

SOME FLUORESCENCE PROPERTIES OF CATARACTOUS EYE
LENSES

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ABSTRACT

The loss of transparency of the ocular lens is caused by the increase of light scattering as a result of structural changes and by the increased absorption of the visible light due to the accumulation of pigments. Following light absorption, these pigments undergo non-radiative and radiative (luminescence) processes which can be monitored spectroscopically. The paper presents some new results concerning the excitation spectra, decay times and polarization of the lenticular fluorescence. Fluorophore heterogeneity manifests itself in all the experimental data. A striking behaviour of the emission anisotropy as a function of temperature is found, particularly for cortical cataract lenses, indicating temperature-induced structural changes at about 20°C.

INTRODUCTION

The photoluminescence of the eye lens was first reported by Regnauld in 1858 [1]. Since then numerous studies of this phenomenon, both *in vivo* and *in vitro*, were performed [2-10]. *In vivo* lens fluorescence can be

utilized for the early detection of cataract [11] and for the evaluation of the anticataract drug efficacy [12].

A number of fluorescent chromophores (fluorophores), also referred to as fluorogens, have already been identified [13-15]. Absorption in the UV region below 300 nm is mainly due to the aromatic amino acids tryptophan, tyrosine and phenylalanine present in lens proteins. In terms of visual capability this absorption is insignificant. Chromophores absorbing in the visible range, however, contribute directly to the loss of the lens transparency. A haze caused by their fluorescence may also interfere with vision [6]. The identified, visible light absorbing fluorophores in the lens are tryptophan derivatives N'-formylkynurenine, L-kynurenine and O- β -D-glucoside of 3-hydroxy-L-kynurenine, as well as 3,3'-bityrosine and anthranilic acid [13-15]. In addition, there is a possibility of the accumulation and retention of numerous extraneous fluorescent compounds, in particular drugs, such as psoralens, phenothiazines, porphyrins or allopurinol [16].

Fluorescence methods are one of the modern, rapidly developing, tools for probing the biomolecular systems [17]. Since fluorescence is sensitive to the properties of the fluorophore's molecular environment, these methods may be used to provide information about it and in particular about its structural and dynamic properties (including rapid nano- and picosecond processes). The extreme single photon sensitivity of modern fluorescence techniques together with the possibility of their *in situ* non-destructive application seem to be a great advantage.

From the point of view of the eye lens

transparency an important property is its absorption spectrum in the visible range from about 380 nanometers to about 700 nanometers. Accurate, direct measurements of the spectral distribution of absorbed light are, owing to strong light scattering and the shape of the lens, very difficult to perform. These difficulties can be avoided either by using the photoacoustic method [3,18] or by recording the fluorescence intensity as a function of the excitation wavelength (the fluorescence excitation spectrum) at a fixed emission wavelength [2]. Such a spectrum is akin to the absorption spectrum and may be, in principle, measured *in situ* as well as other fluorescence characteristics (including its emission spectrum, polarization and decay).

MATERIALS AND METHODS

A total of 102 lenses obtained as a result of intracapsular cataract extraction from the Eye Department of the Joint Provincial Hospital in Toruń were studied. The age of patients ranged from 0 to 87. Intact lenses were placed in a special holder between two quartz windows of 0.5 mm thickness containing no fluids and measurements were performed (unless otherwise stated) at room temperature about one hour after the operation. In some cases the lenses were stored for several days at 0°C. Lens extracts were prepared according to the following procedure. Decapsulated lenses were dessicated in the presence of P_2O_5 for 4 days, cut into four parts and pounded. Each part was then mixed with a solvent (tris buffer pH 7.5 with 0.02 % NaN_3 , cyclohexane, ethanol or acetonitrile),

the mixture was homogenized for 10 minutes in an ultrasonic bath and centrifuged. Aqueous solutions were additionally dialysed to reduce light scattering, otherwise interfering with absorption measurements, and to study the effect of removal of proteins and other macromolecular material on their fluorescence.

Absorption spectra were measured using a Specord UV-VIS spectrophotometer from C. Zeiss, Jena (East Germany). Fluorescence excitation and emission spectra were recorded on a home built versatile computer-controlled photon-counting spectrofluorimeter equipped with polarizers. Measurements of fluorescence excitation spectra required the use of the quantum counter solution of rhodamine B in ethylene glycol (3g/l) in the reference channel of the instrument. With this quantum counter the highest accuracy is obtained in the range of excitation wavelengths limited to 360-600 nm. Emission spectra were not corrected for the spectral sensitivity of the detecting system. Fluorescence decay curves were measured with a home built time-correlated single photon nanosecond fluorometer using a nitrogen-filled flashlamp. The finite time-widths of the exciting light pulse and the instrumental response (an overall halfwidth of about 2 nanoseconds) required the use of a deconvolution procedure to obtain the pure response of the fluorescence intensity to an infinitely short excitation pulse (the so called impulse response). A least squares fitting deconvolution procedure was used to recover the fluorescence decay parameters [19]. In all excitation-emission studies, front face excitation of the anterior part of the lens was applied. The illuminated area, about 2 mm², was centered on the optical axis of the lens. The exciting beam

direction made an angle of 45° with the lens axis and the fluorescence emission was observed along this axis. Construction details of our instruments are available on request.

RESULTS

Let us first present the results obtained for cataractous lens extracts. Typical absorption spectra of such extracts are shown in ~~Figs. 1 and 2~~. Acetonitrile extracts exhibited no measurable absorption in the 200 - 800 nm range. The strongest absorption was found for aqueous extracts and appears to be mainly due to the presence of the aromatic amino acids in the extracted substance. The spectra of ethanolic and cyclohexane extracts are similar to each other, but differ strongly in their intensity, shape and position with respect to the spectra of aqueous extracts. It is interesting to note that the aqueous extracts spectra markedly extend into the visible range for nuclear and mixed cataracts (Fig. 1), but not for the sugar cataract (Fig. 2).

The fluorescence spectra were measured for all of the prepared extracts, using two excitation wavelengths: 366 nm and 436 nm. Only the aqueous extracts exhibited measurable fluorescence under these conditions and the position and shape of the emission band were found to be excitation wavelength-dependent (Fig. 3). The same figure also shows the pronounced effect of dialysis on the fluorescence of the studied extracts. In particular dialysis leads to a several-fold increase of the emission intensity and shifts the maximum of the emission excited with the 436 nm light from about 500

nm to 520 nm.

All of the following results were obtained for whole intact lenses. Figure 4 shows examples of normalized fluorescence spectra of three lenses. These spectra are strongly excitation wavelength-dependent, revealing the heterogeneity of fluorophores. Figure 5 displays the same spectra, but with relative intensities preserved for each excitation wavelength.

Figure 6 presents typical fluorescence excitation spectra (the ratio of the number of emitted photons to the number of incident photons as a function of the excitation wavelength) of transparent (a) and cataractous (b,c) lenses. These excitation spectra strongly depend on the wavelength used to observe fluorescence (500,550,600,650 and 700 nm) which is again an indication of the heterogeneity of the fluorophore population (particularly evident in Fig. 6 b and c). An interesting finding is that 5 out of 6 sugar cataract lenses are characterized by the same spectra shown in Fig. 6c.

Figure 7 presents fluorescence excitation spectra and the emission anisotropy defined as $(I^{\parallel} - I^{\perp}) / (I^{\parallel} + 2I^{\perp})$, where I^{\parallel} and I^{\perp} are the parallel and the perpendicular polarization components of the fluorescence intensity in the case of a linearly polarized excitation light, for two typical cases of cataractous and transparent lenses. The emission anisotropy appears to be essentially constant across the excitation spectrum. A slight increase with rising excitation wavelength may probably be attributed to some leakage of the strongly scattered excitation light through the emission monochromator. This effect is more pronounced for the cataractous lens.

Figures 8,9 and 10 display the temperature

dependence of the emission anisotropy, obtained on heating (Figs. 8,9) and both on heating and cooling.

Typical results of the fluorescence decay measurements for lenses no. 6, 7, and 8 (see excitation spectra in figures 4 and 5) obtained using the 337 nm excitation are shown in Table 1. They represent the impulse response of fluorescence, which was assumed to be a sum of three exponentials: $\sum_{i=1}^3 \alpha_i e^{-t/\tau_i}$.

TABLE 1

Amplitudes α (relative) and Decay Times τ of the Fluorescence Decay for Lenses no. 6, 7 and 8 measured at different Emission Wavelengths λ_{em} . Excitation Wavelength: 337 nm.

lens no.	λ_{em} [nm]	α_1	τ_1 [ns]	α_2	τ_2 [ns]	α_3	τ_3 [ns]
6	398	0.82	0.7	0.24	3.5	0.04	13.6
	423	0.94	0.8	0.26	3.7	0.05	11.1
	451	1.16	0.7	0.25	3.4	0.04	9.4
	483	1.34	0.7	0.17	3.4	0.02	10.3
	542	1.97	0.5	0.12	3.2	0.01	12.6
7	398	0.62	1.8	0.19	5.7	0.003	27.8
	423	0.56	1.4	0.27	4.4	0.04	10.9
	483	0.55	2.2	0.19	7.5	0.01	27.1
	542	0.52	2.2	0.20	8.3	0.004	86.2
8	398	0.70	0.6	0.41	2.7	0.09	7.7
	451	0.55	1.5	0.25	5.1	0.04	12.8
	542	0.50	1.3	0.26	4.5	0.09	11.8

The results shown in Table 1 indicate that the fluorescence decay of a transparent lens no.5 may indeed be represented by three exponentials with decay times essentially independent of the emission wavelength, but in the case of the lenses no.6 and 7 more components are required to adequately represent the data.

DISCUSSION

Fluorescence heterogeneity of cataractous eye lenses is manifested in all of the results presented in the foregoing section. Dialysis did not remove the excitation wavelength dependence of the aqueous extracts, which indicates that the main factor in the observed heterogeneity is the presence of various fluorophores, while the effect of the fluorophore site heterogeneity is less important. The influence of the macromolecular environment (absent in dialysed extracts) manifests itself, however, in the quenching of the fluorescence emission in Figure 3. A bathochromic shift of the emission band after dialysis, in Figure 3b, indicates that the environment of the fluorophores selectively excited with the 436 nm light is in non-dialyzed extracts most likely hydrophobic.

Fluorescence spectra shown in Figure 4 demonstrate that the fluorophore population of the transparent neonatal lens is less heterogeneous compared with mature and senile cataract lenses. This conclusion is supported by the decay time data shown in Table 1.

The fluorescence of the transparent lens is of relatively small intensity (Fig.5). In particular, comparing the intensities in Figure 5c one can judge that the concentration of fluorophores in the transparent lens, excited with the 405 nm light, is about two orders of magnitude smaller than in the two other lenses. This confirms earlier findings [15] that light absorption of the aging lens is progressively extending into longer wavelength regions of the spectrum.

Similar remarks can be made about the fluorescence excitation spectra in the visible range shown in Figure 6. In addition, however, one observes a distinct spectral pattern for sugar cataract lenses (Fig.6c).

Results of the emission anisotropy measurements cannot be unequivocally interpreted. Its value is influenced by too many factors involving the fluorophores and their environment, which are beyond control. These include the structural ordering, the intrinsic angles between absorption and emission dipoles of fluorophores, their rotational mobility and internal flexibility on the nanosecond time-scale, the electronic excitation energy transfer [17] and also light scattering and birefringence [20] of the lens.

The obtained temperature dependence of the emission anisotropy is quite challenging (Figs.8,9 and 10). For instance, the increase of the emission anisotropy with rising temperature (Figs.8 and 10) is inexplicable. One would rather expect its value to decrease, which is the usual property of the fluorescence anisotropy of solutions [17] and is caused by a more rapid randomization of photoselected molecular orientations due to faster rotational diffusion at elevated temperatures. A striking

behaviour of the emission anisotropy is observed for cortical cataract lenses (Fig.9). It appears that a sort of phase transition takes place in these lenses at about 20°C. A different pattern is observed for a sugar cataract lens (Fig.10) whose emission anisotropy displays a minimum, also at about 20°C, on heating. This minimum does not appear on cooling.

In view of the above findings it seems appropriate to draw the attention of other investigators to the necessity of the temperature control (within 1°C) in their studies of physical properties of the lens.

The data shown in Table 1 were analyzed in terms of the fractional contributions $f_i(\lambda_{em}) = \alpha_i \tau_i / \sum \alpha_i \tau_i$ ($i = 1,2,3$) of each of the three components to the total fluorescence intensity. This unique, three component decomposition was possible only for the transparent neonatal lens no. 5. Results of such an analysis are shown in Figure 11. The fractional contributions $f_i(\lambda_{em})$ were then used to resolve the composite emission spectrum of the lens no. 5 (the solid line in Fig.4a) into three components. This was accomplished by simply multiplying the composite spectrum by f_i and the resulting component spectra are shown in Figure 12. These spectra correspond presumably to three different fluorescent species.

Such a unique three component decomposition was not possible for lenses no. 7 and no. 8, which indicates a larger heterogeneity of the fluorophore population (more than three species) in opacified lenses.

Since the presented results raise more questions than provide answers, further studies of the eye lens fluorescence are needed. The author hopes that this opinion will be shared by the reader.

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Figure captions

FIGURE 1

Absorption spectra of cataractous lens extracts for a 2 cm optical path: a) tris buffer pH 7.5 with 0.02 % NaN₃ added, b) ethanol, c) cyclohexane.

Lens no.1 - nuclear cataract, male 31, lens no.2 - mixed cataract, female 85.

FIGURE 2

Same as in figure 1 for sugar cataract lenses.

Lens no.3 - f.76, lens no.4 - f.65.

FIGURE 3

Influence of dialysis on the fluorescence of the aqueous extract of the lens no.1 excited at two wavelengths: a) 366 nm and b) 436 nm.

FIGURE 4

Normalized fluorescence spectra of lenses obtained at various excitation wavelengths: 302 nm - dotted line, 334 nm - broken line, 405 nm - solid line.

No.5 - transparent neonatal lens, no.6 - partially opaque lens from an enucleated eye, no.8 - senile cataract lens.

FIGURE 5

Same as in figure 4, but with relative intensities preserved.

FIGURE 6

Fluorescence excitation spectra obtained at fixed emission wavelengths for : a) transparent lens, b) and c) cataractous lenses.

FIGURE 7

Typical fluorescence excitation spectra obtained for the 550 nm emission and the corresponding spectra of the emission anisotropy.

FIGURE 8

Temperature dependence of the fluorescence anisotropy of four lenses: a) f.66 and b) f.82 - mixed cataract lenses, c) f.61 - almost transparent non-cataractous lens, d) f.68 - nuclear cataract lens.

FIGURE 9

Temperature dependence of the fluorescence anisotropy for two cortical cataract lenses: a) f.68, b) m.59.

FIGURE 10

Temperature dependence of the fluorescence anisotropy for a sugar cataract lens (f.78): a) on the day of the extraction, b) five days later.

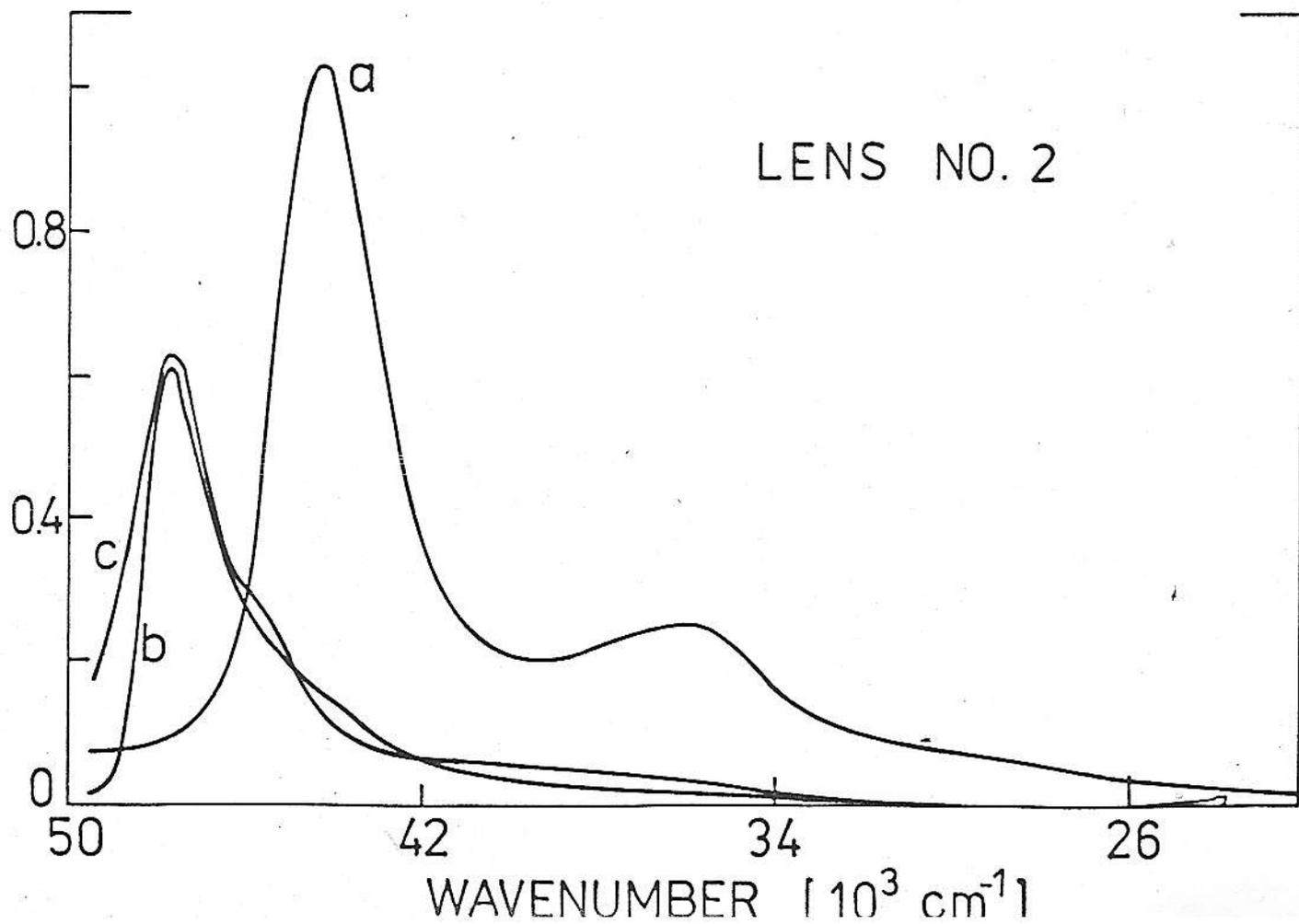
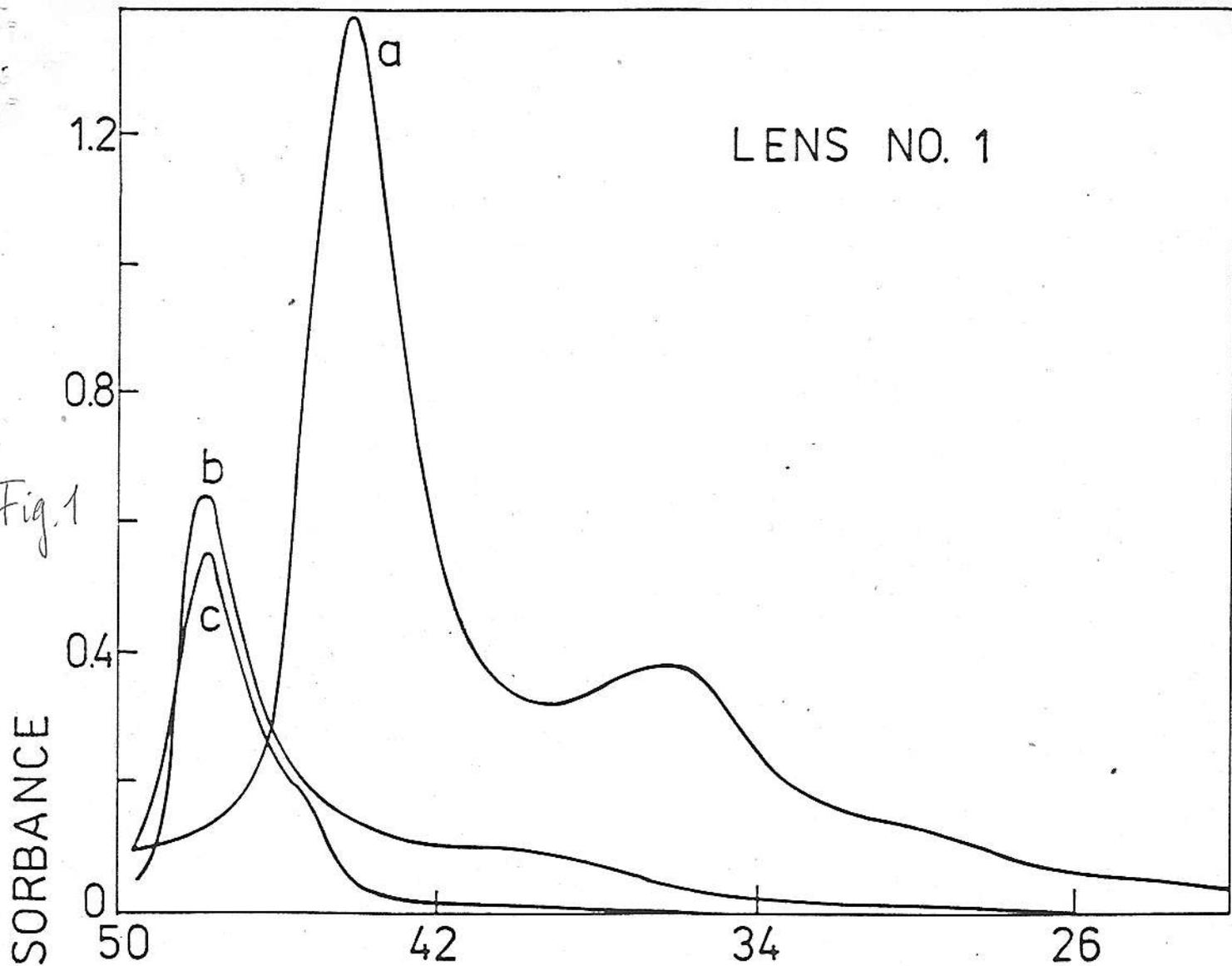
FIGURE 11

Fractional intensities of the three fluorescence decay components: full circles - τ_1 , open circles - τ_2 , crosses - τ_3 (see Table 1).

FIGURE 12

Lifetime-resolved spectral components of the UV excited (334 nm) fluorescence emission of the transparent neonatal lens (the decomposition of the solid line spectrum in Fig.4a).

Fig. 1



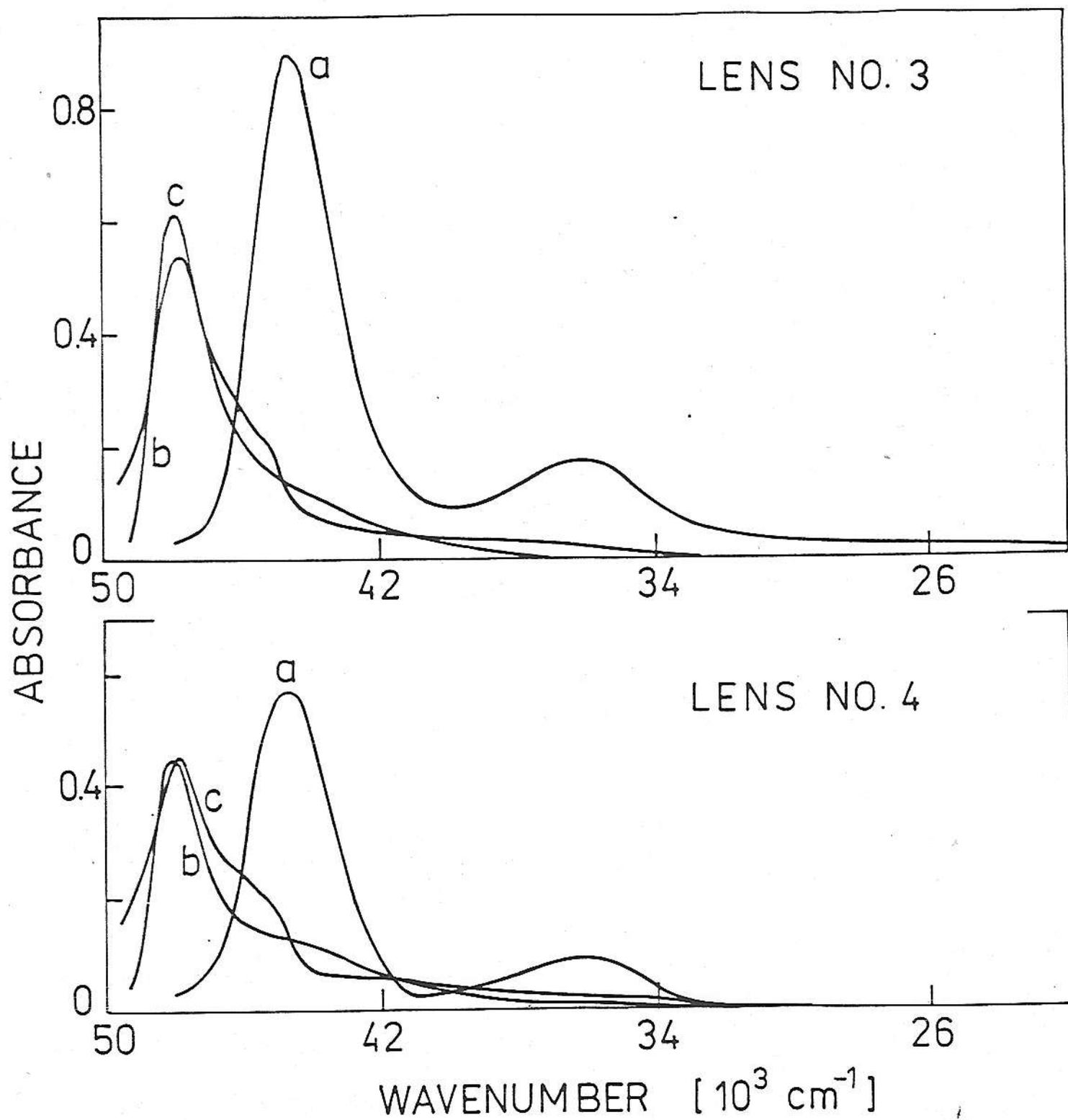


Fig. 2

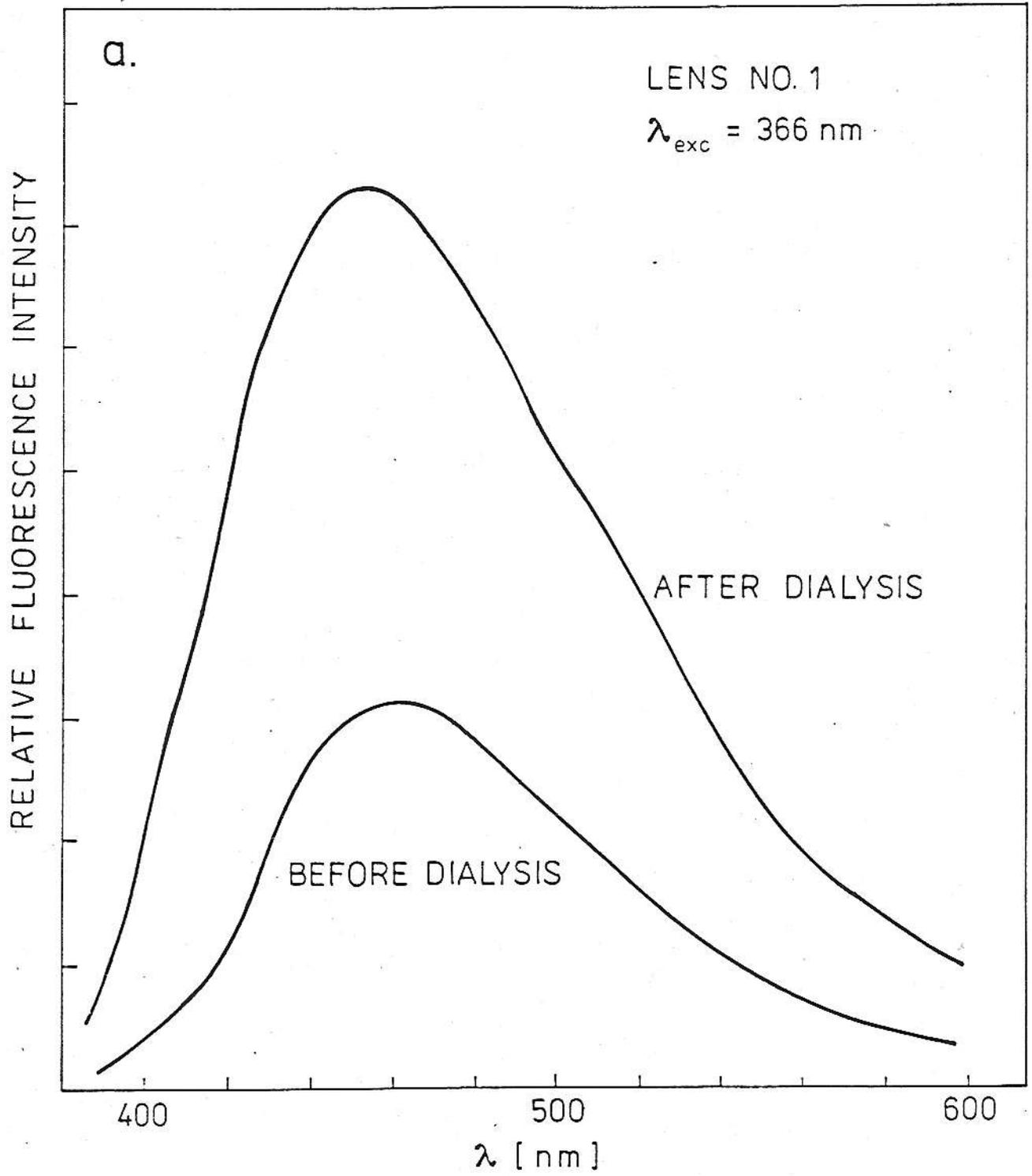


Fig. 3 a F. Balter

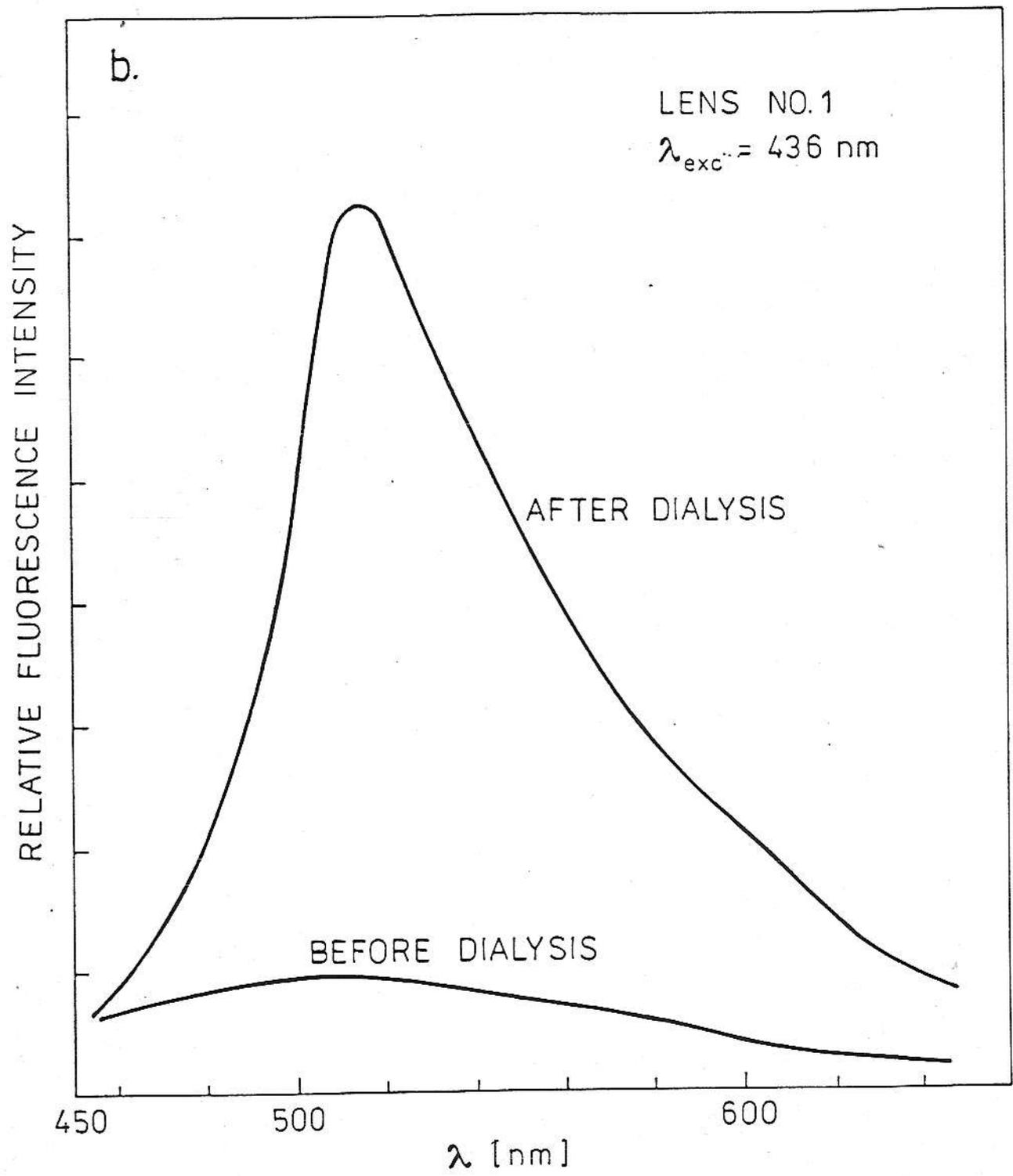


Fig. 3 b. A. Gollor

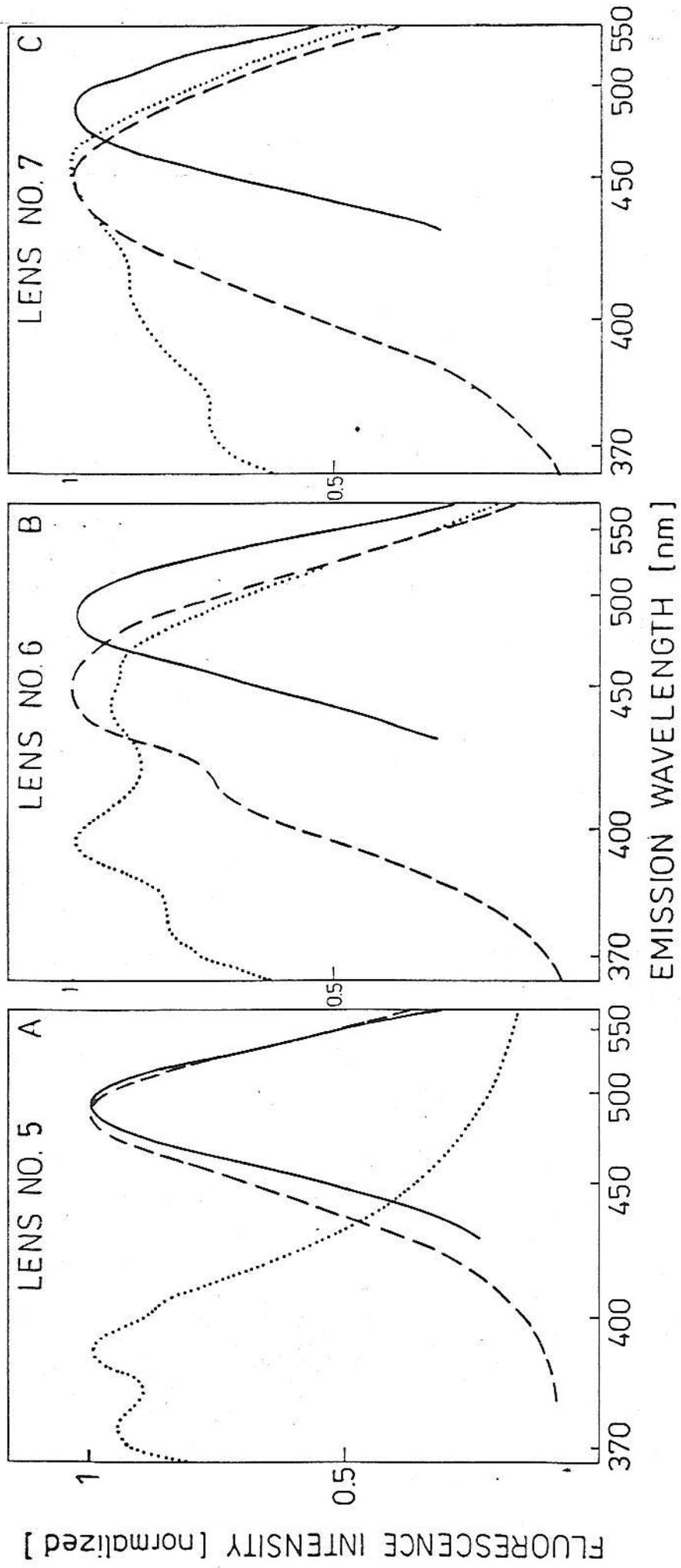


Fig. A A. Butler

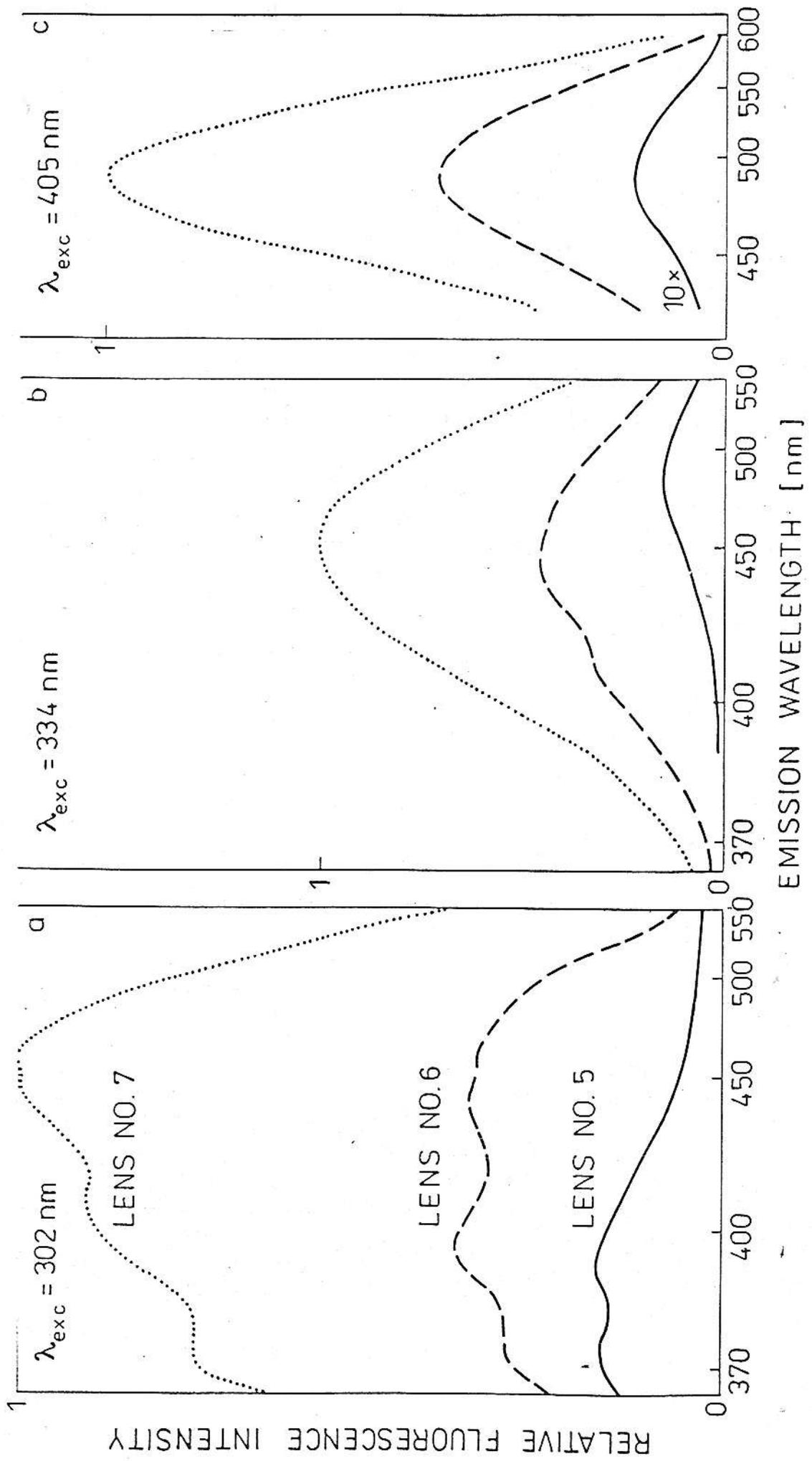


Fig. 5 A. Ballman

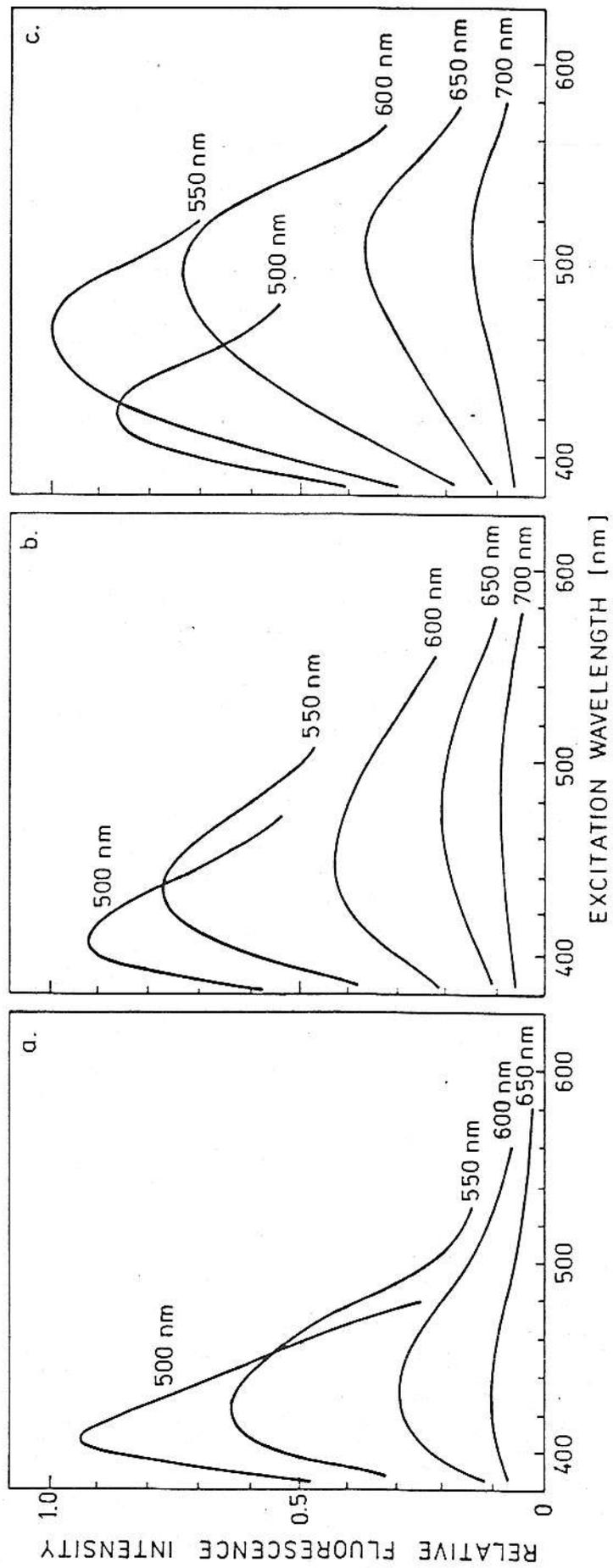


Fig 6 A. Patton

RELATIVE FLUORESCENCE INTENSITY
EMISSION ANISOTROPY (EA)

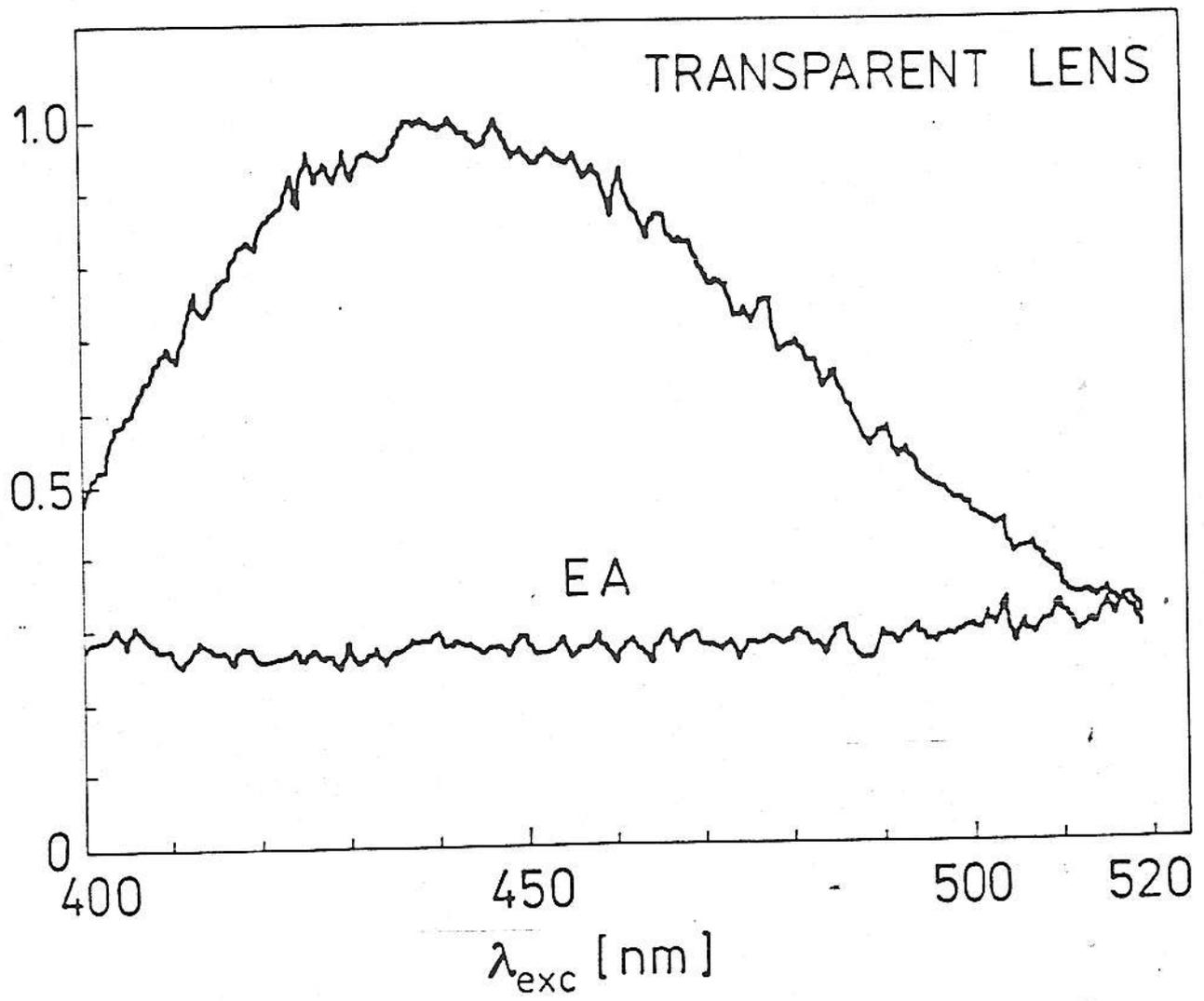
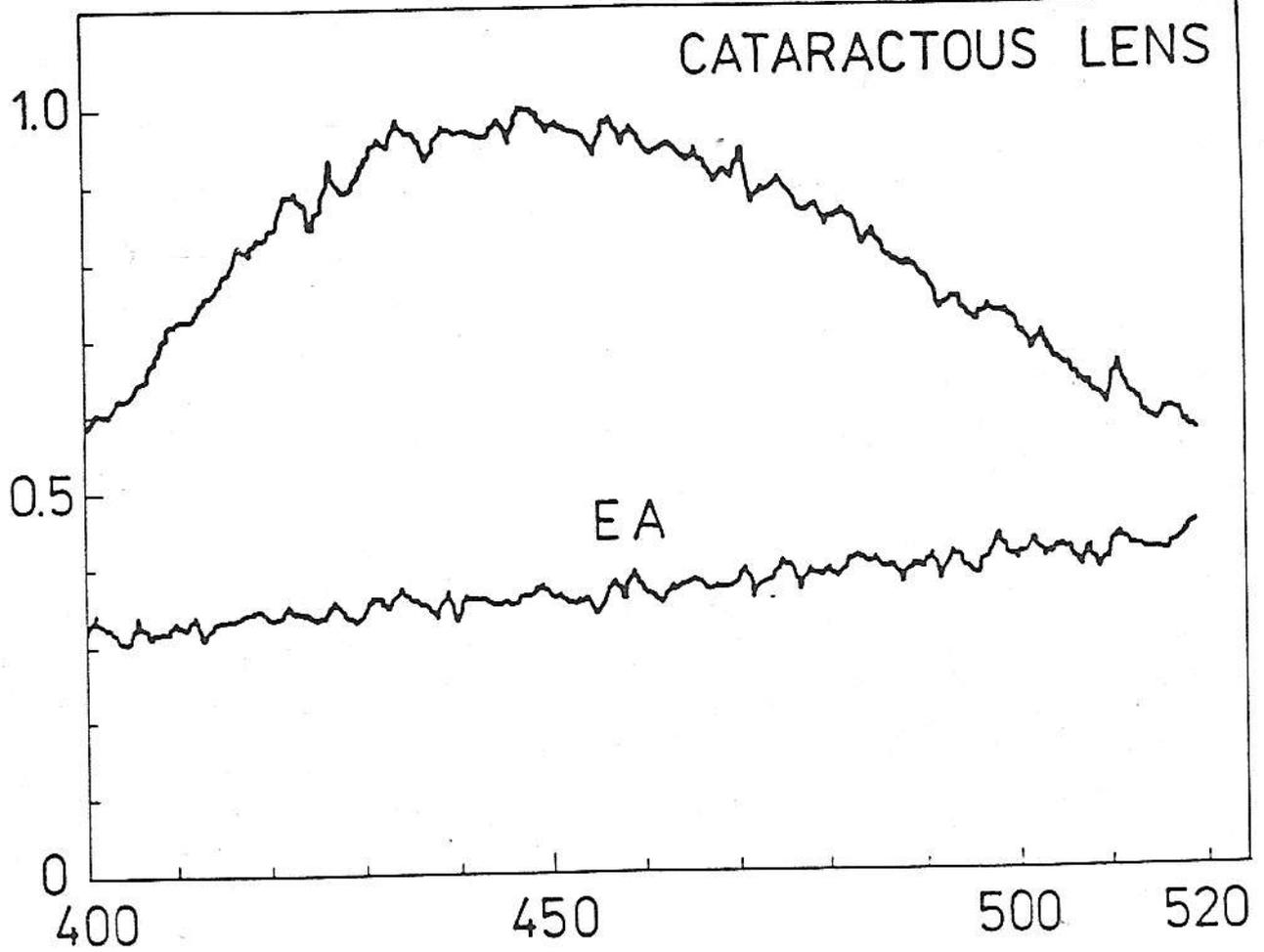
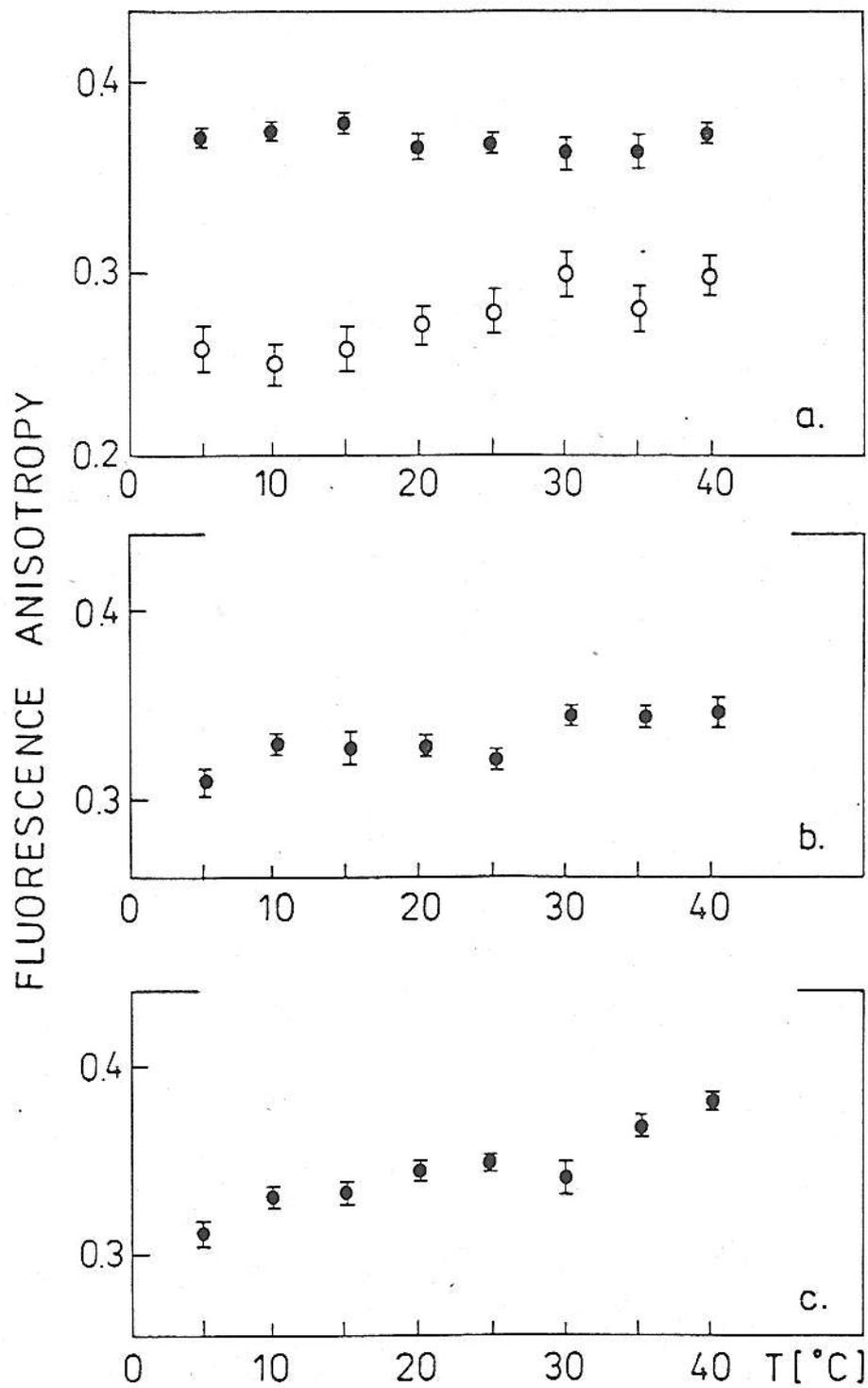


Fig 7 e. Balter



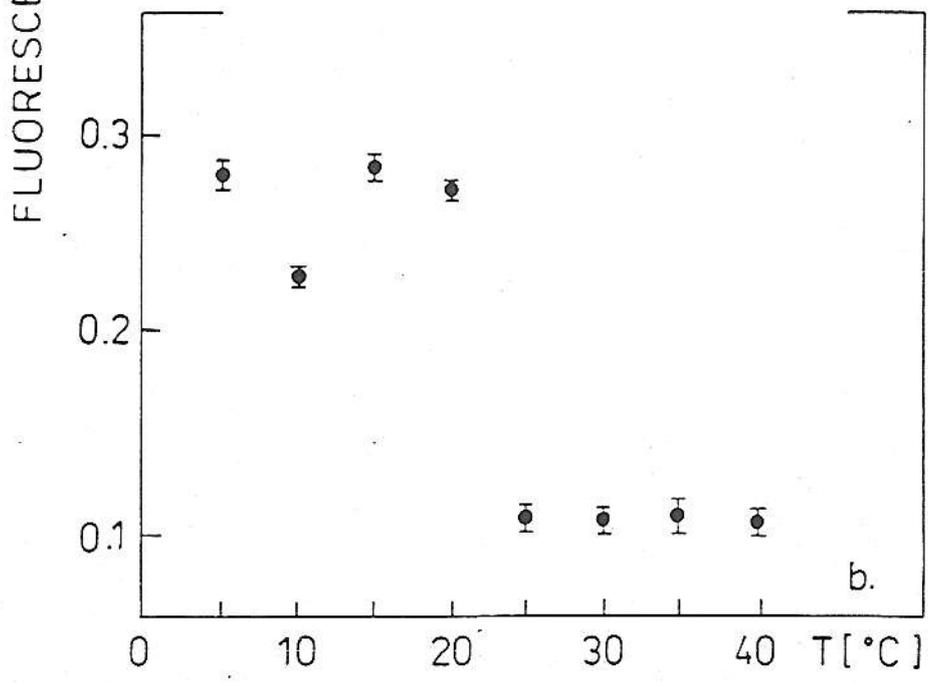
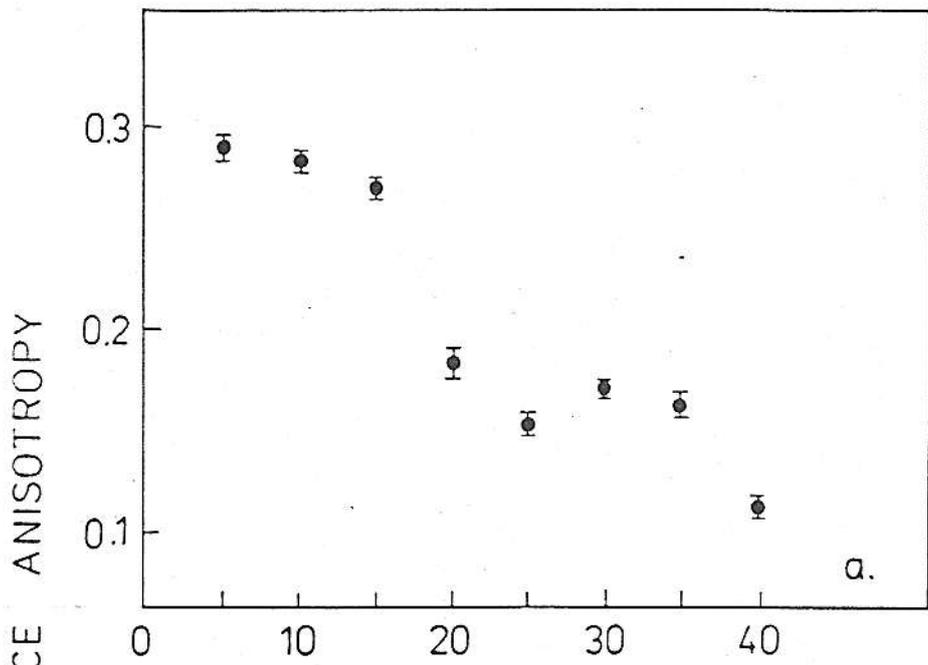


Fig 9 A-Bellows

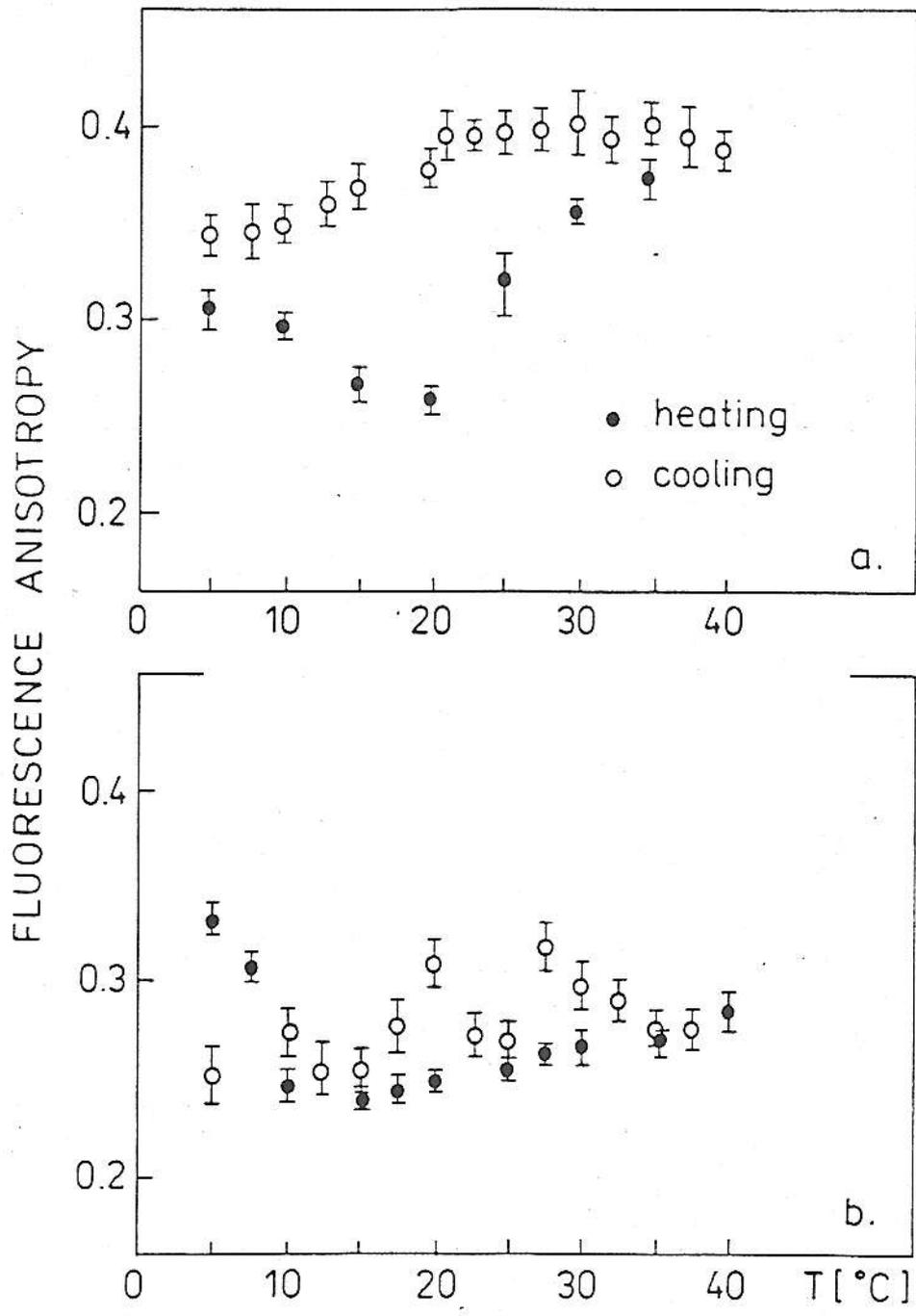


Fig. 10 A. Belter

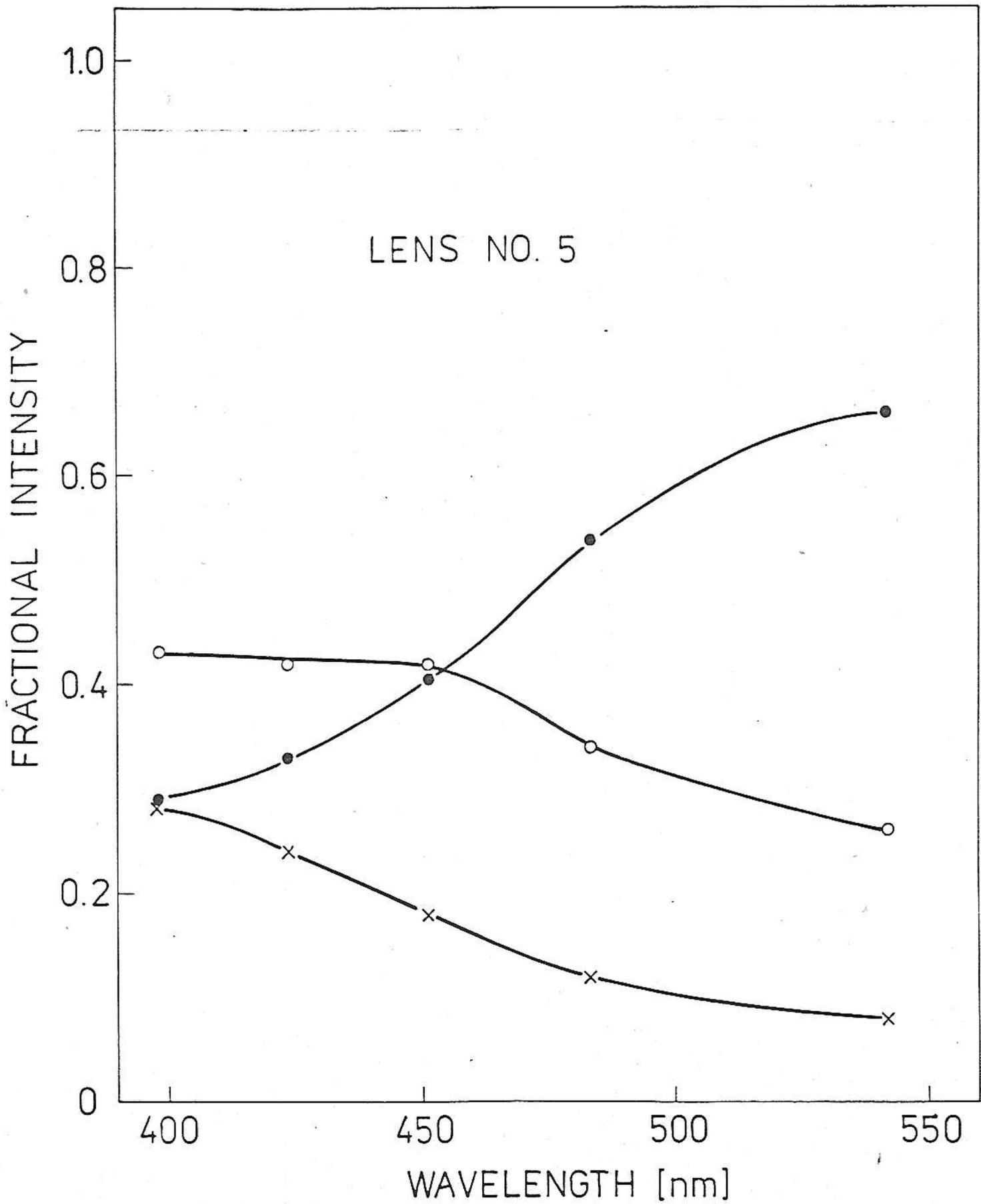


Fig. 11 A. D. Boller

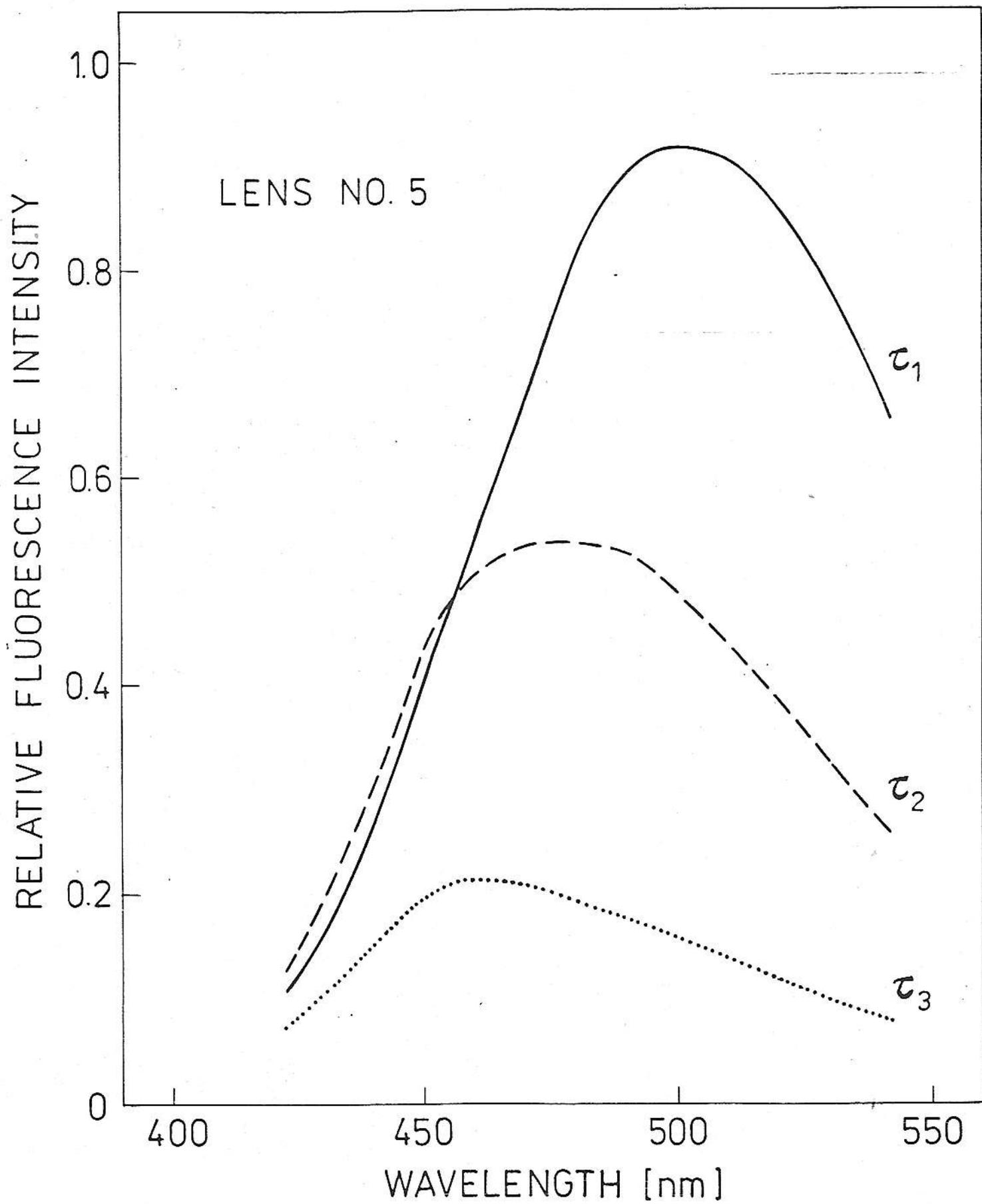


Fig. 12 A. Ballo