

# The

# SPEX

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# Speaker

## SPEX FLUOROLOG: SOME ILLUMINATING ASPECTS

Although many substances fluoresce brightly, spectrofluorometry has found its niche in ultratrace analysis where the intensity of emitted light of the substance sought is correspondingly low. A part per  $10^9$  of an enzyme in a biological fluid may be sufficient to trigger a physiological reaction. To spot that ultratrace substance an instrumental method must be geared to attaining maximum response: The sample must be soaked with intense monochromatic excitation; the emitted light must be transferred with minimum loss to a photomultiplier possessing the highest possible quantum efficiency and the lowest possible noise; the resulting photoelectrons must be directed to an amplifier with optimum response characteristics.

### Limit of Detection

Responsivity of the SPEX FLUOROLOG spectrofluorometer was discussed in *The SPEX Speaker*, Vol. XX-No.4, 1975: the high optical speed ( $f/4$ ) resulting from its aspherical reflecting surfaces which gather and concentrate the light with the fewest number of elements; digital, photon-counting electronics, the signal-to-noise of which is about three times that of conventional analog amplification; digital processing of the signal with boxcar integration (over up to 500 seconds) which statistically enhances the signal-to-noise ratio by a factor equal to the square root of the factor by which the integration time is changed.

The optional high-performance detector, a thermoelectrically cooled GaAs cathode PMT already in hundreds of SPEX RAMALOG Raman systems, provides yet another boost to the signal level. A diagram showing typical response of this PMT is Fig 1. Note that its quantum efficiency, averaging nearly 15%, is relatively uniform throughout the visible and uv and that its response extends beyond 900 nm. Not shown is its remarkably low dark count which is less than one pulse/sec for some of the tubes SPEX has selected. The PMT nearest in performance is a so-called extended S-20 whose dark is a fair match, but relative response in the red and near it is less than a tenth. Specifically, the signal-to-noise of our cooled GaAs photomultiplier is around 3 times better than that of any other reasonably priced photomultiplier and perhaps 1000 times better than that of any uncooled PMT.

An exemplary performance is the ease with which the FLUOROLOG detects ultratrace levels of quinine sulfate. When run in accordance with the ASTM standardized procedure the FLUOROLOG can detect almost unbelievable parts per  $10^{12}$  of quinine; see Table (mol. wt. = 746.93). Under the more realistic conditions required to record recognizable spectra rather than merely concentration levels, the FLUOROLOG's performance is still impressive. Fig 2 shows the spectrum of 0.6 parts per  $10^9$  and, more importantly, the relationship of the spectrum to the water Raman band at 396 nm.

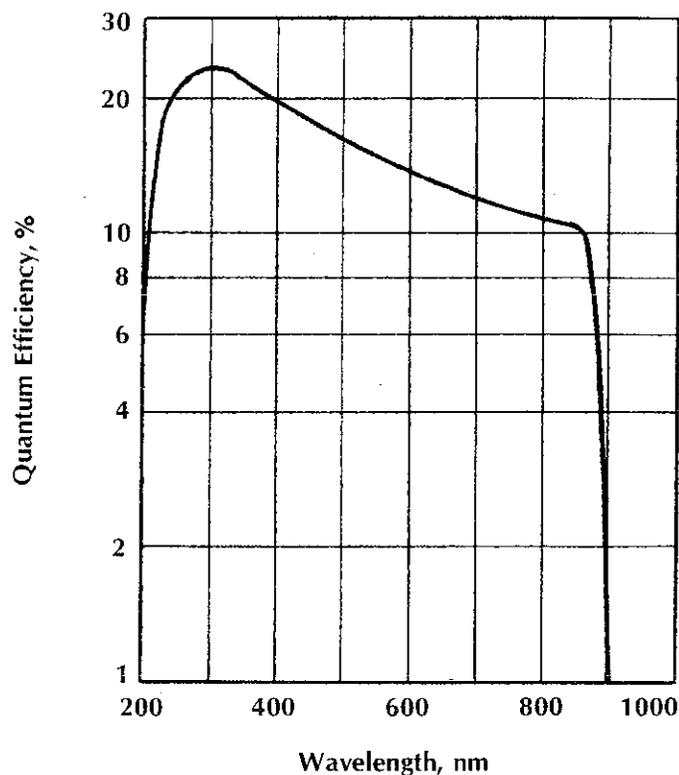


Fig 1 Typical response of a GaAs photomultiplier. Such a detector, cooled, is ideal for fluorescence.

Table  
Limit of detection for quinine sulfate

Concentration of quinine sulfate, molar	Intensity, pps (mean of observed values x full scale)	Range of observed values, pps	Limit of detection, molar, calculated by equation*
0 (blank)	$0.579 \times 20\ 000$	$0.006 \times 20\ 000$	—
$2.6 \times 10^{-10}$	$0.731 \times 20\ 000$	$0.008 \times 20\ 000$	$2.05 \times 10^{-12}$
$5.5 \times 10^{-10}$	$0.905 \times 20\ 000$	$0.009 \times 20\ 000$	$2.02 \times 10^{-12}$
$8.0 \times 10^{-9}$	$0.484 \times 200\ 000$	$0.004 \times 200\ 000$	$2.25 \times 10^{-12}$

\*Limit of detection =  $\frac{\text{concentration} \times \text{peak-to-peak noise in blank}}{\text{intensity (observed-blank)} \times 5}$

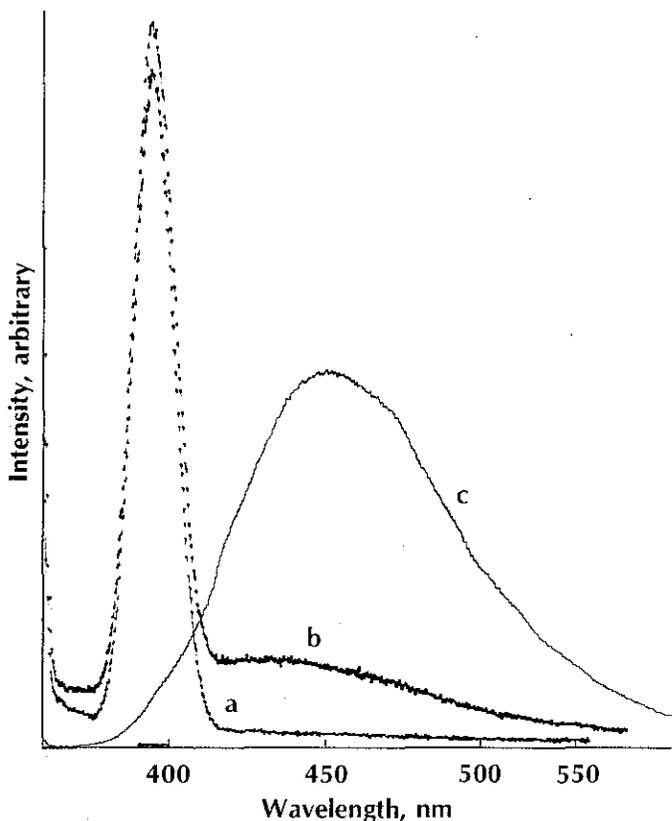


Fig 2 Emission spectra of quinine sulfate in 0.1 N sulfuric acid with excitation at 350 nm, emittance (E/R) mode. a: sulfuric acid background, full scale = 1000; b: 0.6 part per  $10^6$  quinine sulfate, full scale = 1000; c: 0.6 part per  $10^6$  quinine sulfate, full scale = 200 000.

This Raman band is often a convenient secondary standard, reproducible and easily applied on a day-to-day basis to correct for the well known change with time of quinine sulfate's photometric traits.

Still another measure of detectivity, and a more reproducible one, is scanning the Raman spectrum of a familiar compound such as carbon tetrachloride. Because of the small shifts involved this is a particularly difficult compound to characterize with a low intensity source and a low resolution instrument. Fig 3 shows the Raman spectrum of carbon tetrachloride (which has lines shifted 218, 314, and 459  $\text{cm}^{-1}$  and a doublet shifted 762-790  $\text{cm}^{-1}$ ) excited at different wavelengths with a 450-W xenon lamp in the FLUOROLOG. The "a" marks the expected position of the center of the doublet, "b" marks the expected position of the 459  $\text{cm}^{-1}$  band, and "c" marks the expected position of the 314  $\text{cm}^{-1}$  band. The doublets are clearly resolved from the other peaks, but the bandpass precludes resolution of the individual peaks. Note that the stray light rejection of the double monochromators gives an extremely sharp cutoff of transmitted light in the overlap region close to the exciting line, making it possible to see a hint of the 314  $\text{cm}^{-1}$  line when exciting at 410 nm.

### Scattered Light

Actually, getting good fluorescence spectra of clear solutions does not strain most commercial spectrofluorometers, but laboratory samples encountered are often much less tractable. Turbid solutions (such as cell or membrane suspensions), shows (formed by solvents when they freeze), and solids (including films and pow-

ders) scatter incident light many thousands of times as strongly as clear solutions. The intensity of stray light increases by a similar factor and in most instruments can obliterate all but the strongest fluorescence.

Since many highly scattering samples are not easily converted to more manageable forms, the remaining countermeasure is finding instrumentation that ignores stray light or at least drastically reduces it. To this end the FLUOROLOG excitation and emission monochromators are doubled; the light is dispersed through two gratings instead of the usual one. Fig 4 depicts the improvement. Stray or nonmonochromatic light is slashed by a factor of approximately 100 in both the excitation and emission monochromators. A remarkable improvement in selectivity of 10 000 is thereby achieved.

To best illustrate the significant effects of this reduction in scattered light it is well to describe the optical design of double spectrometers. This is a topic of special interest to SPEX engineers because the configuration of a double spectrometer that is optimum for Raman (one of SPEX's earliest specialties) differs from that which is optimum for fluorescence. The reason is that Raman features can be quite narrow, but most fluorescence spectral features are relatively broad. In the SPEX RAMALOG the two gratings are constrained to rotate in the same direction to provide additive dispersion (and therefore a distinct increase in luminosity). In both the excitation and emission monochromators of the FLUOROLOG the two gratings are rotated in opposite directions to achieve subtractive dispersion. Actually, zero dispersion is achieved since the groove density of the tandem gratings is identical and their dispersions cancel one another.

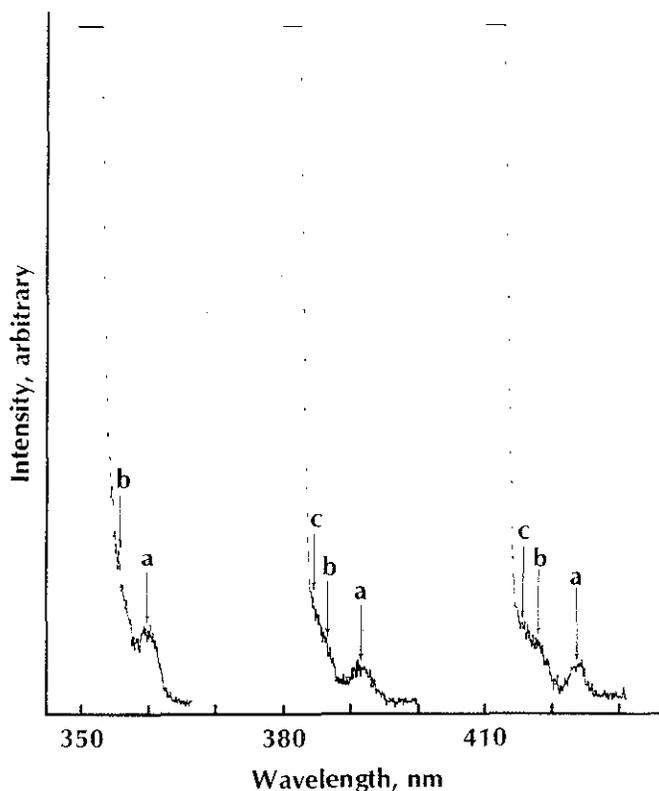


Fig 3 Raman spectra of carbon tetrachloride excited at different wavelengths demonstrates resolution of FLUOROLOG. a: expected position of 760-792  $\text{cm}^{-1}$  Raman doublet; b: expected position of 459  $\text{cm}^{-1}$  band; c: expected position of 314  $\text{cm}^{-1}$  band. Excitation wavelengths were 350, 380, and 410 nm.

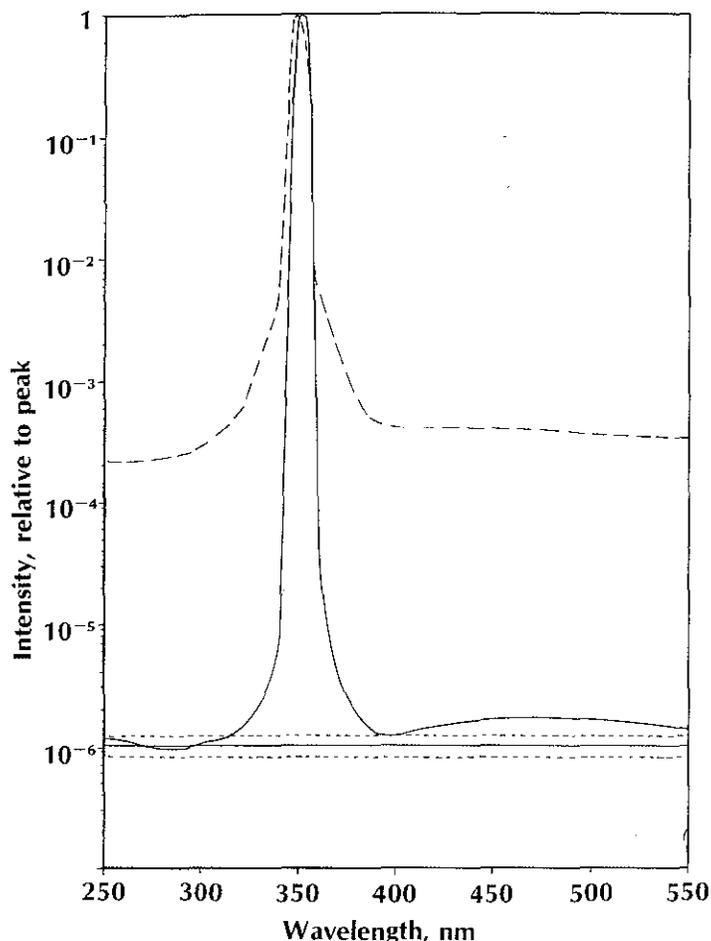


Fig 4 Comparison of emission stray light levels in FLUOROLOGs with single (dashed curve) and double (solid curve) monochromators with excitation at 350 nm. The line at about  $10^{-6}$  is the dark count; its standard deviation is indicated by dotted lines.

Fig 5 illustrates one optical effect of the two kinds of double monochromator. In the diagrams the letters B, G, Y, and R stand for blue, green, yellow, and red. Note that in the additive case light at the exit slit is dispersed (the second grating actually doubles the separation of wavelengths) so that at one jaw of the slit the wavelength is different from that at the other. In the zero case, however, the light at the exit slit is homogenized with respect to wavelength; there is no wavelength difference across the width of the slit. In the excitation monochromator this assures that the entire sample is uniformly illuminated for reproducible, quantitative fluorometry. In the emission monochromator the zero dispersion assures uniform spectral illumination of the cathode of the photomultiplier. With a wide bandpass it is especially poor practice to illuminate the cathode of a PMT with dispersed light because its wavelength responsivity may vary considerably across its surface. The dramatic results of double monochromatization in the FLUOROLOG are readily evident when plane mirrors are substituted for the second grating in both excitation and emission monochromators and comparison spectra are generated.

Model membranes and membrane structure have been investigated with the fluorophore perylene [1]. When perylene is dissolved in mixed micelles of cetyltrimethylammonium bromide (CTABr) and cholesterol, the solution becomes quite cloudy with a combined Rayleigh and Tyndall scatter more than a thousand times that of distilled water. Fluorescence spectra of various concentrations of perylene in mixed CTABr-cholesterol micelles (shown in

Fig 6) were run on a FLUOROLOG modified to contain only single-grating monochromators. The small peaks at 468 and 473 nm in the blank probably arise from scatter of xenon lines at those wavelengths. At a perylene concentration of  $7 \times 10^{-9}$  M the emission spectrum can no longer be resolved. In contrast, spectra from a standard (double-monochromator) FLUOROLOG under similar conditions are shown in Fig 7. At  $7 \times 10^{-9}$  M the perylene spectrum gives better results with the FLUOROLOG than a ten times as concentrated solution does with a single-monochromator instrument. A spectrum can even be detected at a concentration less than 1/25 the minimum concentration possible with a single-monochromator instrument—a concentration at which the Raman scatter is larger than the fluorescence. The limiting factor is the dark count of the photomultiplier, not the background scatter. Increasing the bandpass of the emission would increase the light level and alleviate the limitation due to dark count. With single monochromators a similar improvement would not occur because the background would be increased by the same factor as the signal.

Rare earths, either as solids or in solution, often exhibit fluorescence. For solids the emission lines are usually quite narrow, requiring excellent resolution from a spectrofluorometer. Frequently these rare earths can be chelated by organic molecules whose delocalized electrons cause much stronger and broader absorption bands than do the rare earths themselves. Absorbed energy transferred intramolecularly from the ligand to the rare earth is then emitted as a spectrum characteristic of the rare earth. With such solids the weak spectral lines at low concentrations require far better stray light rejection than is obtained from single monochromators.

As an example, consider the emission spectra of small (6-mm diam) pellets of different concentrations of tetrakis (2,2'-bipyridine—1,1'-dioxide) europium(III) perchlorate in graphite. Graphite was chosen for its especially strong scattering characteristics and its ease of mixing and pellet formation. Pellets were mounted in a #1933 Solid Sample Holder and viewed with front-face orientation. The emission spectra of 0.075%, by weight, of the europium(III) complex in graphite (0.0095% europium(III)) and of 9.1%, by weight, of the complex (1.2% europium(III)) are shown in

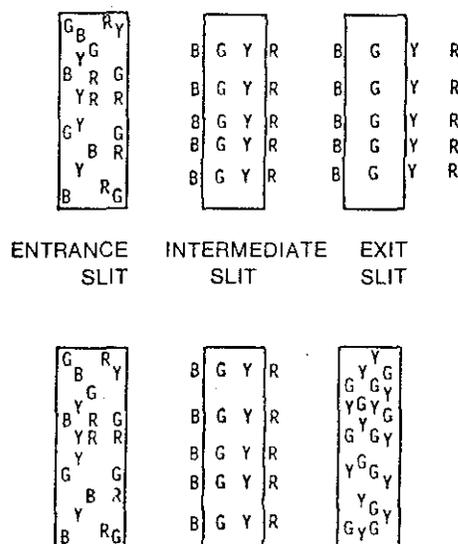


Fig 5 Difference between additive (top row) and subtractive (bottom row) dispersion double monochromators. Subtractive is preferred in spectrofluorometry because it illuminates both sample and photocathode with spectrally homogenized radiation. Letters stand for Blue, Green, Yellow, and Red.

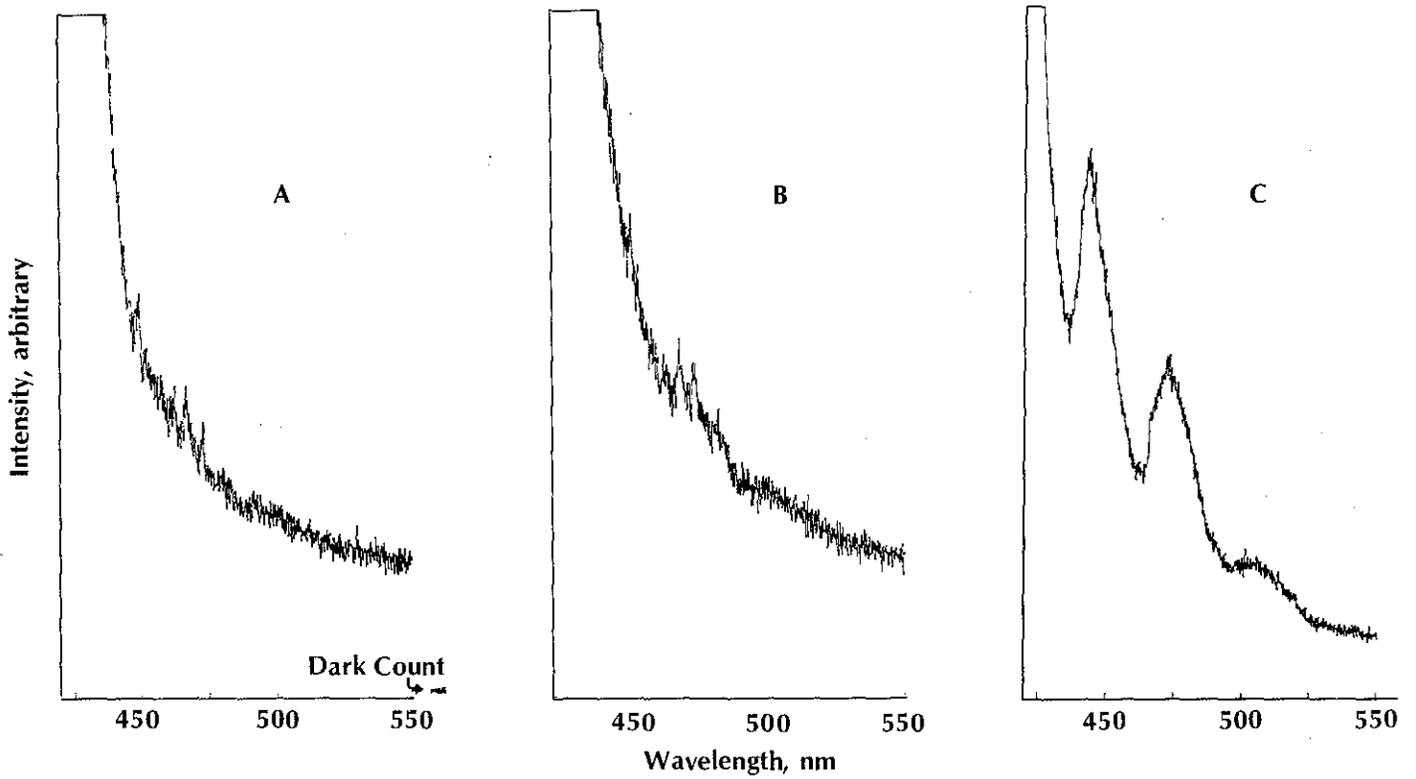
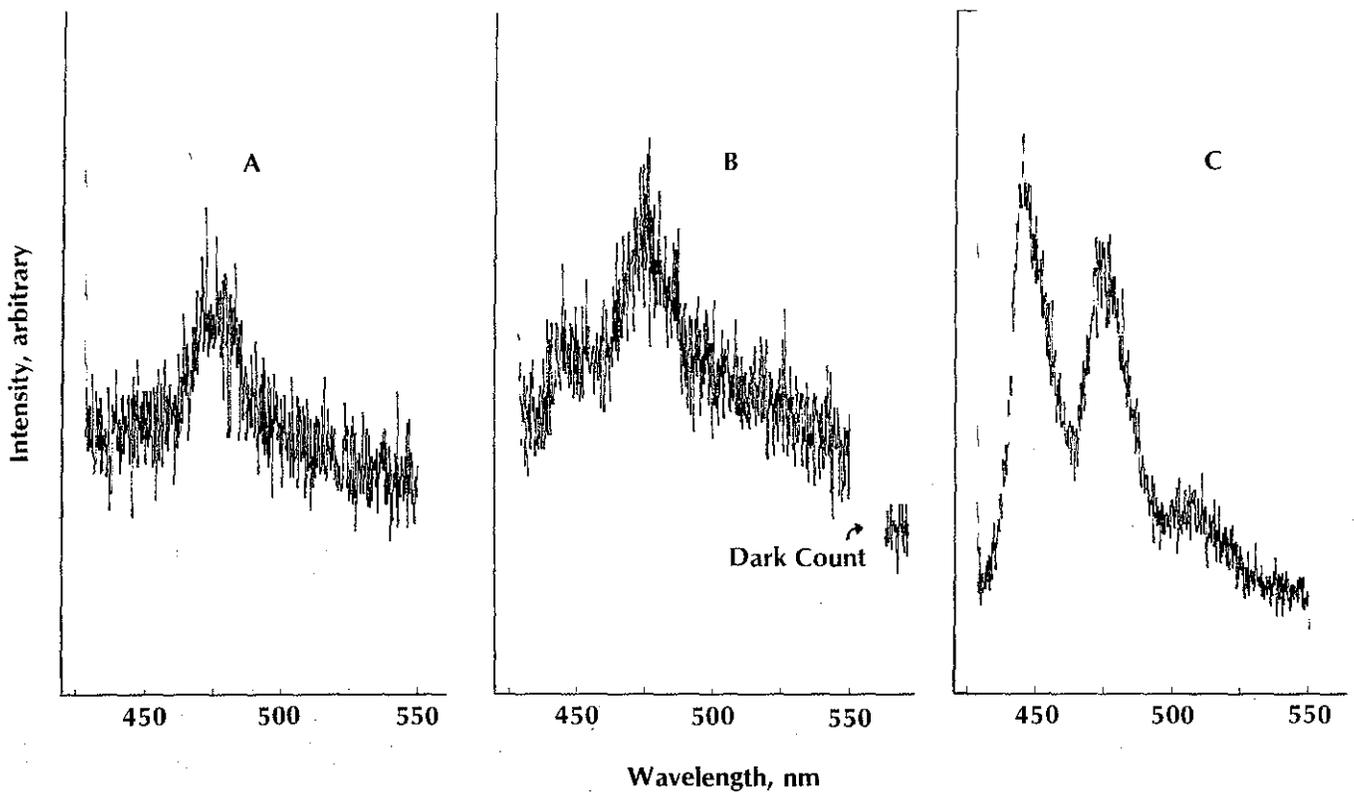


Fig 6 Emission spectra of turbid solution, perylene in CTABr-cholesterol micelles, with single-monochromator FLUOROLOG, excitation at 410 nm. A: micelle blank, full scale = 1000; B:  $7 \times 10^{-9}$  M perylene in micelles, full scale = 1000; C:  $7 \times 10^{-8}$  M perylene in micelles, full scale = 2500.

Fig 7 Emission spectra of turbid solution, perylene in CTABr-cholesterol micelles, with double-monochromator FLUOROLOG, excitation at 410 nm. A: micelle blank, full scale = 40; B:  $3 \times 10^{-11}$  M perylene in micelles, full scale = 40; C:  $7 \times 10^{-9}$  M perylene in micelles, full scale = 100.



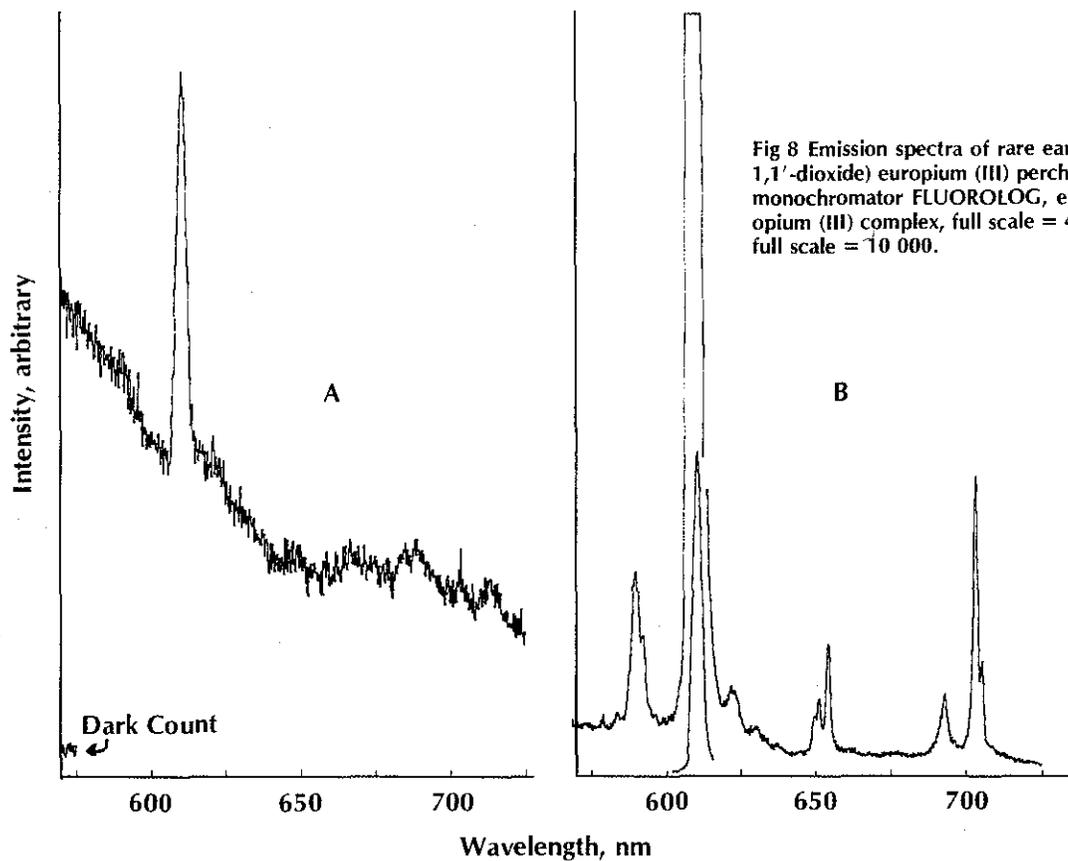


Fig 8 Emission spectra of rare earth complex, tetrakis (2,2'-bipyridine-1,1'-dioxide) europium (III) perchlorate in graphite pellets, with single-monochromator FLUOROLOG, excitation at 280 nm. A: 0.075% europium (III) complex, full scale = 400; B: 9.1% europium (III) complex, full scale = 10 000.

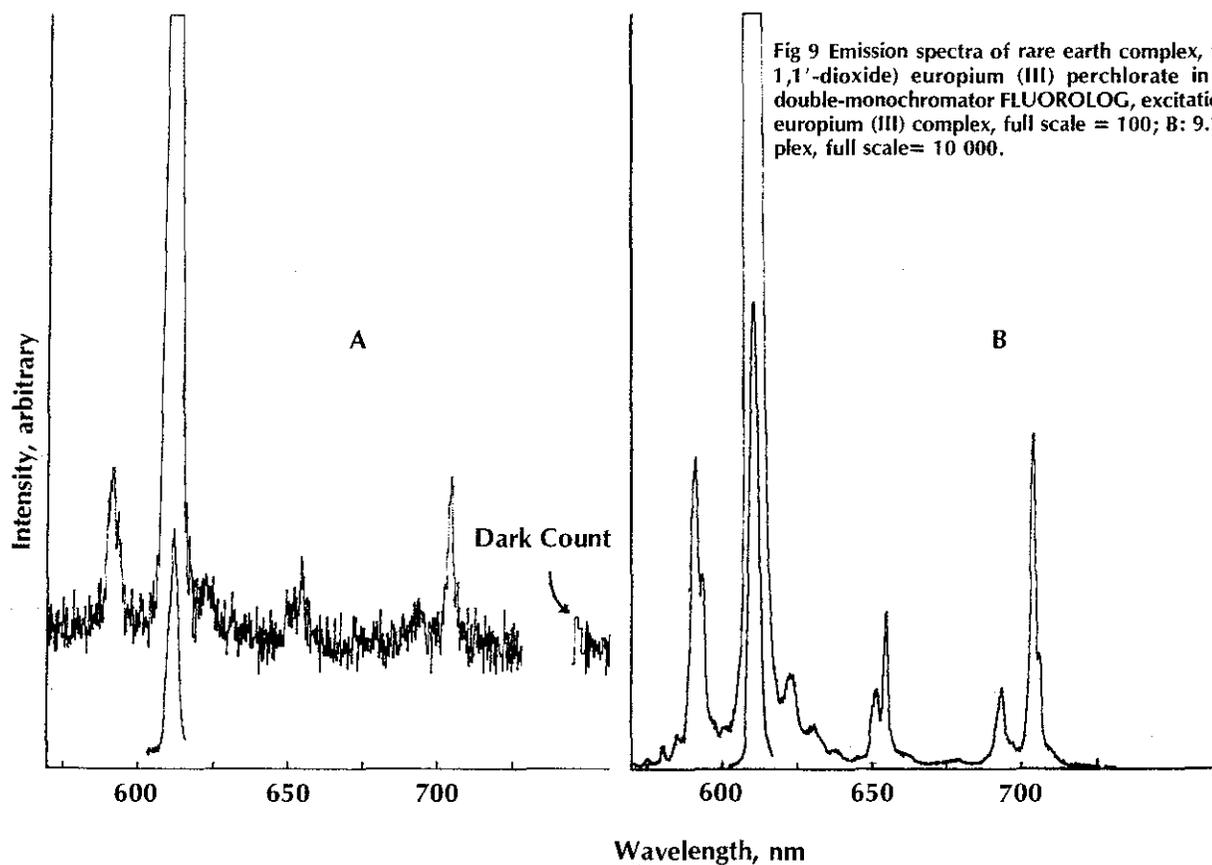


Fig 9 Emission spectra of rare earth complex, tetrakis (2,2'-bipyridine-1,1'-dioxide) europium (III) perchlorate in graphite pellets, with double-monochromator FLUOROLOG, excitation at 280 nm. A: 0.075% europium (III) complex, full scale = 100; B: 9.1% (europium (III) complex, full scale= 10 000.

Fig 8 and Fig 9. In the former the Rayleigh and Tyndall scatter was 5000 times the intensity of the 590 nm emission line. With a single-monochromator configuration it was not possible to measure the emission spectrum of the dilute sample because the high stray light levels masked the signal. Fluorescence of more concentrated samples could still be measured but was distorted by background signal. Wherever possible spectra were recorded on a scale expanded to 10 times that necessary to show the 610 nm line.

### Polarization

Obtaining meaningful polarization measurements is another problem with high-level scatterers. In studies as diverse as energy transfer and molecular structure determination, polarization is an indispensable tool. A measure of the preferential orientation of electric vectors of emitted light, the polarization provides information about the motion and the hydrodynamic shape of a molecule. For right-angle viewing of emission with polarized exciting light, fluoroscopists define polarization as  $(I_a - I_b)/(I_a + I_b)$ , where  $I_a$  is the intensity of light polarized parallel to the exciting light and  $I_b$  is the intensity of light polarized in one plane perpendicular to the exciting light [2]. If the exciting light is polarized and the molecule rotates little (including "very little" and "none") during the fluorescence lifetime, then the emission is also polarized. Polarization is a measure of the time necessary for the molecule's orientation to change by an angle of  $(1/e)(\pi/2)$  radians, where  $e$  is the base for the natural logs.

To measure the rotation of a molecule its polarization in a frozen solution is compared to its polarization in a nonfrozen one [3]. The binding of molecules in solution is described by observing changes in polarization due to the different sizes of the molecules as compared to the complex. Changes in viscosity in the vicinity of the fluorophore are detected through polarization changes, as in studies of membranes reacting to stimuli [4]. Energy transfers between different fluorophores, and their orientations when both are coupled to the same large molecule, are investigated with polarization. The orientations of absorption and emission dipoles for different transitions of the same fluorophore may be deduced from polarization information combined with data about lifetimes. Polarization also characterizes the effects of solvents or of substitutions of ligands.

An excitation polarization spectrum of rhodamine 6G in propylene glycol is shown in Fig 10. The SC4 SPEXCOMP spectrometric computer system did the calculations by subtracting a blank and then applying a form of the polarization equation:

$$P = ((VV/VH)(HH/HV) - 1) / ((VV/VH)(HH/HV) + 1),$$

where VV is the intensity of light when excitation and emission polarizers are both vertical, calculated as VV of sample - VV of blank;

VH is the intensity when the excitation polarizer is vertical and the emission one is horizontal, calculated as VH of sample - VH of blank;

HV is the intensity when the excitation polarizer is horizontal and the emission one is vertical, calculated as HV of sample - HV of blank; and

HH is the intensity when both polarizers are horizontal, calculated as HH of sample - HH of blank.

### Excitation Correction

Illustrative of the universality of the FLUOROLOG is its versatility in operation. A quantum counter—rhodamine B—provides excitation correction. Well known because its quantum efficiency is invariant with respect to wavelength, rhodamine B's emission can serve as a means of correcting for intensity fluctuations of the source as well as for variations in the efficiency of the grating and other optical components with wavelength.

In Fig 11 are two spectra of a 0.2 part per  $10^6$  solution of anthracene in cyclohexane recorded by the FLUOROLOG. The uncorrected excitation spectrum was generated by measuring the emission at 400 nm while the excitation was scanned from 220 to 400 nm. For the excitation correction the emittance, the ratio of the emission intensity to the rhodamine reference intensity (E/R), was plotted in the same manner.

Proof of proper correction of an excitation spectrum is its match with an absorbance spectrum. The FLUOROLOG is able to plot directly either transmittance (T/R) or absorbance (log R/T). An absorbance spectrum of  $4 \times 10^{-6}$  M anthracene is Fig 12. The scale was chosen so that all peaks, including the uv feature at 252 nm, would remain on scale so their intensities could be compared. The absorption spectrum in Fig 13—one produced for reference as part of the Documentation for Molecular Spectroscopy series [5]—is reproduced for comparison. The ratio of peak heights of 252 nm to 374 nm in our absorbance and corrected excitation modes was about 26:1, in close agreement with that in the DMS spectrum.

### Emission Correction

Although fluorescence has been an established research tool for more than a decade, direct comparison of fluorescence emission spectra from different laboratories, or even from different instruments within the same laboratory, has been tedious and often impossible. Correction for variations in fluorescence (due to the monochromator and detector efficiencies' dependence on wavelength) is a prerequisite for direct comparison. Fluorescence emission is inherently not correctable by instrumental techniques such as the double beam referencing in absorbance and transmittance. Historically correction methods have ranged from manual calculations with values read from a graph to on-line correction by computer-controlled instruments like the FLUOROCOMP.

The #1936 Programmable Emission Corrector offers most of the versatility and convenience of computerized systems but with a much smaller investment. The #1936 comprises a programmable calculator with magnetic tape cassette and a special interface. Through the Data Log connector built into the FLUOROLOG the calculator has access to the data displayed on the FLUOROLOG's panel meter. Through the same connector the calculator instructs the emission monochromator to scan and turns the data registers on and off. Recorder output is routed through the interface rather than taken directly from the FLUOROLOG. A block diagram of the system is shown in Fig 14.

A basic fluorescence emission correction program is permanently stored on magnetic tape; correction factors based on a standard lamp are stored on the same tape so that they need not be

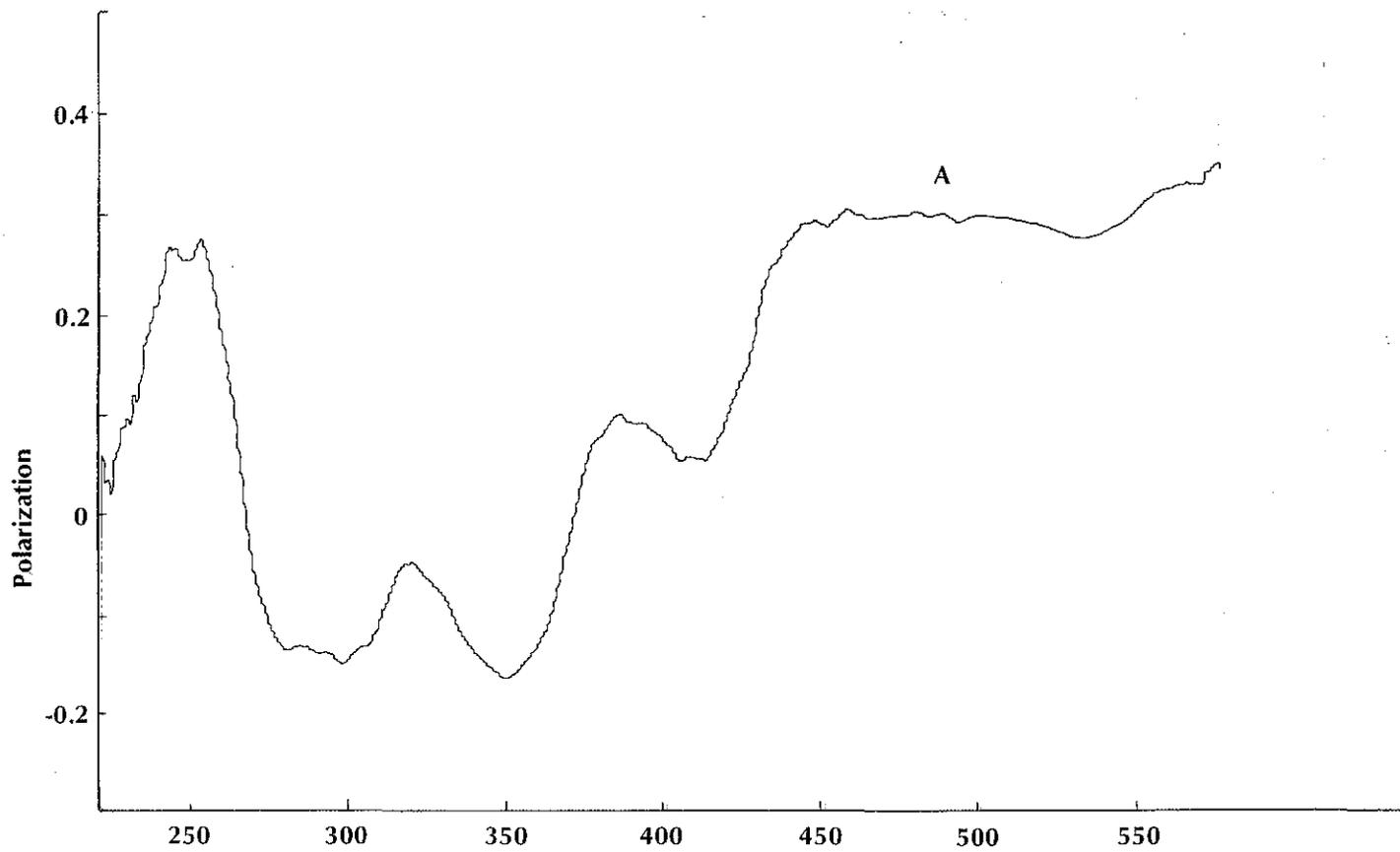


Fig 10 Excitation polarization spectrum of rhodamine 6G in propylene glycol. A: polarization spectrum calculated by the SC4 SPEXCOMP spectrometric computer system; B: uncorrected excitation spectrum, emission wavelength = 580 nm.

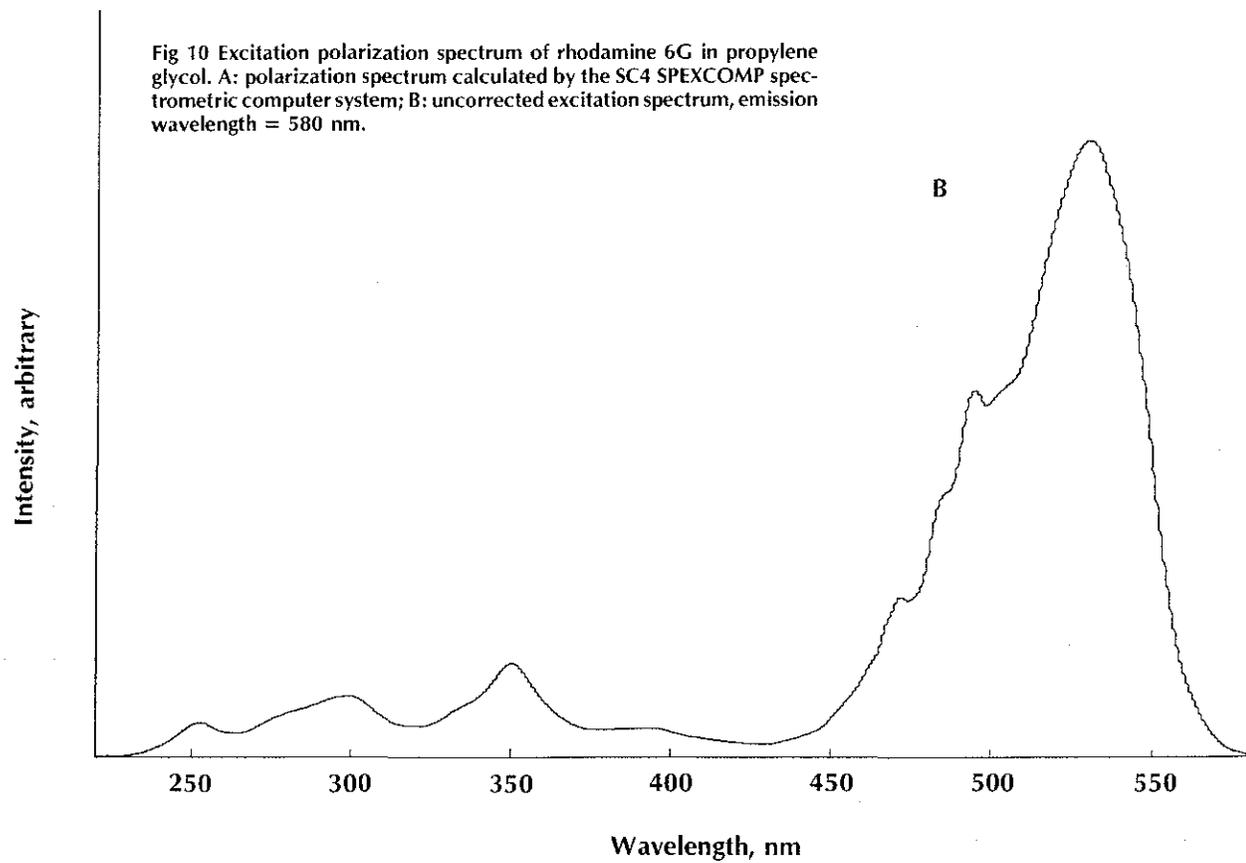


Fig 11 Excitation spectra of 0.2 part per 10<sup>6</sup> anthracene in cyclohexane. A: uncorrected spectrum (emission mode); B: corrected spectrum (emittance mode).

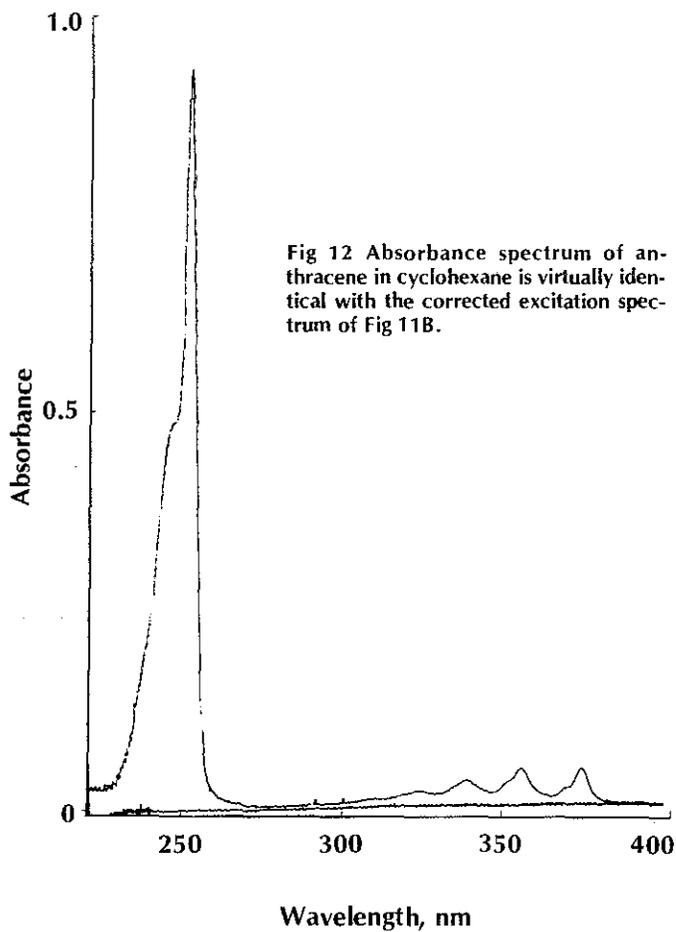
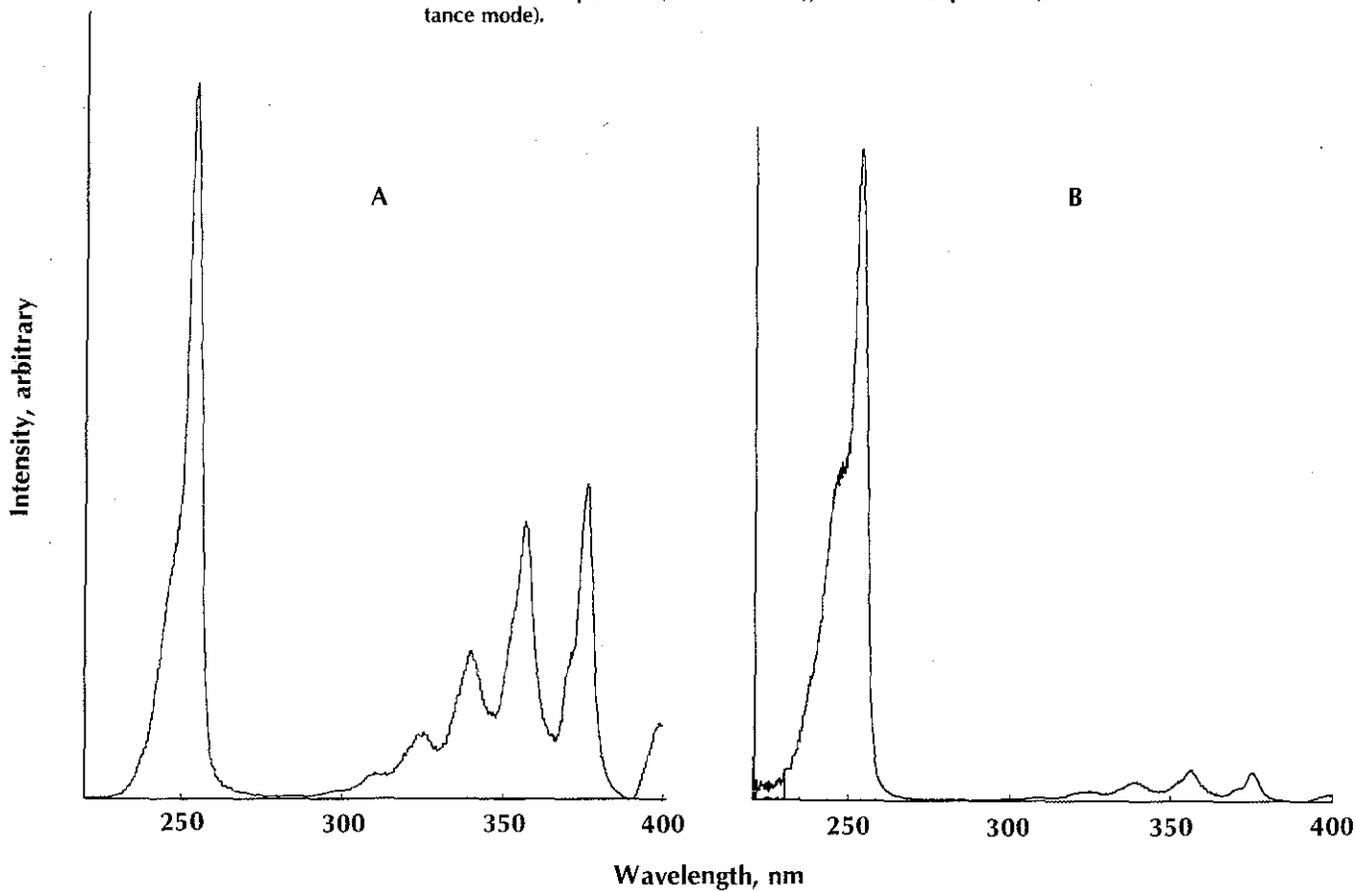


Fig 12 Absorbance spectrum of anthracene in cyclohexane is virtually identical with the corrected excitation spectrum of Fig 11B.

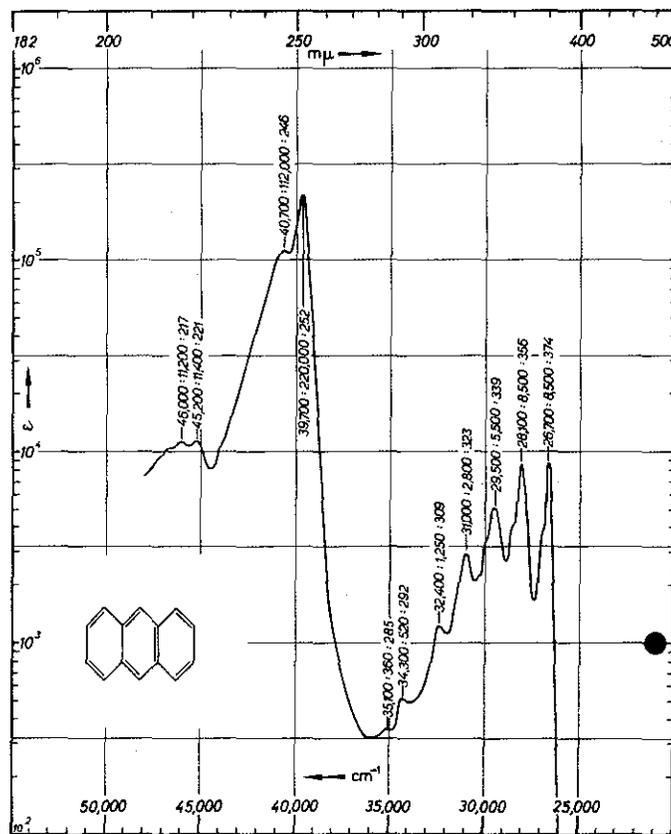


Fig 13 DMS reference absorption spectrum of anthracene in hexane [5].

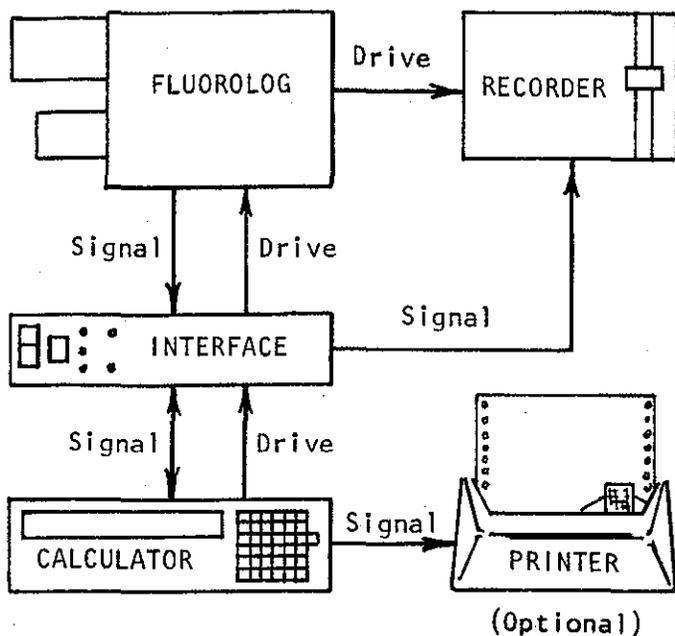


Fig 14 Block diagram of FLUOROLOG with #1936 Programmable Emission Corrector.

reinitialized each time power is interrupted. The program sequence is: A start signal is keyed into the calculator, as are the initial and final wavelengths. The calculator reads the data available at the Data Log connector, turns off the data registers, and signals the monochromator to move one increment. The four-digit datum is multiplied by the appropriate correction factor for the wavelength. The result is returned to the interface for conversion to an analog recorder signal. Wavelength position is updated, then the calculator turns on the data registers and checks whether the end of the scan has been reached. If not, the cycle is repeated. Fig 15 is a flow chart of the program.

The programmable calculator offers several advantages over other real-time corrections. Changing to a different correction takes only the few seconds necessary to load a new program from magnetic tape. Expanded capability, such as subtraction of a stored spectrum (perhaps a blank), is attained with optional add-on memory. Magnetic tape storage means that correction factors need only be generated as often as dictated by aging of the photomultiplier and optics, usually only a few times a year. Programs are included to generate correction factors either in the region from 220 to 600 nm (with a rhodamine B quantum counter and a white scatterer) or in the region from 250 to 1000 nm (with a standard quartz-tungsten lamp). Also, none of these activities interfere with the calculator's usual functions.

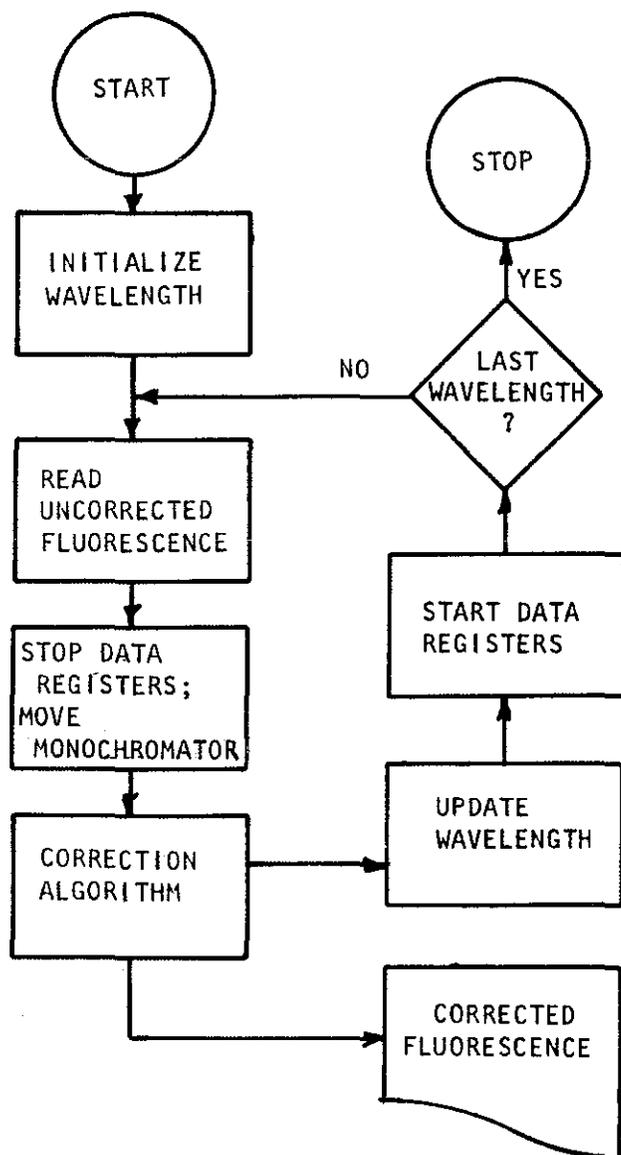


Fig 15 Flow Chart of correction program in #1936 Programmable Emission Corrector.

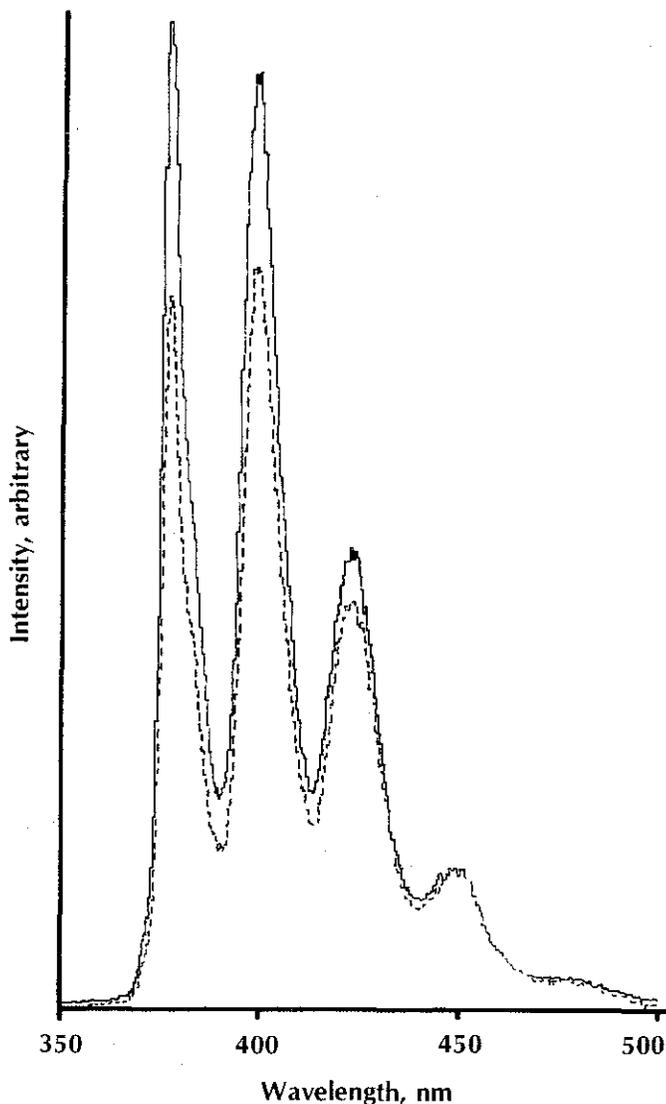


Fig 16 Emission spectra of 100 parts per 10<sup>9</sup> anthracene in cyclohexane, uncorrected (dashed) and corrected (solid line).

As an example of the performance of the #1936 Programmable Emission Corrector both the uncorrected and corrected emission spectra of 100 parts per  $10^9$  anthracene in cyclohexane are shown in Fig 16. Except for correction all parameters are the same in the two spectra. A cooled photomultiplier with GaAs photocathode was the detector. A quartz scrambler wedge (from the #1935 Polarization Accessory) was inserted ahead of the emission monochromator to remove polarization effects. Reference for the correction was a tungsten standard lamp calibrated against National Bureau of Standards sources.

A second example (Fig 17) of emission correction is shown in the spectra of 500 parts per  $10^9$  coproporphyrin III in 0.1 N HCl. These porphyrin spectra illustrate the excellent (and flat) red response of the GaAs photomultiplier as well as the effectiveness of the emission corrector.

### Integration

Not to be confused with the integration (actually quadrature) of the SC4, the FLUOROLOG itself integrates the signal to obtain the data it displays. In sharp contrast to the resistance-capacitance integration which is usual in analog systems, the digital nature of the FLUOROLOG provides boxcar integration. There is no carry-over of signal from one integration interval to the next; *all* of the signal observed during the integration period is included, without weighting, and *no other* signal is allowed to intrude. The great advantage here is the opportunity to integrate a given spectral region and then close the shutters and integrate the dark count over an equal period of time. The difference between the two values (which may be considered to be the area lying between two curves) is directly proportional to the concentration of the compound of interest. In the same fashion the total integral of the emission band, necessary to determine quantum yield, is recorded automatically rather than by tedious manual methods. Fig 18 is an example of such integration on a mixture of anthracene and rubrene in benzene.

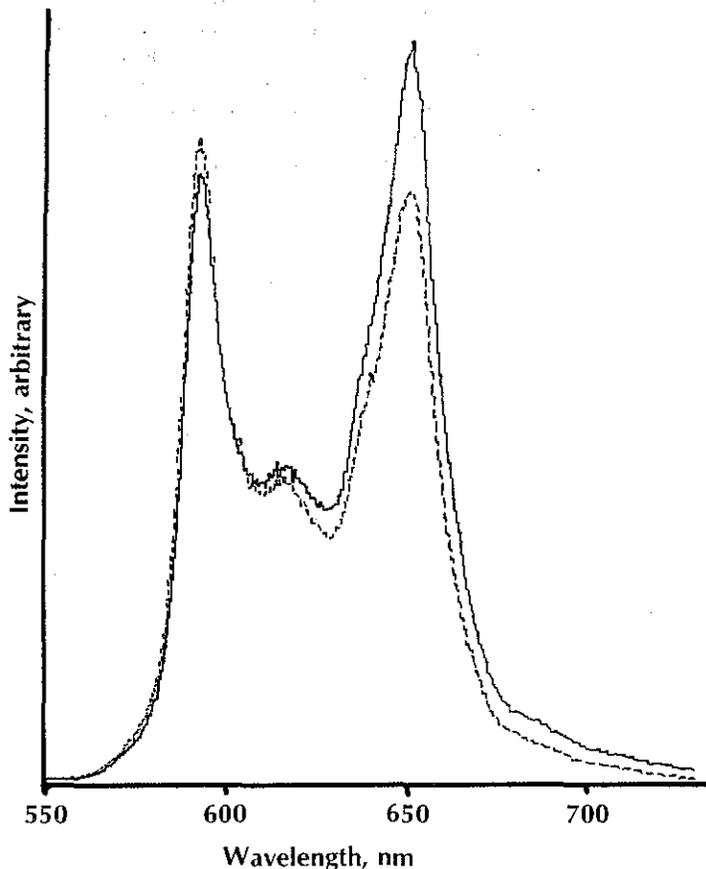
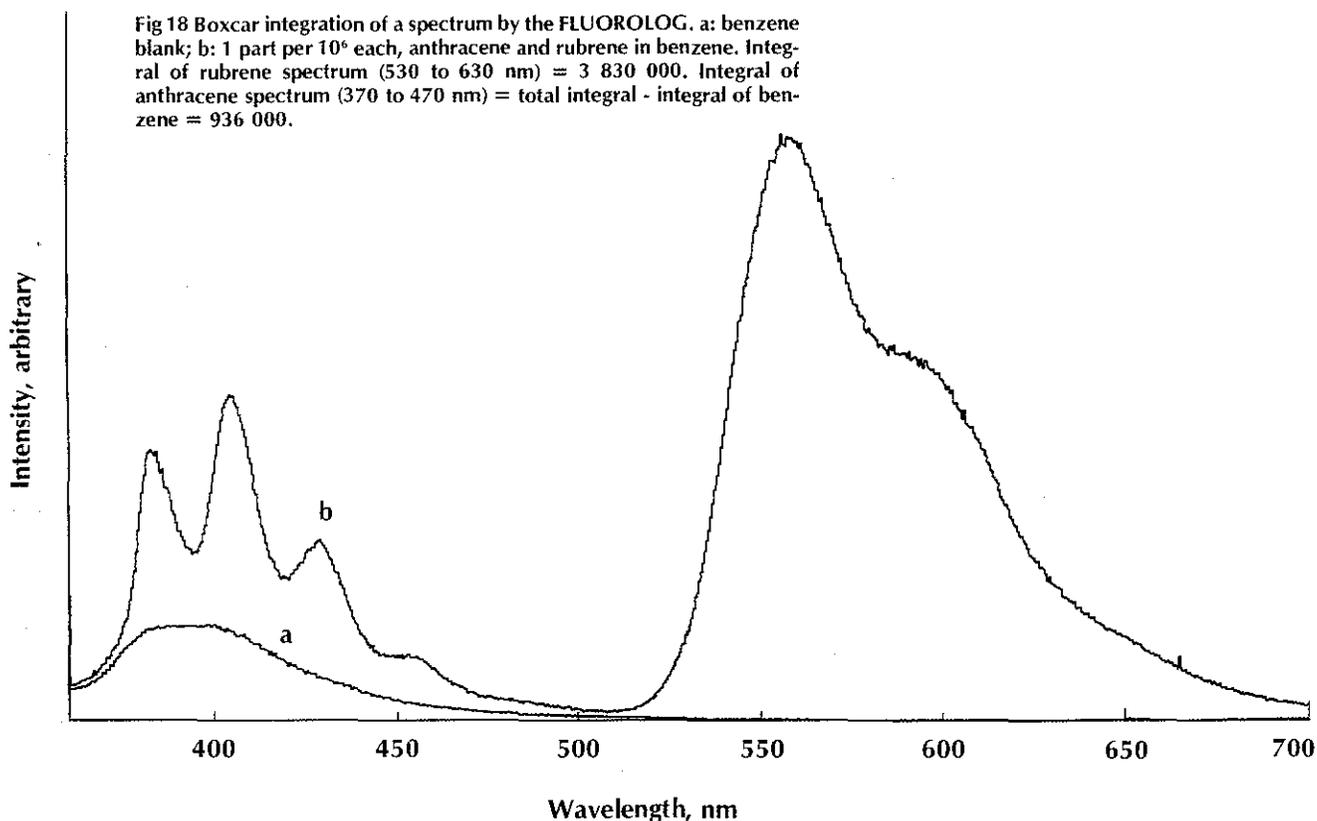


Fig 17 Emission spectra of 500 parts per  $10^9$  coproporphyrin III in 0.1 N hydrochloric acid, uncorrected (dashed) and corrected (solid line).

Fig 18 Boxcar integration of a spectrum by the FLUOROLOG. a: benzene blank; b: 1 part per  $10^6$  each, anthracene and rubrene in benzene. Integral of rubrene spectrum (530 to 630 nm) = 3 830 000. Integral of anthracene spectrum (370 to 470 nm) = total integral - integral of benzene = 936 000.



## Preview of Coming Attractions

To meet a post-deadline for this issue of the Speaker and, even more importantly, to expedite deliveries of FLUOROLOGs, which suffer (as do all youngsters) from growing pains, only preliminary information on the #1932 Variable Temperature Accessory (Fig 19 and the #1934 Phosphorimetry Attachment (Fig 20) are included. More data will be forthcoming shortly on these and on three other accessories on our drawing boards. One involves the substitution of a laser for the normal xenon source. It wouldn't surprise us to find that a powerful laser is able to push back the limit of detection by a decimal point or two.

The second device is our new microprocessed TOMCA (Television Optical MultiChannel Analyzer). Its Raman powers already demonstrated, the TOMCA with its Fellgett advantage should be able to record a complete fluorescence region on a TV screen in about the same time that it now takes to obtain a single point. Unfortunately, the zero dispersion emission double spectrometer, ideal for point-by-point measurements, is inapplicable for TOMCA (or photographic) recording. For this reason the TOMCA's camera must be positioned to monitor the dispersed spectrum after the first, rather than the second, grating in the emission side of the FLUOROLOG.

To be announced shortly is a Double Beam Attachment. Replacing the normal sample compartment, this unit will permit the measurement of the difference in emission intensity of two samples. Obviously, one application is in correcting for the fluorescence of a solvent. Another is in following the reaction of an enzyme.

To be continued in the next episode of this cliff-hanger series are some fascinating results of processing and massaging fluorescence data through our SC4 SPEXCOMP minicomputer system. We promise to keep you posted.

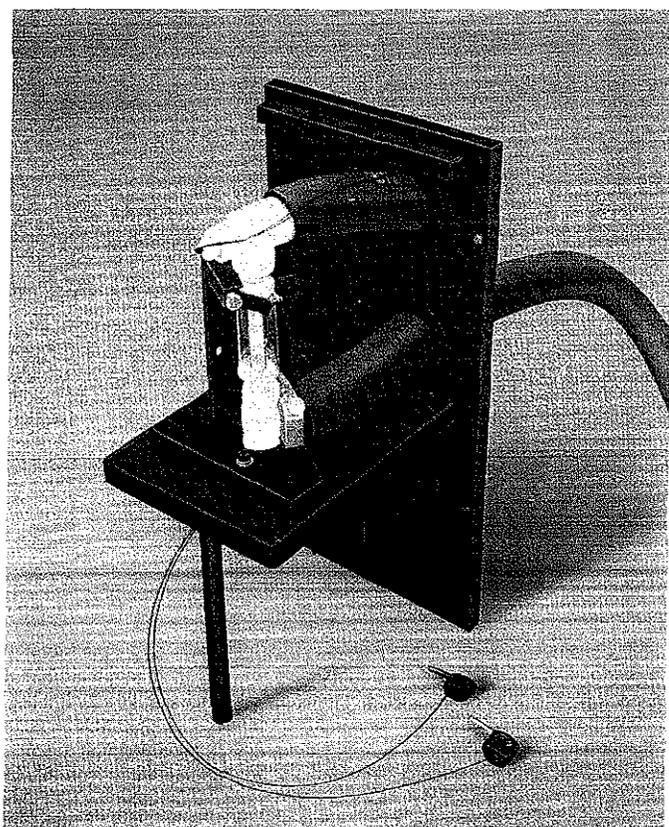


Fig 19 #1932 Variable Temperature Accessory for FLUOROLOG.

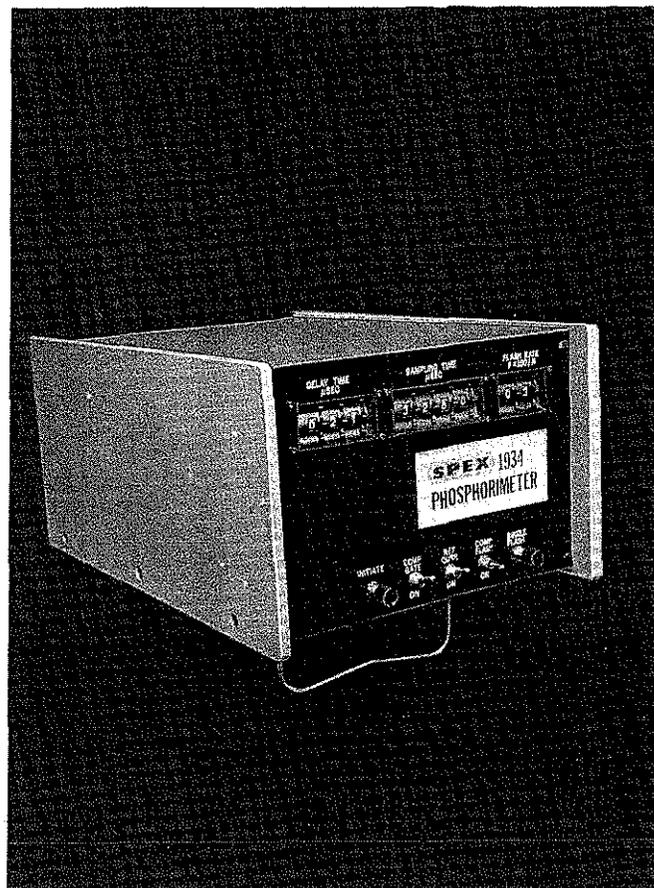


Fig 20 #1934 Phosphorimetry Attachment for FLUOROLOG.

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2. G. Weber, *Biochem. J.*, **51**, 155 (1952)
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## ERRATA

Vol. XXI, No. 2, June 1967

(At Dr. Lewis's request the following note appears exactly as he wrote it.—Ed)

Publication deadlines forced the editor to publish the manuscript "Insight into Eyesight through Resonance Raman" without Professor Lewis reviewing the proofs. Thus the following amendments should be made in the article:

p. 3, column 1, paragraph 3, line 2: nanoseconds to  $\mu$ secs

p. 3, column 1, paragraph 5:

(a) A. Mandelstam to L. Madelsham

(b) momentums to momenta

p. 4, column 1, paragraph 1: Reference 7 to 6

(This same amendment should be made on:

p. 4, col. 2, para. 2

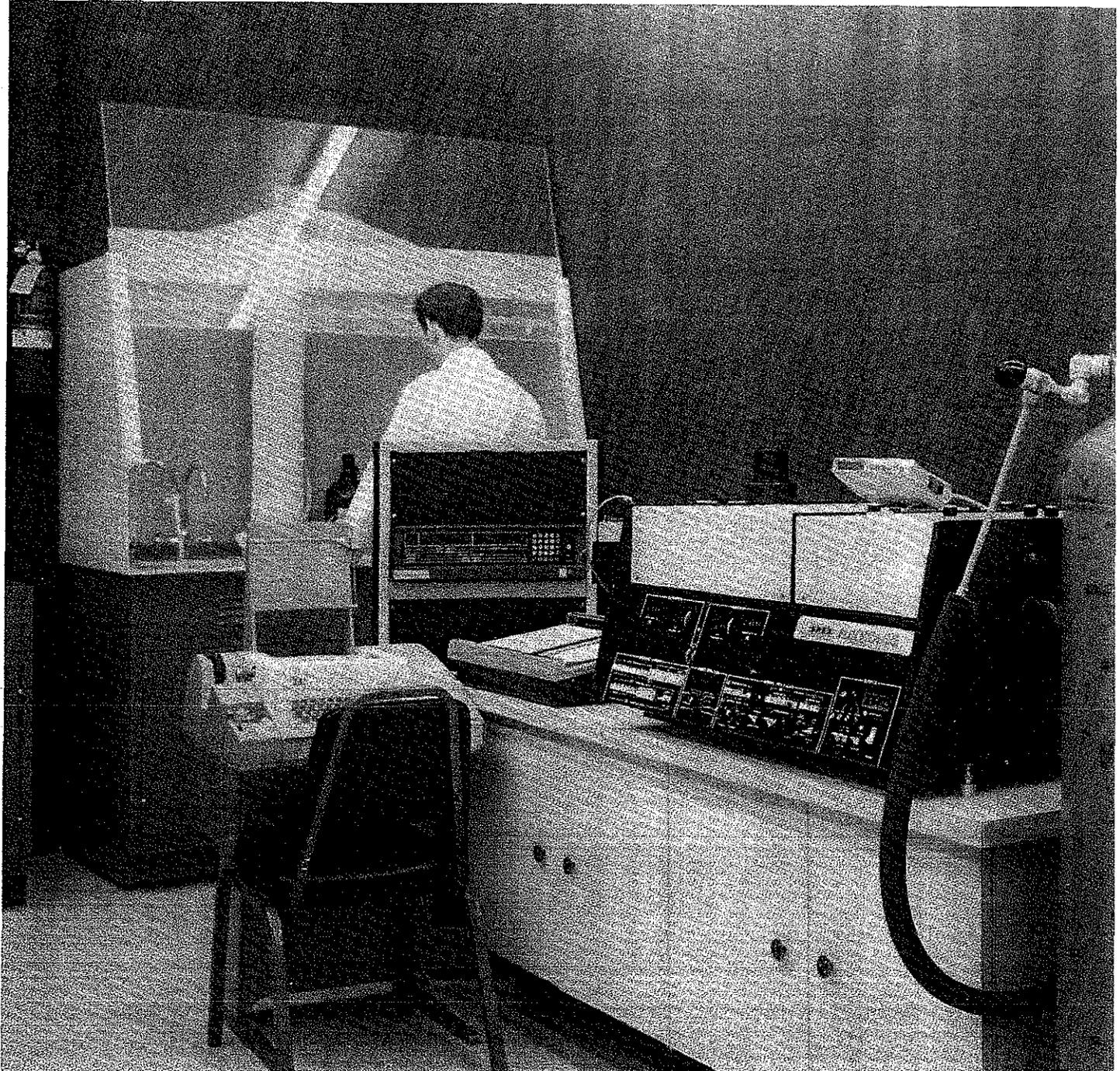
p. 5, col. 1, para. 4

p. 6, col. 1, para. 1

p. 6, col. 2, para. 1).

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