Variations in the fluorescence spectra of chlortetracycline in different spectrofluorometers

Chlortetracycline (CTC) is one of the tetracycline antibiotics that has been used widely to detect fluxes of calcium concentration at its intracellular stores[1–4]. It has emission bands at 420 nm and 520 nm (ref. 5). The latter band is broader, sensitive to divalent cations and to polarity of the media[6–8]. We observed that there was a marked variation in the spectral pattern of the 520 nm emission band of CTC in different spectrofluorometers. There were two peaks observed for the 520 nm emission in one spectrofluorometer (instrument I) but a single peak was seen in the second (instrument II)[9]. The latter pattern was reported in all earlier papers[1,5,7]. Both the instruments are now widely used. All the results given here were done in instrument I. We show here that the broad nature of the 520 nm emission band of chlortetracycline and variations in the transmittance of the emission monochromator to vertically and horizontally polarized light resulted in differences in the emission spectra of CTC in different spectrofluorometers.

It was observed that polarization of light changed the pattern of spectra in instrument I. The sample was excited with horizontally or vertically polarized light. The corresponding emission spectra were recorded through the emission polarizer oriented vertically and then horizontally (Figure 1 *a*). Whenever the emitted light was horizontally polarized, there were two peaks. But only a single peak appeared when emitted light was vertically polarized. The position of the excitation polarizer did not influence the pattern of the emission spectra. Also, the single peak was at a wavelength which was in between that of the double peaks. The unpolarized emission spectra in instrument I and in instrument II resembled the one seen when the emitted light was horizontally and vertically polarized, respectively. But these changes in spectral pattern were not observed with fluorescein (Figure 1 *b*), which has an emission band overlapping the 520 nm band of CTC.

This was further examined by exciting the sample at 0 position (light falls directly on the sample from the xenon lamp without being diffracted by the excitation monochromator). In the absence of dye, the spectra of scattered light were recorded by scanning with emission monochromator (Figure 2 *a* and *b*). When the polarizers were oriented at HV position, one band was seen. But two bands were seen when the polarizing filters were in the HH position. When CTC was added to the sample, its fluorescence was superimposed on the spectra of the scattered light but the pattern remained the same having a single peak or double peak (Figure 2 *a*). The pattern did not change even at very high intensities of CTC fluorescence as seen in methanol with 1 mM calcium (data not shown). It could be inferred from Figures 1 and 2 *a* that the transmittance of emission monochromator to vertically or horizontally polarized light changed the pattern of spectra.

When experiments in Figure 2 *a* were repeated with fluorescein and with polarizers at HH position (Figure 2 *b*), it was observed that at lower concentrations of the dye, the two peaks were seen. But there was a gradual change to single peak at higher concentrations of the dye. The latter observation was in contrast to that seen with CTC in Figure 2 *a*. To understand the contrasting patterns of spectra seen at higher intensities of fluorescence of the dyes in HH position of filters, the normalized emission spectra of the dyes at higher concentrations and the spectra of scattered light were compared (Figure 2 *c*). All samples were excited at 0 position and filters were oriented at HH. The double peaks of CTC emission completely overlapped the double 'band' seen with scattered light. Fluorescein band was narrower and only partially overlapped the double band of the spectra of scattered light. Therefore, variation in the transmittance of the emission monochromator was fully carried on to emission band of CTC, but the fluorescein band was too narrow to reflect it. It could be inferred from Figure 2 *c* that the broad nature of the 520 nm emission band of CTC also contributed to the variations in the emission spectra.

The transmission efficiency of light by grating monochromators varies with wavelength of light, the type of grating (planar or concave), the polarization of light and the blaze angle[10]. Monochromators have higher efficiency of transmission to horizontally polarized light at wavelengths longer than their maximum efficiency of transmission[10]. The wavelength of the maximum efficiency of

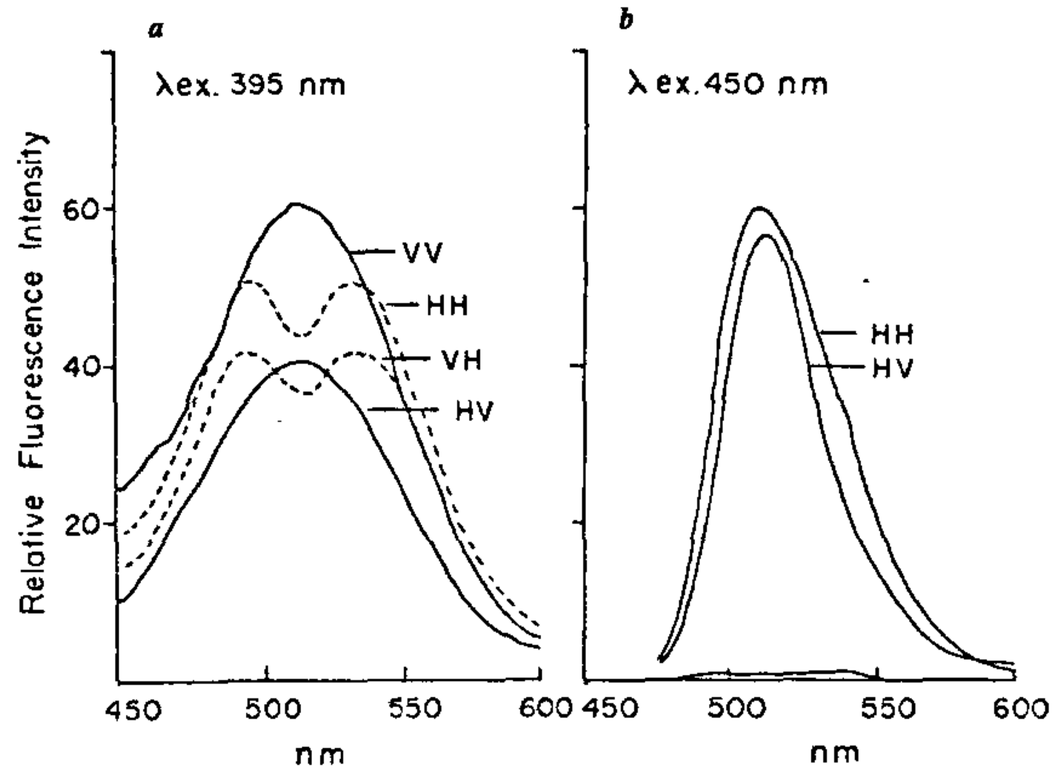

Figure 1. Emission spectra of CTC and fluorescein at horizontal and vertical positions of polarizing filters recorded in instrument I. *a*, 10 μM CTC and 10 mM Ca^{2+} in 10 mM Hepes buffer pH 7.4; *b*, 250 nM fluorescein in 0.05 N NaOH. VV, VH, HH and HV are orientations of the excitation and emission polarizers, respectively.

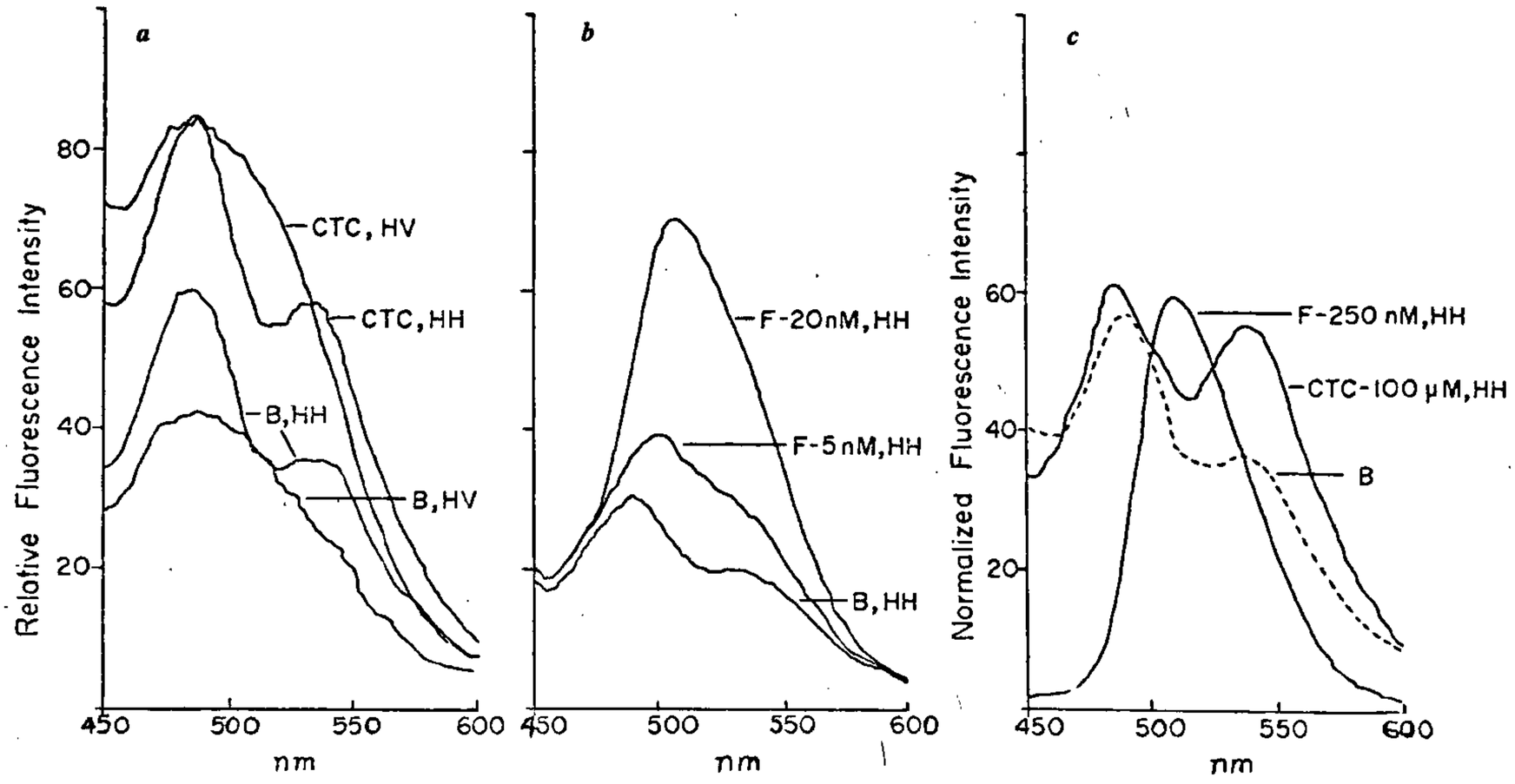

Figure 2. Spectra of scattered light and fluorescence of CTC and fluorescein (F) excited at 0 position in instrument I, when light falls directly on the sample from the xenon lamp, without being diffracted by the excitation monochromator. Spectra were recorded in the absence (B) or presence (as labelled) of the dyes. Solutions of dyes and their concentrations were as in Figure 1, unless mentioned otherwise. HH and HV are orientations of the excitation and emission polarizers, respectively. In (*a*) and (*b*) the fluorescence spectra of the dyes are compared with that of the blank (B). (*c*) is the normalized emission spectra of the three samples.

transmission of monochromators can be varied by blazing. In instrument I, the emission monochromator is blazed at 450 nm. Therefore, when the unpolarized 520 nm band of CTC passes through the emission monochromator, the light coming out will be predominated by horizontally polarized light. So the unpolarized spectra of CTC in instrument I will resemble the one seen at HH or VH orientation of polarizers (Figure 1 *a*), where double peaks are seen.

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9. *Instrument I*: Kontron SFM 25, monochromators with concave holographic gratings, 1200 lines/mm, ex. monochromator blazed at 250 nm and em. monochromator blazed at 450 nm. The instrument was set in the ratio mode, at an excitation bandwidth of 10 nm, emission bandwidth of 20 nm, response time of 1 s and scan speed of 100 nm/min; *Instrument II*: Hitachi Model 650-10, monochromators have concave diffraction gratings of 600 lines/mm. Instrument settings were the same as for I except scan speed set at 80 nm/min.
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