. The Raman intensities were evaluated from the integrated areas of the individual Raman bands, recorded on the $x-y$-plotter.

3. *Quantitative spectroscopic trace analysis applying Raman scattering of solvents as internal standard.* We deluted the aqueous fluorescein and the dansyl tryptophan in ethanol to a concentration of $3\cdot10^{-9}$ M. A number of corrected spectra of these solutions are shown in Fig. 5 (λ_e=337 nm; la, 2a — 10^{-8} M; 1b, 2b — $3\cdot10^{-9}$ M). The measured and the calculated (by Eg. 1a-b) ratios of the fluorescence and Raman bands (at concentration of 10^{-8} M) are also collected in Table I.

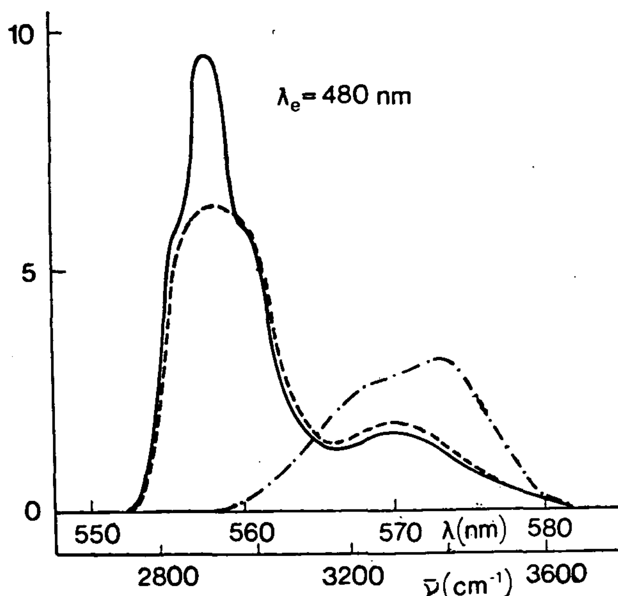

Fig. 4. Raman spectra of water (— · —), ethanol (—) and methanol (exciting wavelength = 480 nm)

Table I

Flourescein 0.01 N NaOH H_2O (MW=332)	λ_e=480 nm		$c=10^{-10}$ M		V=10 μl		5 μJ/pulse	
	ε (1/M cm)	σ_a (cm²/molecule)	$\sigma_{fl,t}$ (cm²/molecule·sr)	η	n_{fl}/n_{H_2O}	N_{fl}/N_R exp.	N_{fl}/N_R cal.	
	$6 \cdot 10^4$	$2.4 \cdot 10^{-16}$	$1.8 \cdot 10^{-17}$	0.93	$1.8 \cdot 10^{-12}$	2.5	—	0.33 pg=1 fmol
	$\sigma_{R,t}$ (cm²/molecule·sr)			λ_e (nm)				
H_2O	$1.2 \cdot 10^{-29}$ $4.9 \cdot 10^{-29}$			480 337				
EtOH	$2.4 \cdot 10^{-29}$ $9.8 \cdot 10^{-29}$			480 337				
	λ_e=337 nm		$c=10^{-8}$ M		V=10 μl		75 μJ/pulse	
Fluorescein 0.01 N NaOH H_2O	$4 \cdot 10^3$	$1.53 \cdot 10^{-17}$	$1.2 \cdot 10^{-18}$		$1.8 \cdot 10^{-10}$	5.3	4.4	
Dns—Trp EtOH (MW=435)	$4.3 \cdot 10^3$	$1.65 \cdot 10^{-17}$	$0.92 \cdot 10^{-18}$	0.70 [16]	$4.6 \cdot 10^{-10}$	3	4.3	43.6 pg 100 fmol

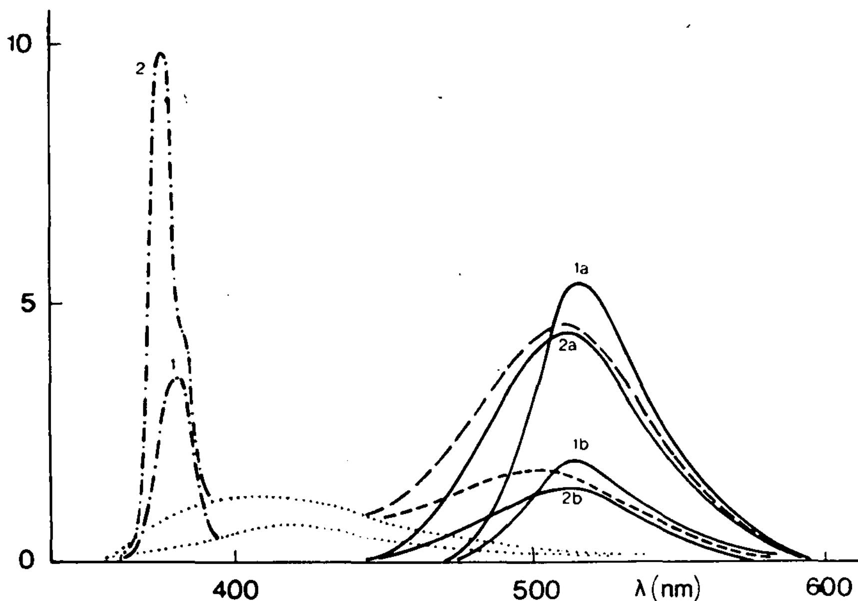

Fig. 5. Total spectra of the aqueous fluorescein (1) and dansyl tryptophan in ethanol (2) at concentrations of 10^{-8} M and $3 \cdot 10^{-9}$ M (a, b), respectively. (..., —·— fluorescence Raman scattering of solvents, — total fluorescence, — fluorescence of Fl and Dns+Trp).

Conclusions

1. We have described a new spectroscopic (pulsed laser spectrofluorimetric) method for determining the spontaneous Stokes—Raman scattering compared with the fluorescence of the "tracing" dye.
2. We applied this spectroscopic method for the quantitative determination of dissolved "traces" (without calibrating curves) by using data of the Raman scattering cross section of solvents at a low concentration of 10^{-8}—10^{-10} M, measuring only a single total spectrum.
3. The spectral detection limit in our measurement was $3 \cdot 10^{-11}$ M for fluorescein and $3 \cdot 10^{-9}$ M for Dns-Trp. The excited volume was 10 μl, so that the mass of fluorescein dye and Dns-Trp was 0.1 pg (0.3 fmol) and 15 pg (30 fmol), respectively and consequently, the number of detected molecules was $2 \cdot 10^7$ and $2 \cdot 10^9$. The total emission spectra can be obtained in a short time (1—2 min).
4. The fact that fluorometric spectral detection of dyes of subfemtomol quantities was achieved can be considered as a good result, but spectrofluorometric detection of dansyl amino acids of fmol has not been reported earlier [17], except for fluorescence derivatization of amino acids by *o*-phthaldialdehyde [18]. This means that this pulsed laser spectrofluorimeter is highly sensitive and may prove a good detector for high performance liquid chromatography (HPLC).

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ОПРЕДЕЛЕНИЕ ПОПЕРЕЧНОГО СЕЧЕНИЯ КОМБИНАЦИОННОГО РАССЕЯНИЯ (КР) ВОДЫ ПРИ ЕГО СРАВНЕНИИ С ФЛУОРЕСЦЕНЦИЕЙ ВОДНЫХ РАСТВОРОВ КРАСИТЕЛЕЙ; КОЛИЧЕСТВЕННОЕ ОПРЕДЕЛЕНИЕ «СЛЕДОВ ВЕЩЕСТВА» ПРИ ИСПОЛЬЗОВАНИИ СИГНАЛА КР В КАЧЕСТВЕ ВНУТРЕННЕГО РЕПЕРА

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При возбуждении излучением с длиной волны 480 нм в импульсном лазерном спектрофлуориметре получена величина $1{,}2\cdot 10^{-29}$ см²/мол. ср для сечения спонтанного стоксового КР $\sigma_{R,t}$ полосы воды (2900—3700 см$^{-1}$) путем сравнения КР с поперечным сечением флуоресценции $\sigma_{Fl,t}$ водного флуорсцеина. Измерены спектры флуоресценции очень малых количеств ($\sim$15 фмол) dansyl-tryptophan-a а также вычислены их величины по спектральным данным $\sigma_{R,t}$ и $\sigma_{Fl,t}$.