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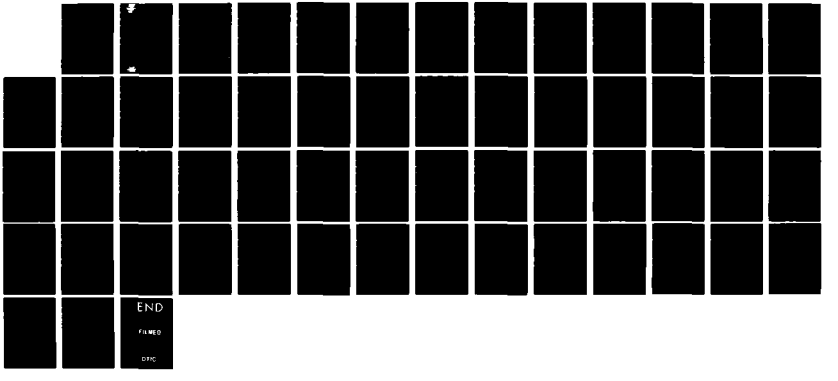

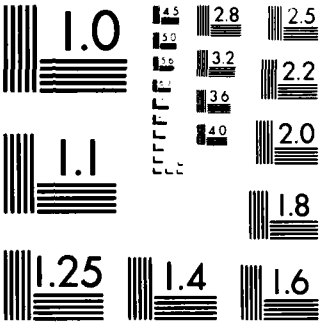

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FLUORESCENT BINDING ASSAYS FOR ANTIGEN DETECTION

1. Introduction

The essential element in analysis is the quantification of a single substance in a complex mixture. Use is made of an unique measureable chemical or physical property of the substance to be quantified which has a well-defined relation to its concentration in the mixture. Analysis is simpler when the substance to be quantified is an abundant constituent of the mixture. However, the pervasive problem in clinical analysis is the determination of substances which are present only in very low concentrations.

Since they were first introduced in 1956, radioimmunoassays have enormously expanded the capabilities of clinical laboratories because of their great sensitivity and selectivity, making possible the quantification of many analytes that cannot be easily determined by other methods. Immunoassays are used to quantify hormones, drugs and other biologically important substances both for purposes of diagnosis, and for purposes of assessing and following the effectiveness of medical treatment. Immunoassays have proven to be very powerful tools in medical practice. As a result, their use has increased dramatically during the past decade and shows every indication of continuing to increase as new uses for existing tests and new tests are developed. Fluorescence is a method of great possible usefulness in clinical chemistry owing to its enormous potential sensitivity. New types of fluorescent assays are being developed as substitute methodologies for radioimmunoassays.

This project involves the development of fluorescent competitive bin-

ding assays for analytes relevant to the immune system of man and animals, and with the goal that such assays can easily be performed not only in clinical chemistry laboratories of major hospitals, but also under field conditions in remote locations. The immunodiagnostic reagents and required instrumentation developed are intended for human and veterinary medical laboratories using fluorescence instead of radioactivity for quantification, and are expected to be more stable, at least as sensitive, easier to perform, and less costly.

2. Advantages of Fluorescent Competitive Binding Assays

At present radioactive isotopes are used as a means of quantification in immunoassays. Radioimmunoassays have a number of shortcoming including:

a. Radioactivity requires special licensing and special precautions in handling to minimize health hazards and contamination. Proper disposal of radioactive wastes is an associated problem. Such a large number of radioimmunoassays are now being performed in many laboratories that the hazard of contamination and the increase in radioactive background has become sufficiently great that many laboratory directors would like to give up radioimmunoassays if substitute methods of comparable quality and cost were available.

b. Standard curves are constantly changing owing to decay of radioactivity and radiodecomposition of constituents.

c. There are considerable batch to batch differences in manufacture.

d. Scintillation counters for measurement of radioactivity are large, expensive, cumbersome, and do not lend themselves to automation.

In addition to eliminating the disadvantages of radioimmunoassays lis-

ted above, fluorescent immunoassays also have the additional potential advantages:

a. Stability of reagents, permitting manufacture in large batches (lower manufacturing costs), and longer life in user laboratories (lower user wastage).

b. Shorter performance time of the assay permitting faster turnaround time for critical assays and increased efficiency in the user laboratory.

c. Fluorescent immunoassays probably will lend themselves better to performance in user laboratories for "stat" assays evenings, weekends, and in emergency situations.

d. Simpler, less cumbersome, and less expensive instrumentation is used that should make these assays more attractive for small hospital laboratories not now performing radioimmunoassays, or under field conditions in remote locations.

e. Standard curves of assays are constant and reproducible over long periods of time obviating the need for frequent restandardization of the assay.

3. Difficulties Encountered in the Development of Fluorescent Immunoassays

The quantification of fluorescence in biological materials involves overcoming a number of problems which may be summarized as follows:

a. The most troublesome problem probably involves light scattering which causes part of the excitation light to be detected in the emission channel. Reduction of light scattering requires filters in the excitation and emission channels that effectively block excitation light from detection. In the case of protein solutions or solid phase assays, the refractive index of the sus-

pension medium should be adjusted to equal as nearly as possible the refractive index of the protein or solid phase material because Rayleigh light scattering is proportional to the fourth power of the difference between the refractive index of the suspension medium and the refractive index of the scattering particles.

b. Serum and other biological fluids have intrinsic fluorescence (1), which is almost entirely at wavelengths shorter than 515 nm. The effects of intrinsic fluorescence of serum can be minimized by using fluorescent probes with excitation maxima at wavelengths greater than 515 nm, or by devising assays where the product to be quantified is removed from the bulk of serum. Alternatively, the intrinsic background fluorescence of serum can be subtracted from the total fluorescence.

c. Bleaching of the fluorophores. Fluorescent probes are chemically modified to a greater or lesser extent by the exciting light to colorless or non-fluorescent compounds. Frequently back-reactions cause the fluorophore to be regenerated during a period of darkness. Bleaching is most marked when ultra-violet excitation is used and less when excitation is at longer wavelengths. Bleaching is also greater in more dilute solutions. Thus, the probes used should be excited at longer wavelengths, and probes with a low intrinsic rate of bleaching should be used.

d. When the emission spectrum of one fluorophore overlaps the excitation spectrum of a second fluorophore, fluorescence energy transfer can occur whereby the excited first fluorophore in turn excites the second fluorophore by its emission. Extrinsic fluorophores can be excited by intrinsic fluorophores in serum leading to erroneous results.

e. Inner filter effect. In more concentrated solutions, exciting light is absorbed by the solutes in solution as it penetrates the solution, causing less excitation of the fluorophore deep in the solution that at the surface.

f. Interference by jaundiced serum, hyperlipemic serum, drugs, etc. These substances fluoresce or cause increased light scattering or both and thereby cause spurious light to register on the photomultiplier and interfere with the assays.

g. Interference by Raman scattering. Light is elastically scattered in solution; in the case of aqueous solutions, Raman scattering occurs approximately 300 wave numbers on either side of the exciting light. The Stokes shift of many fluorescent markers is approximately 300 wave numbers, and thus, Raman scattering is registered by the photomultiplier using the excitation and emission filters for the fluorescent marker. Raman scattering can be minimized by using fluorescent markers with large Stokes shifts.

h. The instability, high noise level and costliness of present commercially available spectrofluorimeters. Particularly in portable applications, and in applications where stat testing is done, rugged dependable intruments would be desireable.

4. The Photon Counter Instrument

In one of his famous 1905 Annalen Physik papers (for which he was awarded the Nobel Prize), Einstein demonstrated that light consists of discrete packets or photons. At low light levels, the individual photons impinging on the cathode of a photomultiplier tube will result in discrete pulses of electron current at the anode. At high light levels, the pulses produced by

individual photons merge into a continuous electron current that is proportional to the incident light flux.

Ordinary fluorimeters are analog instruments in which the anode current of the photomultiplier tube is proportional to the light intensity on the photocathode. The anode circuit of the photomultiplier is designed to have a long time constant thereby averaging short term fluctuations. Thus, the unitary character of photons of light is lost in ordinary fluorimeters by pulse pile-up and the averaging process.

Another method of quantifying light intensity involves counting the discrete electrical pulses produced at the anode of the photomultiplier by the individual photons falling on the cathode (2). Photon counting bears the analagous relation to ordinary flux fluorimeters that scintillation counting bears to the old rate meters used for measuring radioactivity. Photon counting has been used in research applications for half a century where the enormous sensitivity, the extremely favorable signal-to-noise ratio, and other advantages have been well-documented. The simplification of photon counting circuitry is nontrivial, but is required to design an easy to use, stable instrument such as that used in this project.

It should be noted that ordinary spectrophotometers used for visible, UV and infrared measurements operate at high light flux levels. A sample that absorbs light is placed between the light source and the detector. The determination involves estimation of the difference between two large numbers, namely the difference in light intensity with the sample out of and in the light path. Thus, because they measure such high light fluxes that the photon pulses merge, photon counting cannot be used in ordinary spectropho-

tometers. Fluorescent substances, on the other hand, actually emit light at a different wavelength than the exciting light. The exciting light can be excluded by observing the emitted fluorescence at right angles to the beam of exciting light and by the use of suitable monochromators or filters. The fluorophore can be regarded as a weak light source and its measurement involves estimation of its intensity directly. Thus, fluorescence lends itself to quantification by photon counting.

The major advantages of a photon counter compared to ordinary analog fluorimeters may be listed as follows (3):

a) Greater sensitivity than analog fluorimeters. Photon counting is particularly suited to quantifying the extremely low light levels that are produced by very low concentrations of fluorophores.

b) The data is inherently digital and can be handled by digital processing without the necessity of D to A conversion or vice versa. The instrument lends itself to microprocessor control and the data to manipulation and calculation of final results by a microprocessor.

c) Photon counting permits a wide range of linear response over six decades or more. This obviates the need for dilution of samples to fit a restricted range of measurement.

d) The effects of stray light, electrical noise, leakage of electric currents, cosmic radiation and instrument instabilities including drift are minimized. These instabilities appear as though they were fluctuations in the anode current of the photomultiplier tube; they are largely eliminated by the unitary nature of photon counting measurements.

Fig. 1 depicts the number of counts for a series of standard fluores-

cein samples; the instrument shows a linear response for at least five decades of fluorophore concentration.

A troublesome problem in fluorimeters is short-term and long-term fluctuations in measurements resulting from variations in the exciting light source intensity. The simplest method of reducing light source variations is the use of a regulated power supply, which reduces short-term fluctuations, particularly those due to variations in the line voltage, but has little effect on long-term fluctuations such as those due to gradual sputtering of lamp filament material. A better method of stabilizing exciting light flux employs optical feedback (4). A portion of the exciting light flux is monitored by a small phototube or photodiode, the output amplified, and negative feedback utilized to maintain relatively constant light output over the short-term and long-term.

Still more sophisticated compensation for exciting light fluctuations is possible with photon counting. A portion of the exciting light is monitored by a photodiode whose output is fed to a voltage to frequency converter, thereby producing pulses proportional in number to the incident light flux. By referring the fluorescence measurements to a predetermined number of these pulses (rather than to a predetermined time), the fluorescence is measured for a constant integrated light flux. If the light intensity increases somewhat, the number of pulses will occur in a shorter time span and vice versa for decreases in light intensity. This method is particularly effective in normalizing fluorescence measurements for both short-term transients as well as for long-term changes in exciting light intensity.

In Fig. 2, changes in measured fluorescence with variations in line

voltage are compared for an unregulated light source power supply, for a light source using a regulated power supply, and for a system employing measurement over total integrated exciting light flux. The latter is shown to provide the best regulation.

Further improvements in assays can be obtained by use of a helium-neon (HeNe) for excitation. The HeNe laser has a single output at approximately 633 nm. When a HeNe laser is used as a light source. an extended range photomultiplier tube (e.g. RCA 4832) must be used for pnoton counting detection. The particularly useful properties of laser excittion light are:

a) monochromaticity; the width of the envelope of emission is only a small fraction of a nanometer,

b) high intensity at the exciting wavelength,

c) concentration and collimation of the light beam, and

d) the light output is largely coherent, i.e., already polarized.

The HeNe laser was used because it is relatively inexpensive and most suitable for a mass-produced clinical laboratory instrument.

5. Two Optical Channels

The instrument used in this project was designed with two optical channels, one for measuring the fluorescence of the unknown, and a reference channel for the measurement of blank or standard fluorescence, or for measurement of fluorescence polarization (see below). The instrument configuration is such that the two optical channels can use a single light source and a single photomultiplier and are time-multiplexed by a chopper to which

the electronics are synchronized (Fig. 3). Thus, the fluorescence is measured alternately in the two channels and each set of photon counts accumulated. Short-term instabilities in the light source and instrumentation are minimized and the use of a single photomultiplier obviates the necessity of dealing with differences in the spectral and intensity responses and other variables of photomultipliers. The electronics enables direct subtraction of one channel from the other or calculates the ratio of one channel to the other.

The two channel system is very convenient in many types of fluorescent assays. Biological materials have a significant intrinsic fluorescence; in a two cnannel system the sample to be measured is placed in one channel and a blank in the other and tne blank (intrinsic fluorescence) is immediately subtracted from the sample. In the case of fluorescence enhancement type assays, the initial sample is placed in one channel and a final sample is measured in the second channel with immediate calculation of the difference. Another kind of assay depends upon quenching of fluorescence. Here again the initial sample is placed in one channel and the final sample in the second with immediate calculation of the difference.

The light from a tungsten-halogen bulb passes through an infrared filter and collimating lens to a beam-splitting prism. The exciting light is monitored by a photodiode through a fiber optic element for measuring integrated exciting light flux. An alternating chopper to which the electronics are synchronized provides time-multiplexing of the two channels. The emitted fluorescence is observed at right angles to the exciting light and conducted to the photomultiplier by a Y-shaped randomized fiber optic element. The

exciting light and emitted fluorescence wavelengths are determined by narrow band-pass, sharp cut-off (10 nm FWHM) 3-cavity dielectric filters at wavelengths selected to minimize light scattering. We have found these filters to be superior to monochromators for clinical laboratory instruments because of the difficulty in setting monochromators back to precisely the same wavelengths and because filters transmit more light of the desired wavelength.

The output pulses of the photomultiplier are amplified and fed into a discriminator which rejects noise. The output of the discriminator is a series of standard pulses each of which corresponds to a photon. The pulses are counted by a microprocessor. The microprocessor is synchronized to the light chopper and ensures that the light windows and number of exposures of each channel are equal. The source light monitor enables the microprocessor to measure fluorescence over a predetermined integrated exciting light flux. Measurement ("through-put") time for clinical assays developed in our laboratory was usually about 12 sec.

The microprocessor counts the photons in each channel. It can display on a LED read-out the number of counts in either channel, the difference in counts between the two channels, the ratio of counts between the two channels, or the slope of the progress curve of an enzyme reaction. Alternatively, the output of the microprocessor can be fed into a standard alphanumeric printer or into a laboratory printer for on-line processing of results.

6. Synthesis of Fluorescent Tracers

There are several desireable characteristics that make fluorescent molecules good probes for clinical assays:

a) excitation maxima greater than 515 nm, or excitation corresponding to the output lines of a laser light source, or if they possess a fluorescent lifetime appreciably greater than 10 nanosec if the excitation maximum is less than 515 nm,

b) high absorbance,

c) high quantum yield,

d) the Stokes shift, i.e., the difference between the wavelenths of the excitation and emission maxima, should be as large as possible; background fluorescence will have relatively small Stokes shifts,

e) have chemically reactive groups that permit easy coupling to functional groups of analytes, as well as having such solubility and other properties that the unconjugated dye can be easily and completely removed from the product,

f) chemical stability,

g) resistance to photobleaching, and

h) when coupled to an analyte, not appreciably alter its immunoreactivity.

Fluorescein is the fluorescent moiety most commonly used in analytical systems. Improved performance of analytical systems could be achieved by improving upon any of the above characteristics. In general, we found that dyes in which the reactive groups was a sulfonyl chloride coupled more "cleanly" to analytes than isothiocyanates, and the unreacted dye could be more completely removed from the reaction mixture. Sulfonic acid residues also increase the hydrophilic character of the dye, making for greater water

solubility and less change in fluorescent excitation and emission maxima as well as fluorescence intensity with changes in the dielectric properties of the solvent or the microenvironment around the fluorescent moiety. Greater substitution on the rings of the dye causes a red shift to longer wavelengths of both the excitation and the emission wavelengths.

Two such compounds synthesized were rhodamine-123 sulfonyl chloride (Fig. 4, **A**), excitation 546 nm and emission 571 nm, and rhodamine-101 sulfonyl chloride (**B**), excitation 595 nm and emission 610 nm. Their quantum yield was close to 1. Of interest is the fact that rhodamine-123 adducts are relatively pH insensitive. These reactive fluorescent compounds combine with amino and mercapto groups; the reaction is carried out at pH 9.0 in bicarbonate or phosphate buffer in the dark. The excess fluorescent probe is reacted with aminoethanol and the product purified by chromatography on Sephadex gels.

Lucifer yellow vinyl sulfone (**E**) was synthesized by the method of Stewart (5). Excitation maximum 430 nm and emission maximum 523 nm (Stokes shift 93 nm). The vinyl sulfone group reacts with amines and mercaptans by the Michael addition; analytes were labeled using the general procedure of Smith (6). For example, to obtain the fluorescent T4 (thyroxine) adduct, approximately 20 mg analyte and 20 mg dye in 2 ml pyridine/water/triethylamine (9:1.5:1) was stirred for 40 hrs in the cold room. The crude product was precipitated by adding 30 ml 0.2 M ammonium acetate buffer, pH 4.0. The product was collected by centrifugation and filtration and was washed with water. It was redissolved in 1 ml ammonium bicarbonate, pH 11.0. The solution was chromatographed on a 1.5 X 39 cm column of Sephadex LH-20-100 equi-

librated with 0.05 M ammonium bicarbonate, pH 9.0. The product was the major band eluted from the column with water.

Coumarins are another attractive group of compounds that were thought to be suitable for a covalently reactive fluorescent probe. Reactive probes were prepared from coumarin-343 (**C**), excitation 467 nm and emission 496 nm, and of the benzthiazole analog of coumarin-6 (**D**), excitation 456 nm and emission 512 nm. Major effort was directed toward the synthesis of a reactive derivative of coumarin-152 (**F**). Following the procedure of Long (7), 886 mg (0.0035 moles) coumarin-152 purchased from Eastman-Kodak was reacted 0.0035 moles diazotized p-nitroanilin prepared by standard methods. The resulting yellow solid was recrystallized from ethanol. The product was then reduced to the amine by a combination of the techniques of Newman (8) and Sippel (9) as follows. A mixture of 463 mg product, 9 mg mercuric chloride and 158 mg aluminum powder were refluxed for 3 hrs in 10 ml ethanol. After cooling, the mixture was filtered and allowed to stand for 12 hrs. The precipitate was collected by filtration and recrystallized from toluene. Excitation 409 nm and emission 519 nm (Stokes shift 110 nm). Pyrenetrisulfonyl adducts are quite resistant to photobleaching.

One new fluorescent probe of great interest and possible utility is 8-acetoxy-1,3,6-pyrenetrisulfonyl chloride (**G**) which was synthesized as follows. 5 g 8-hydroxy-1,3,6-pyrenetrisulfonic acid was refluxed in 50 ml acetic anhydride for 1.5 hrs. A light yellow solid precipitated that was collected by filtration, washed with ethylene chloride, and dried in a descicator. 1.5 g of this material dissolved in 1 ml dimethylformamide was added to 10 ml thionyl chloride with constant stirring for 20 hrs in a fume hood. The

mixture was then poured onto about 20 g crushed ice. After the vigorous reaction had subsided, the aqueous solution was transferred to a separatory funnel and extracted three times with 20 ml portions of chloroform. The organic layers were combined and dried over anhydrous magnesium sulfate. The clear orange solution was filtered and the volume reduced to 15 ml by evaporation. Hexane was added until cloudiness of the solution appeared. After storage at 2 degrees, golden brown crystals formed which were harvested. Yield = 31 %. The excitation and emission spectra of the product itself are shown in Fig. 5. For steric reasons, only one molecule of analyte couples to each fluorescent probe molecule; the remaining two sulfonyl chloride groups are hydrolyzed. Excitation maximum of the adduct with bovine serum albumin is at 467 nm and emission maximum at 569 nm (Stokes shift 102 nm).

An attempt was made to synthesize a reactive derivative of a rare earth chelate, the terbium chelate of p-aminodipicolinic acid, in an effort to investigate the possible usefulness of such compounds as fluorescent probes in assay systems. 0.85 g m-chlorodipicolinic acid and 8 ml concentrated ammonium hydroxide were sealed in a glass tube and heated at 150 degrees for 24 hrs. After cooling, the tube was opening and the white solid removed by washing with 20 ml water. The solution was evaporated to near dryness and then solid formed was recrystallized from 15 ml water. This amino compound was converted to maleimide, iodoacetamide or isothiocyanate derivatives by standard techniques (9,10). The excitation and emission spectra of the product are shown in Fig. 6.

Some characteristics of these dyes are summarized in Table 1. Fluorescein succinic acid (excitation 494/nm, emission 516 nm), prepared by

reacting 5-aminofluorescein with a slight excess of succinic anhydride, was the index fluorophore with which the new fluorophores were compared. In general, these dyes had larger Stokes shifts than fluorescein, and the excitation and emission maxima were at longer wavelengths. These properties lessened the interference from background biological fluorescence and light scattering. Furthermore, all of the compounds had greater resistance to photobleaching than fluorescein, increasing the fluorescence intensity stability during an analysis. However, none of the dyes had as great fluorescence sensitivity as fluorescein. Fluorescence sensitivity is the product of absorbance at the excitation maximum wavelength and quantum yield; it is a relative measure of the detectability of a dye (11). Thus, the fluorescence intensity produced by these dye adducts for a given concentration of analyte is less than for fluorescein adducts. The dyes with the most favorable characteristics were (**F**) and (**G**).

7. Synthesis of Long Wavelength Fluorescent Probes for Use with a Helium-Neon Laser

In order to take advantage of using a Helium-Neon laser as an excitation source, it was necessary to synthesize fluorescent probes with an excitation envelope that included the single output light band of the He-Ne laser, namely 633 nm. The ideal probe would also possess the properties of high quantum yield, high extinction coefficient, large Stokes shift, and resistance to photobleaching. In addition, the probes must have a reactive functional group to permit covalent coupling to the analyte to be quantified.

The first group of such long wavelength fluorescent probes studied were

the phenoxazine (Nile Blue) family of dyes of which Nile Blue A (Fig. 7) was the index compound in our experiments. The most important properties of Nile Blue A are listed in Table 2. Nile Blue A has a low quantum yield and does not have a reactive functional group. Therefore, we undertook to overcome these deficiencies by attempting the synthesis of Nile Blue Iodoacetamide, Nile Blue Acetic Acid and Juloidine Nile Blue (compounds **B**, **C**, and **D** in Fig. 7). The synthetic approaches are outlined below. These compounds contain reactive groups, **B** an iodoacetamide that reacts with sulfhydryl groups, and **C** with primary amines. It is well known that the quantum yield of fluorescent compounds containing a dialkylamino group can be increased by making this groups relatively immobile; we attempted to increase the quantum yield of the Nile Blue dyes by synthesizing compound **D** in which the dialkylamino group is immobilized by fusing it into the ring system. Unfortunately this attempted synthesis did not succeed.

Nile Blue Iodoacetamide was synthesized as follows. 3.88 g (17 mmoles) Nitroso-m-diethylaminophenol, prepared by treating m-diethylaminophenol with sodium nitrite and HCl on ice, was mixed with 2.59 g (10 mmoles) N-aminoethyl-1-naphthylamine in 60 ml isopropanol and the mixture refluxed for several hours. The dark blue mixture was then filtered and washed twice with cold methanol to yield 2.77 g aminoethyl-Nile Blue A. This material was mixed with 2.6 g chloracetic anhydride in 4 ml triethylamine and 60 ml dimethylformamide for several hours. The reaction mixture was filtered and approximately 3 g product was collected as a blue-black solid. TLC of the product in methanol:chloroform 3:1 showed a single blue spot, Rf = 0.85. The iodoacetamide was prepared <u>in situ</u> by dissolving the material in 20 ml ace-

tone and 4 ml dimethylformamide and allowing it to react overnight with 0.24 g sodium iodide.

Nile Blue Acetic Acid was prepared by refluxing 0.575 g nitroso-m-diethylaminophenol and 0.5 g 1-naphthylamine acetic acid in 30 ml isopropanol containing 3 drops 6 M HCl for several hours. After cooling, 0.33 g product was collected as a black crystalline powder.

A more promising class of dyes that could be used as laser excited probes are the cyanines. A prototype cyanine dye is compound **A** in Fig. 8. The cyanines are especially interesting because many features of the general structure can be varied to give probes with particular physical properties. For example, increasing the length of the methine chain between the heterocyclic rings results in a progressive bathochromic (red) shift in spectral properties. We investigated a dicarbocyanine dye (compound **B** in Fig. 8) designated DiIC3(5) in the nomenclature system of Waggoner and coworkers (12) as a potentially useful He-Ne laser excited probe. The spectral properties of this dye are listed in Table 2; this model dicarbocyanine dye has the desired spectral properties but lacks a reactive functional group. Furthermore, although the quantum yield is better than the Nile Blue compounds studied, it is still probably inadequate. However, in the case of the dicarbocyanine dye, the very high extinction coefficient helps offset the low quantum yield, and we were able to detect this dye in low concentrations as will be discussed below.

A derivative of DiIC3(5) containing a reactive group was prepared in our lab by incorporating a carboxylic acid into the alkyl side chains of the dye (compound **C** in Fig. 8). The spectral properties of this dye designated

DiIC3(5)COOH are also listed in Table 2 and showed very little difference from the parent compound, and were quite as good. Thus, DiIC3(5)COOH was a potentially useful probe that could be excited by a He-Ne laser and that could be coupled to analytes with primary amines.

DiIC3(5)COOH was prepared by refluxing 4.8 g 5-amino-2-methylbenzothiazole and 7.65 g 2-bromacetic acid in 35 ml cyclohexane for 15 hrs. After cooling, the dark red tar that formed was used without further purification. The product (about 10 g) was refluxed with 3.3 g 1,3,3-trimethoxypeopene in 30 ml pyridine for several hours. The initially red solution turned blue almost immediately. After cooling to room temperature, 100 ml 3 M HCl was added to precipitate approximately 6 g product as golden-green iridescent crystals that was collected by filtration. The product was dissolved in 120 ml 0.5 M NaOH and recrystallized.

8. Principles of Assays

Immunoassays are performed by adding a predetermined amount of a labeled analyte and specific antibody to serum or other biological fluid containing an unknown amount of analyte. Labeled and unlabeled analyte compete for binding by the specific antibody. Quantification of bound and/or unbound labeled analyte permits calculation of the amount of unlabeled analyte in the unknown sample. In fluorescent immunoassays, analytes are covalently coupled to a fluorescent compound of desired excitation and emission wavelength and high quantum yield at a site on the analyte that is not an antigenic determinant.

Two methods of quanitification were used. In the first method fluores-

cence intensity was measured to determine the amount of analyte. When the fluorescence intensity method is used for measurement, it is usually necessary to separate free analyte from analyte bound to antibody by one means or another.

In the second method, quantification was from measurement of fluorescence polarization as described by Dandliker and others (13-15). The mixture of unknown analyte, fluorescent analyte and specific antibody is measured in an instrument described below. Fluorescence emission intensity of a fluorophore excited by plane polarized light is measured with polarizers in the analyzer parallel to, and perpendicular to the plane of polarization of the exciting light. These data permit calculation of the amount of analyte in the unknown sample.

Briefly, the principle of fluorescence polarization assay is as follows. When a fluorescent material is excited by polarized light, the fluorescent emission is also polarized. However, there is a finite time between the instant of excitation and the instant of fluorescence emission during which fluorophore molecules in solution tumble owing to Brownian motion. Because of this molecular motion, the polarization of fluorescent emission is "scrambled." Polarization, the fundamental parameter measured in polarization assays is defined in terms of the fluorescence intensity measured with the polarizers oriented parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the excitation polarizer. P is then calculated from the relation

$$P = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$$

Because molecular tumbling is less the greater the molecular weight, a solution of low molecular weight free fluorescently tagged analyte will have a low polarization, while fluorescent analyte bound to high molecular weight antibody will have a high polarization, and mixtures of free fluorescent analyte and fluorescent analyte-antibody complexes will have intermediate P values. As the amount of analyte in a sample is increased, the lower the P value (Fig. 9). Thus, a homogeneous mixture of free and antibody-bound analyte can be quantified by the polarization of the solution.

It should be noted that all solutions prepared for use in photon counting fluorescence measurements must be passed through a 8 micron Millipore filter to remove dust and other particulate matter that would cause interference with the measurements.

9. Determination of Immunoglobulin Isotypes by a Heterogeneous Immobilized Immunoabsorbent Fluorescent Assay

Many analytes can be quantified by heterogeneous immobilized solid-phase immunoabsorbent assays (16-20); the immunoglobulin isotypes were studied as index fluorescent assays of this kind. These assays were performed by incubating the unknown sample with an excess of isotype-specific antibody immobilized on agarose beads which absorbed the immunoglobulin antigen in the sample. An excess of fluorescent labeled antibody was then added and the mixture incubated further. The second fluorescent antibody was bound mole for mole to the antigen absorbed on the immobilized first antibody forming a "sandwich", while the excess fluorescent antibody remained in solution. The solid phase immune complex was removed by centrifugation, washed, resuspen-

ded, and the fluorescence of the suspension measured in the photon counter. All procedures were carried out in PBS to minimize nonspecific protein absorbtion. Because only the analyte of interest was removed with the solid phase complex and the remainder of the serum was discarded with the supernatant fraction, and because the fluorescent probe emitted at longer wavelengths than the intrinsic fluorescence of serum, the contribution of background fluoresence of serum was minimized. The fluorescent second antibody in the immobilized immune complex was quantitatively proportional to the amount of antigen in the solid-phase sample giving a linear curve of fluorescence intensity versus concentration.

Agarose beads (SeaSep AC beads obtained from Marine Colloids Division, FMC Corp., Rockland, ME) were activated with cyanogen bromide (21) and coupled (22) to immunoglobulin isotype-specific rabbit antihuman antibody (Dako, Accurate Chemical Co., Hicksville, NY) at neutral pH. The remaining reactive groups on the derivatized beads were inactivated by incubation with ethanolamine and the antibody-coated beads were then well washed with PBS. Isotype-specific goat antihuman antibody was obtained from Cappel Laboratories (Cochranville, PA) and labeled with rhodamine-101 sulfonyl chloride so that the final molar dye to protein ratio was approximately two.

The immobilized and fluorescent labeled antibodies were titrated with human immunoglobulin isotype fractions that had been standardized against International Reference Preparations from the World Health Organization (Meloy Laboratories, Springfield, VA) by radial immunodiffusion. The antibody derivatized beads and fluorescein labeled antibodies were diluted with PBS to give equivalent binding capacities of 60, 8 and 8 mg% for IgG, IgA and

IgM respectively. Sodium azide, 0.03 % final concentration, was added to all reagents as a preservative. Progress curves of antigen binding by both immobilized antibodies and fluorescent labeled antibodies showed the reaction to be essentially complete after incubation for 20 min at 37°.

Unknown sera were diluted 1:50 with PBS. To 2.0 ml bead immobilized antibody suspension in disposable test tubes was added 100 ul diluted sera, standards or PBS for blanks. The mixture was vortexed and incubated for 45 min at 37°. Fluorescent-labeled antibody solution, 100 ul was added and the mixture incubated for an additional 45 min. The beads were sedimented by centrifugation at 2,000 X g for 7 min, the supernatant fraction discarded without disturbing the pellet, and the tubes drained by inversion. 2.0 ml PBS was added to each tube and the mixture vortexed. The beads were again sedimented by centrifugation at 2,000 X g for 7 min, the supernatant discarded, and the tubes drained by inversion. Exactly 2.0 ml of 0.6 g/L NaCl and 7.4 g/L dextrose in 20 mM borate buffer, pH 8.8 was added and the mixture vortexed to disperse the beads. This dispersion buffer was chosen to minimize light scattering and settling of the beads. The fluorescence was measured in the photon counter. Because the photon counter is a two channel instrument, the blank is counted in one channel and the unknown in the other; the difference, which is the corrected fluorescence intensity, is automatically subtracted and displayed on the read-out.

Plots of serum immunoglobulin isotype concentration versus photon counts of fluorescence gave straight lines for serum concentrations as high as 2400, 400 and 400 mg% for IgG, IgA and IgM respectively. Each immunoglobulin isotype was determined for four secondary serum standards calibrated

against International Reference Preparations from the World Health Organization as well as twelve patient serums, four with low values, for normals, and four highs. The average correlation coefficient for the regression curves of six replicate assays were 0.966, 0.987 and 0.984 for IgG, IgA and IgM respectively. The interassay coefficients of variation (n = 6) lay between about 2 % for the high protein assays to about 5 % for the low protein assays. The cross correlation coefficients for 24 determinations of each isotype by this fluorescent immunoassay and by radial immunodiffusion were 0.828, 0.660 and 0.982 for IgG, IgA and IgM respectively. It is known that determination of IgA by radial immunodiffusion also give low cross correlation coefficients with nephelometry (23).

10. Fluorescence Polarization Assay for Thyroxine

A simple fluorescence polarization assay for free thyroxine was developed using several of the fluorophores. In this assay, 3,5-dichloro-3',5'-diiodothyronine (T_4Cl_2) adducts were used as the fluorescent tracers. This compound was used in place of thyroxine in forming the adducts because the heavy iodine atoms in ring A of thyroxine quench fluorescence. T_4Cl_2 was coupled to Lucifer Yellow vinyl sulfone as follows. To a solution of 20 mg T_4Cl_2 (obtained from Calbiochem) in 1 ml pyridine:water:triethylamine 9:1.5:0.1 was added a solution of 20 mg Lucifer Yellow vinyl sulfone in 1 ml of the same solvent and the reaction mixture was stirred in the cold room in the dark for 40 hrs. The crude material was precipitated from the reaction mixture by adding 26 ml 0.2 M ammonium acetate, pH 4.0, collected by centrifugation, and finally washed with 30 ml cold water. The crude material was

dissolved in 1 ml 0.05 M ammonium bicarbonate, pH 9.0, and applied to a 1.5 X 39 cm column of Sephadex LH 20-100 column. The column was first eluted with 700 ml 0.05 M ammonium bicarbonate, pH 9.0, followed by 50 ml water. The desired product was eluted by the water as a green fluorescent band and was verified by absorption measurements at both 338 nm and 430 nm indicating that thyronine and Lucifer Yellow were present in equimolar amounts. Adducts with other reactive fluorescent probes were similarly prepared, except when using acetoxypyreneacetyltrisulfonyl chloride which was quite unreactive and did not form a satisfactory adduct.

Sheep antithyroxine serum was obtained from Windsor Chemicals, Dallas. The optimum antiserum dilution was determined by finding the dilution of antibody that gave approximately 85 % of the maximum polarization value with 10^{-7} M fluorescent tracer. The fluorescence polarization assay was developed in the usual way by generating a calibration curve using standards prepared by adding known amounts of l-thyroxine to pooled human serum. Assays were carried out in the presence of 0.05 M salicylate to minimize nonspecific adsorption. The normal range of thyroxine concentrations in human serum is 55 to 115 ng/ml. The data showed that although the assay was adequate in the euthyroid and hyperthyroid range of serum values, the sensitivity was barely adequate in the hypothyroid range.

11. Assays of Therapeutic Drug Concentrations

Assays for therapeutic drugs offered a set of good tests for evaluating our new dyes and measurement techniques. The assay of gentamycin using fluorescein as the fluorescent probe served as an index assay. Fluorescein

labeled gentamycin was prepared as follows. Solutions of 10 mg gentamycin sulfate dissolved in 1 ml 0.01 M phosphate buffer, pH 7.5, and 10 mg fluorescein isothiocyanate isomer-1 in 1 ml 0.01 M carbonate buffer, pH 9.0 were mixed and stirred for 1 hr. Alternatively, fluorescein succinic acid was coupled to gentamycin using a carbodiimide. The fluorescein adduct of gentamycin was separated on a 2.5 X 40 cm column of Sephadex G-25. The separation was poor; the central part of the green fluorescent band was collected discarding the leading and tail parts of the elution. The concentration of fluorescein-labeled gentamycin was determined by measuring the absorption and assuming an extinction coefficient of 65,000. The solution was then diluted to 10^{-9} in 0.05 M phosphate buffer, pH 7.5, containing 0.1 M sodium azide and 0.01 % bovine serum albumin. The gentamycin concentrations of interest are in the 0 to 20 mcg/ml range. Standards were prepared by addition known final concentrations of 2.0, 4.0, 8.0 and 16.0 mcg/ml gentamycin to pooled human serum.

The gentamycin antibody used in our experiments was purchased from Western Chemical Research Corp., Ft. Collins, Colorado. The final antibody concentration used in the assay system was chosen to give 90 % of the maximum polarization when used with the lowest concentration standard, i.e., 2 mcg/ml. With this antibody, a 100X stock solution turned out to be a 1:225 dilution of the neat serum in 0.05 M phosphate buffer, pH 7.5, containing 0.1 M sodium azide and 0.01 % bovine plasma albumin. The optimum antibody dilution will vary with each batch of antibody. It should be noted that the most satisfactory antibodies are those raised using antigen coupled to adjuvant protein at the same chemical position as the fluorophore is coupled to

analyte in the tracer. Not all antisera are satisfactory in fluorescence polarization assays; a high titer good antibody for radioimmunoassays may be a poor antiserum for fluorescence polarization assays. Each antiserum must be evaluated by making a polarization titration curve.

A typical assay mixture contains 25 mcl tracer stock solution, 25 mcl antibody stock solution, 25 mcl standard or unknown solution, and 2.0 ml 0.05 M phosphate buffer. After incubation, the parallel and perpendicular fluorescence intensities were determined and P calculated. These data were used to generate a standard curve in which P is plotted against analyte concentration are shown in Fig. 10.

For comparison, a heterogeneous solid phase assay for gentamycin was used. Gentamycin antibody was coated on the lower inner wall of polystyrene test tubes. An incubation mixture containing diluted unknown serum or standard analyte solution and free rhodamine-101 labeled gentamycin was incubated in the tubes. The optimal analyte concentration was found to be approximately 2×10^{-7} M. The supernatant solution was then removed and its fluorescence intensity measured. A standard curve such as that shown in Fig. 11 was obtained by plotting gentamycin concentrations against fluorescence counts or Rx against fluorescence counts where

$$Rx = (Fx - Fo) / (Ft - Fo)$$

and Ft is the total fluorescence counts in the absence of antibody, Fx the fluorescence counts for sample x, and Fo the fluorescence counts for a serum blank. Unknown values were obtained by interpolation on the standard curve.

The accuracy and precision of the heterogeneous assay was about the same as for the polarization assay but the heterogeneous assay involved more steps and was more complicated and time consuming to run.

A similar assay was devised for phenytoin for which the range of concentrations of interest is 0 to 20 mcg/ml. Tracer was prepared by coupling fluorescein succinic acid using a carbodiimide, or by coupling rhodamine-101 sulfonyl chloride to diphenylglycine. A standard curve in which P is plotted against analyte concentration are shown in Fig. 10.

Efforts were then made to develop a gentamycin assay using DiIC3(5)COOH labeled gentamycin and a HeNe laser for excitation. Therapeutic drugs generally are found in serum in the micromolar concentration range. The assay requirements, therefore, are much less stringent than for hormone and protein assays where the analytes are present in the nanomolar range. Because the concentration of fluorophore is being measured in a fluorescence immunoassay, we determined the sensitivity of the laser excited photon counting instrument for the fluorescent dye DiIC3(5)COOH. The results which are given in Table 3 show that a 100 mcl 1 X 10^{-10} M dye sample can be confidently distinguished from a serum blank. For comparison, Fig. 13 shows plots of fluorescence counts against dye concentration for fluorescein, acetoxypyrenetrisulfonic acid, and DiIC3(5)COOH from which the minimum detectable concentration can be determined. Thus, this dye taken together with the instrumentation are more than adequate for therapeutic drug assays, but borderline or not quite sufficiently sensitive for hormone assays that can easily be performed using radioimmunoassay techniques.

After mixing 1 ml ice-sold 0.075 M DiIC3(5)COONa in water and 1 ml ice-

cold 0.15 M gentamycin sulfate in water, the pH was adjusted to 3.8 and 0.1 g 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate was added. The mixture was stirred in the cold room for 24 hrs. After filtering the solution through a 8 micron Millipore membrane, 2 ml of the filtrate was loaded on a 1.5 X 22 cm column of Sephadex G-25. Unreacted dye was eluted first by 0.05 M ammonium formate, after which the dye-gentamycin complex was eluted with 1.8 M ammonium formate. Tests on this fraction indicated that the gentamycin:dye ratio was about 1.3.

In developing a gentamycin assay system, we first selected the best antiserum and the optimum antibody dilution by generating antibody titration curves from data such as those listed in Table 4 obtained by adding different amounts of antibody to a constant amount of dye and determining P. Contrary to expectations, there was very little variation in P for different antibody concentrations. In fact, P for the free dye was as great as it was for dye-labeled gentamycin with an excess of antibody. These results are not typical for a low molecular weight fluorophore, and probably indicate aggregation of the dye molecules possibly through stacking interactions. In any event, aggregation or other cause for the high P values of free fluorophores must be overcome before a satisfactory assay system can be developed.

The terbium complex that we synthesized, although it had a higher energy transfer efficiency and quantum yield than complexes with benzyl EDTA or B-diketone was still not sufficiently fluorescent to be as good a probe as fluorescein or rhodamine-101 in workable assays (Fig. 14).

12. Photobleaching

Fluorescein and many other fluorescent probes suffer from photobleaching which causes a progressive reduction in fluorescence intensity with continued exposure to exciting light. Photobleaching is worse for more dilute solutions and is one of the factors limiting detection sensitivity of fluorescent probes. One approach that we studied in an effort to overcome this problem was to synthesize probes that were intrinsically resistant to photobleaching; examples are rhodamine-101 and acetoxypyrenetrisulfonyl chloride. Another approach was to carry out assays in the presence of free radical scavengers such as aminoethylthiouronium chloride (AET). These compounds were found to inhibit photobleaching significantly. For example, under conditions when a 0.1 nanomolar solution of fluorescein showed a 6.6 % reduction in fluorescence intensity in 1 hr, bleaching was reduced to 1.7 % in the presence of 0.01 M AET and was not detectable in the presence of 0.1 M AET.

13. Quantification of Cell Surface Markers

Exploratory studies were performed aimed at using our fluorescent dyes to quantify cell surface receptors and markers using a cell sorter. These experiments were carried out in collaboration with Drs. K. Gulliya and A. Khan at the Wadley Insitute for Molecular Biology in Dallas. In initial experiments, REH cells were used, a cell line of human lymphoblastic leukemia cells grown in suspension culture with a specific surface antigen for which a mouse monoclonal antibody, 81G, has been obtained (24). In the assay system, the first antibody 81G interacted with and was bound to the cell surface. Then, a sheep anti-mouse second antibody labeled with rhodamine-101 was added. The cell suspension was assayed in a cell sorter and the fluorescence

quantified. Fig. 15 is a histogram that shows labeling by the fluorescent probe while controls without the first antibody or controls using other cell lines that do not have this particular surface marker were not labeled. Similar experiments using the rhodamine-101 adduct of pindolol, a specific beta-adrenergic antagonist with a very high binding affinity, permits quanitification of beta-adrenergic receptors on the cell surface of different cell lines.

14. Conclusions

These experiments show promise of leading to simple, homogeneous assays using fluorescence for analytes present in very low concentrations. The primary stumbling block at this time that needs to be overcome is the development of suitable fluorescent probes. The instrumentation using photon counting is quite adequate for such assays that have relevance in immunological and cell identification assays.

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Dye	λ_{abs}[a] (nm)	absorbtivity ($M^{-1}cm^{-1}$)	λ_{ex} (nm)	λ_{em} (nm)	Stokes Shift (nm)	Ab. Fluor. Sensitivity[b]
PATC	458	1.32×10^4	460	512	52	3.6×10^3
Lucifer Yellow VS	430	1.0×10^4	430	523	93	2.8×10^2
Coumarin 152	404	1.0×10^4	409	519	110	1.0×10^3
$Tb(DPAC1)_3$	273	8.77×10^3	273, 282	491, 545	209-263	3.5×10^1
FITC	495	7.5×10^4	492	525	33	3.6×10^4

a) All spectral properties are for the dyes in aqueous media such as .05M $NaHCO_3$ (pH=8.1).

b) The product of the quantum yield and the absorbtivity ($\phi\varepsilon$) gives the absolute fluorescence sensitivity (see ref. 11).

Table 1. Spectral Properties of Fluorescein Substitutes

Dye	ε ($M^{-1}cm^{-1}$)	λ_{ex} (nm)	λ_{em} (nm)	Net Quant. Yield[a]	Reactive Group	Photo-decomposition
Nile BlueA	2.99×10^4	635(broad)	665	low	no	9%/hour
$DiIC_3(5)$	1.80×10^5	636	657	moderate	no	1%/hour
$DiIC_3(5)$-COOH	1.85×10^5	638	654	moderate	yes	1%/hour

a) The "net quantum yield" is a relative term that includes the inefficiency of the photomultiplier tube to long wavelength detection.

Table 2. Spectral Properties of Long Wavelength Dyes.

sample concentration	counts[a,b]	standard deviation
1×10^{-10}M	1.34×10^{2}	12.9
1×10^{-9}	6.73×10^{2}	16.5
1×10^{-8}	3.29×10^{4}	2.2×10^{3}
1×10^{-7}	1.15×10^{6}	7.1×10^{3}
blank[c]	32.4	11.2
dark count	38.2	7.1

a) average of five readings
b) PMT voltage = 1200V
c) .05M $NaHCO_3$ (pH=8.1)

Table 3. Detection response data for $DiIC_3(5)COOH$ using He-Ne laser excitation.

sample	Ab titer	dye conc.	P
1 (tracer)	1/125	2.14×10^{-8}M	.15±.01
2 "	1/250	"	.16±.02
3 "	1/500	"	.17±.02
4 "	0	"	.19±.01
5 (dye)	0	1.0×10^{-8}	.18±.01

Table 4. Antiserum Polarization Titration Results.

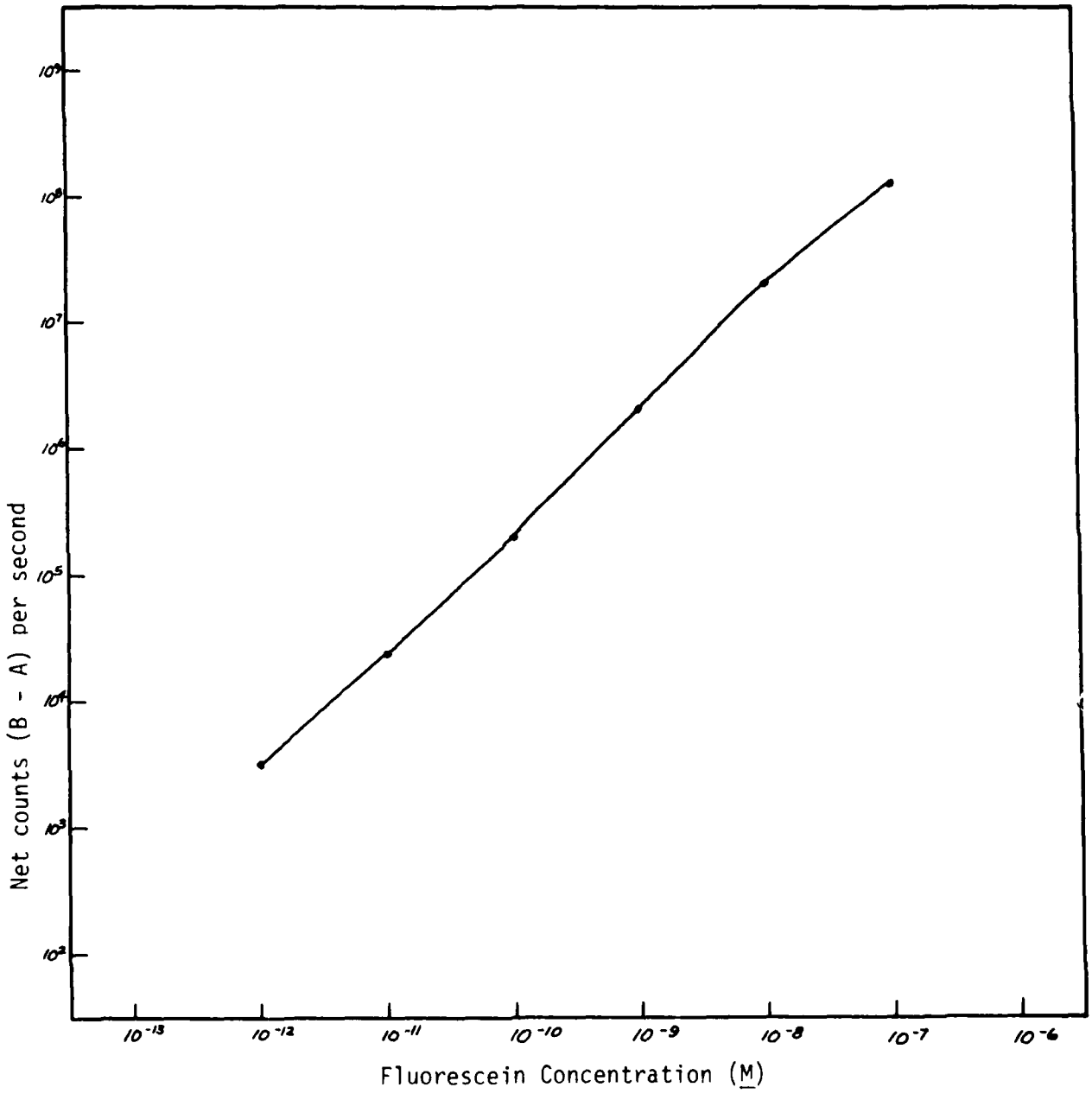


Fig. 1. Photon counts vs. concentration of fluorescein in 20 mM borate buffer, pH 9.0.

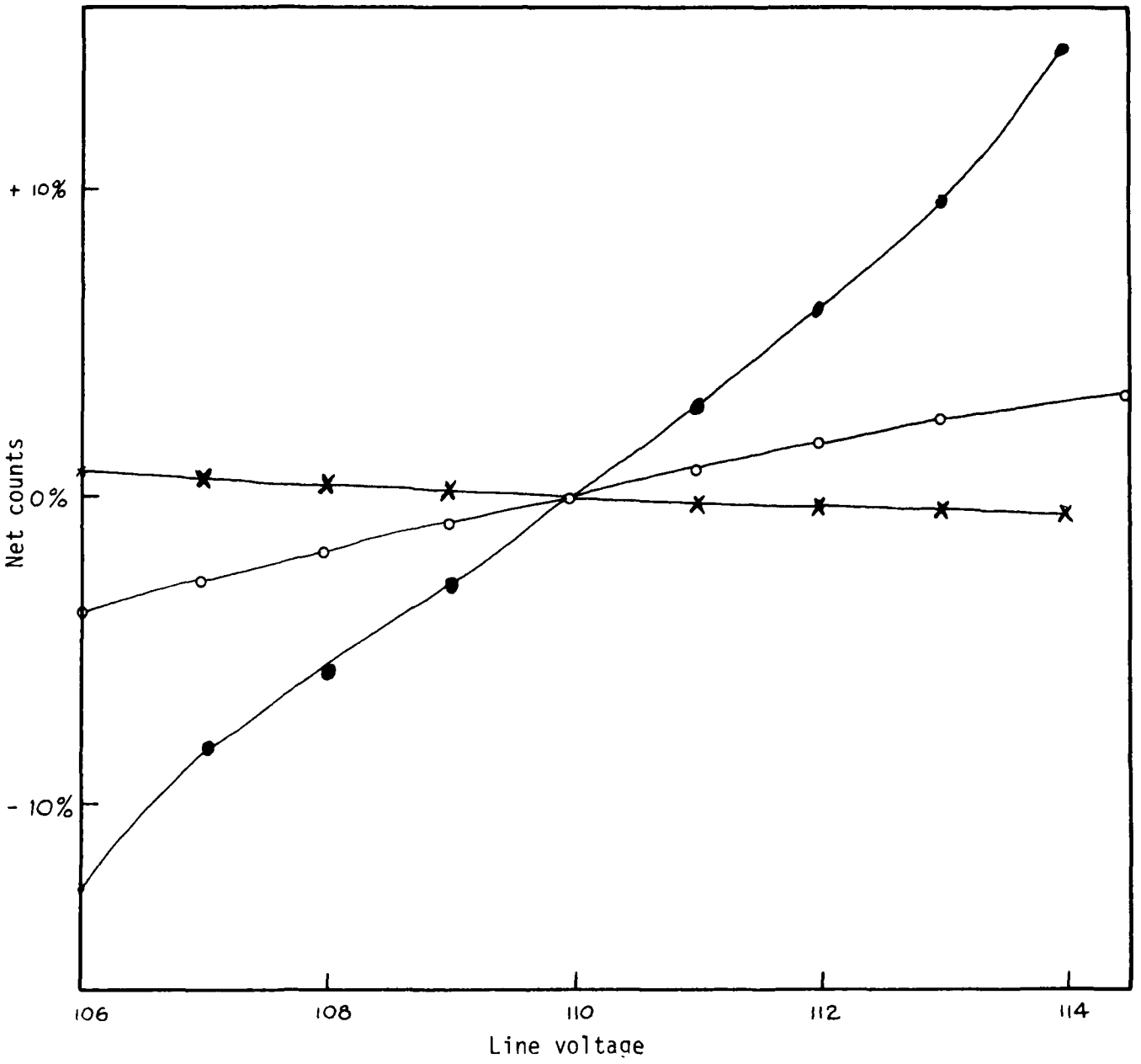


Fig. 2. Comparison of the fluctuation of net counts for a 10^{-9} fluorescein solution at 20° C in 20 mM borate buffer, pH 9.0: ● no regulation, o constant voltage regulation, x constant light flux regulation.

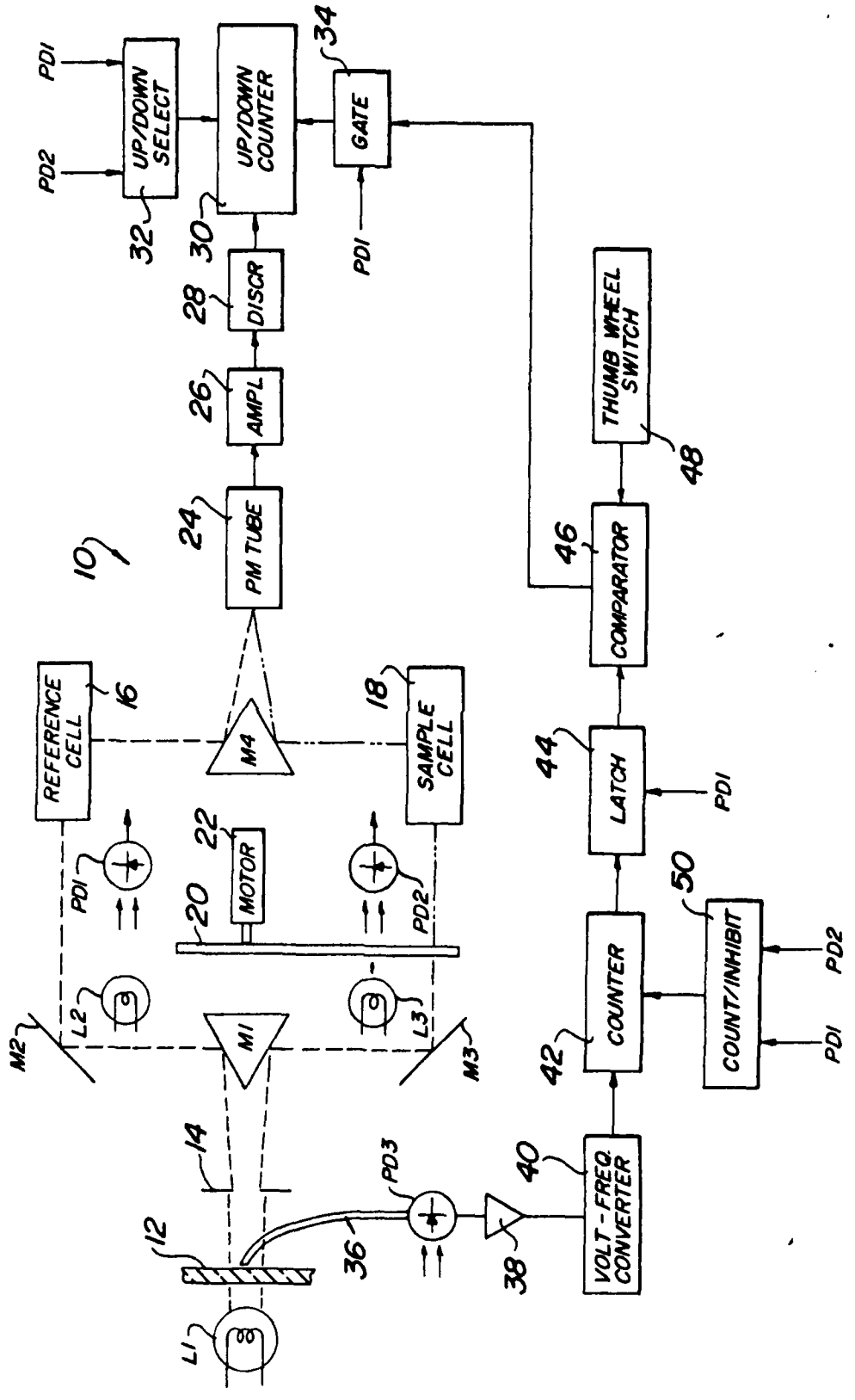
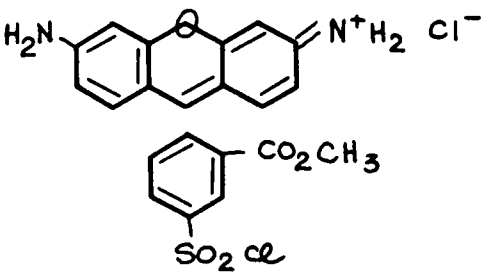


Fig. 3. Block diagram of photon counting instrument.

Fig. 4

Rhodamine 123 Sulfonyl Chloride

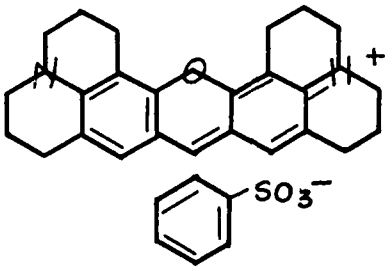


(A)

λ excitation 504 546

λ emission 523 571

Rhodamine 101 Sulfonyl Chloride

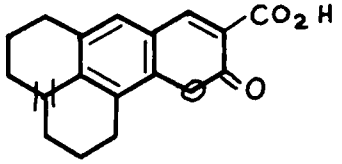


(B)

λ exc 595

λ emis 610

Coumarin 343



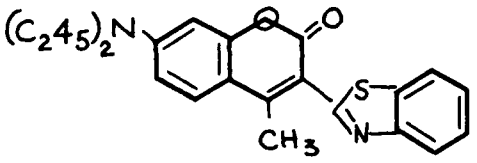
(C)

λ exc 467

λ au 496

coupled to BSA 478 515

Coumarin 6-benrthiazole



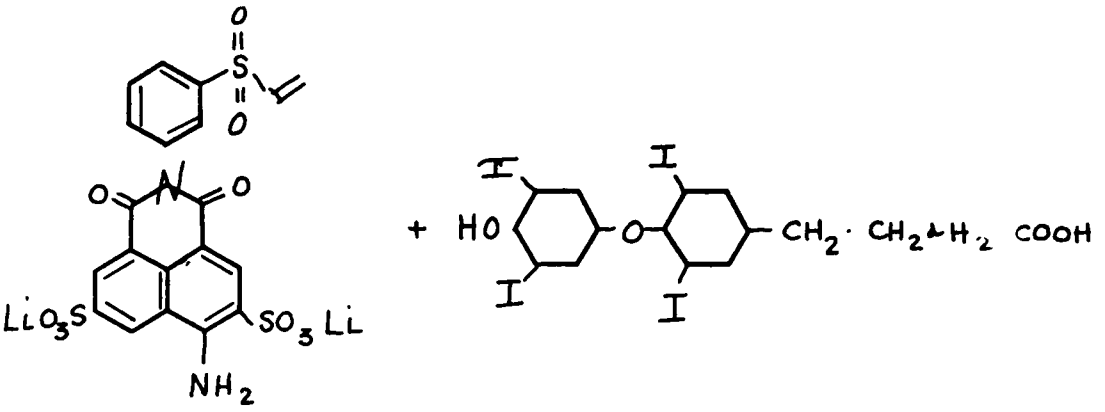
(D)

456 456 512

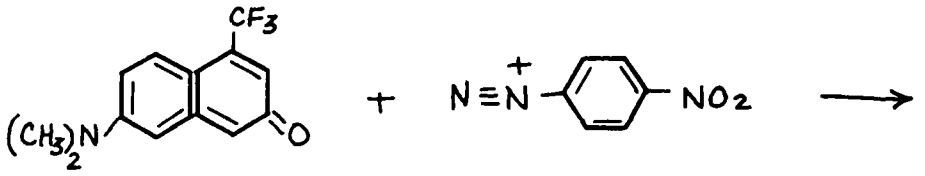
512

Lucifer Yellow Vinyl Sulfone

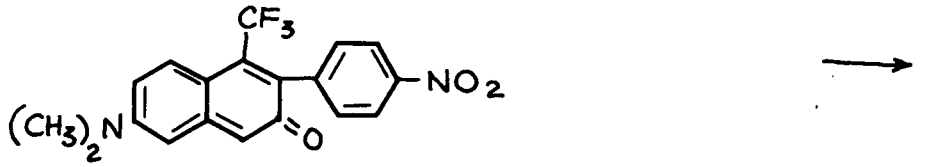
(E)

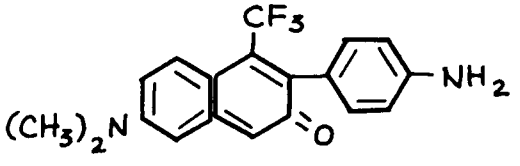


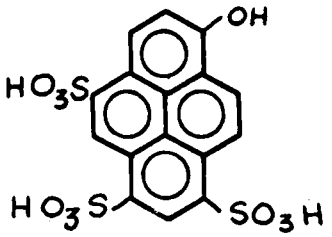
Coumarin 152

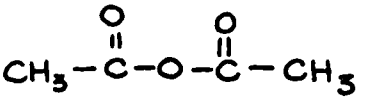


(F)





(G) 

+ 

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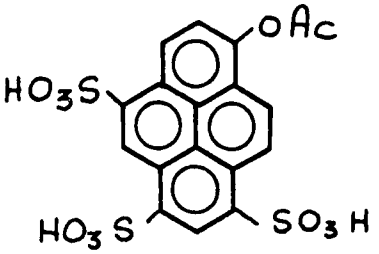
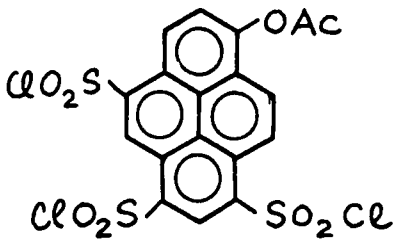


$\xrightarrow[DMF]{SOCl_2}$



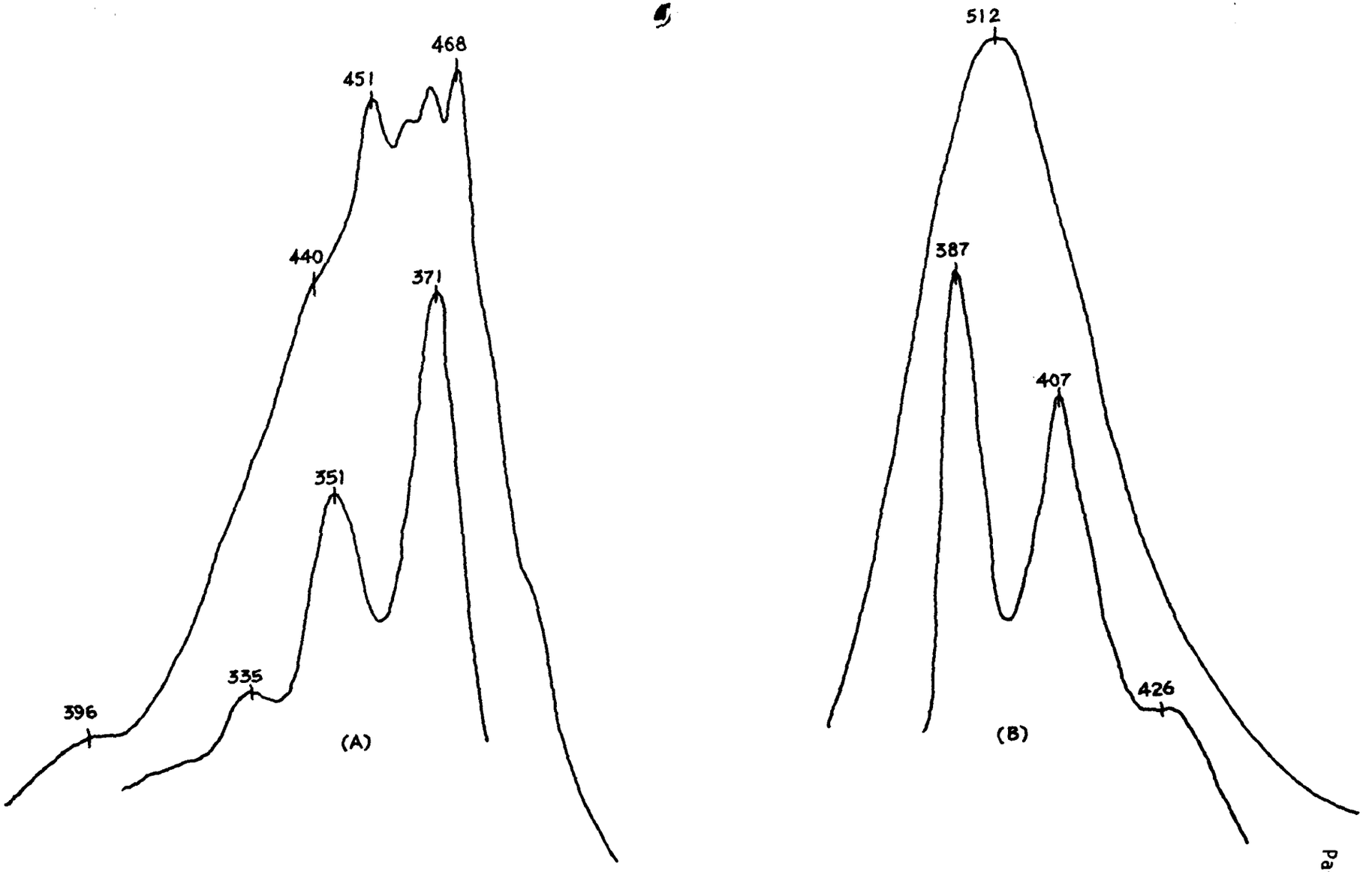


Fig. 5. Fluorescence excitation (A) and emission (B) spectra of 8-acetoxy-1,3,6-pyrenetrisulfonyl chloride in aqueous buffer (upper curves) and in dimethylformamide, concentration 1 X 10^{-7} M.

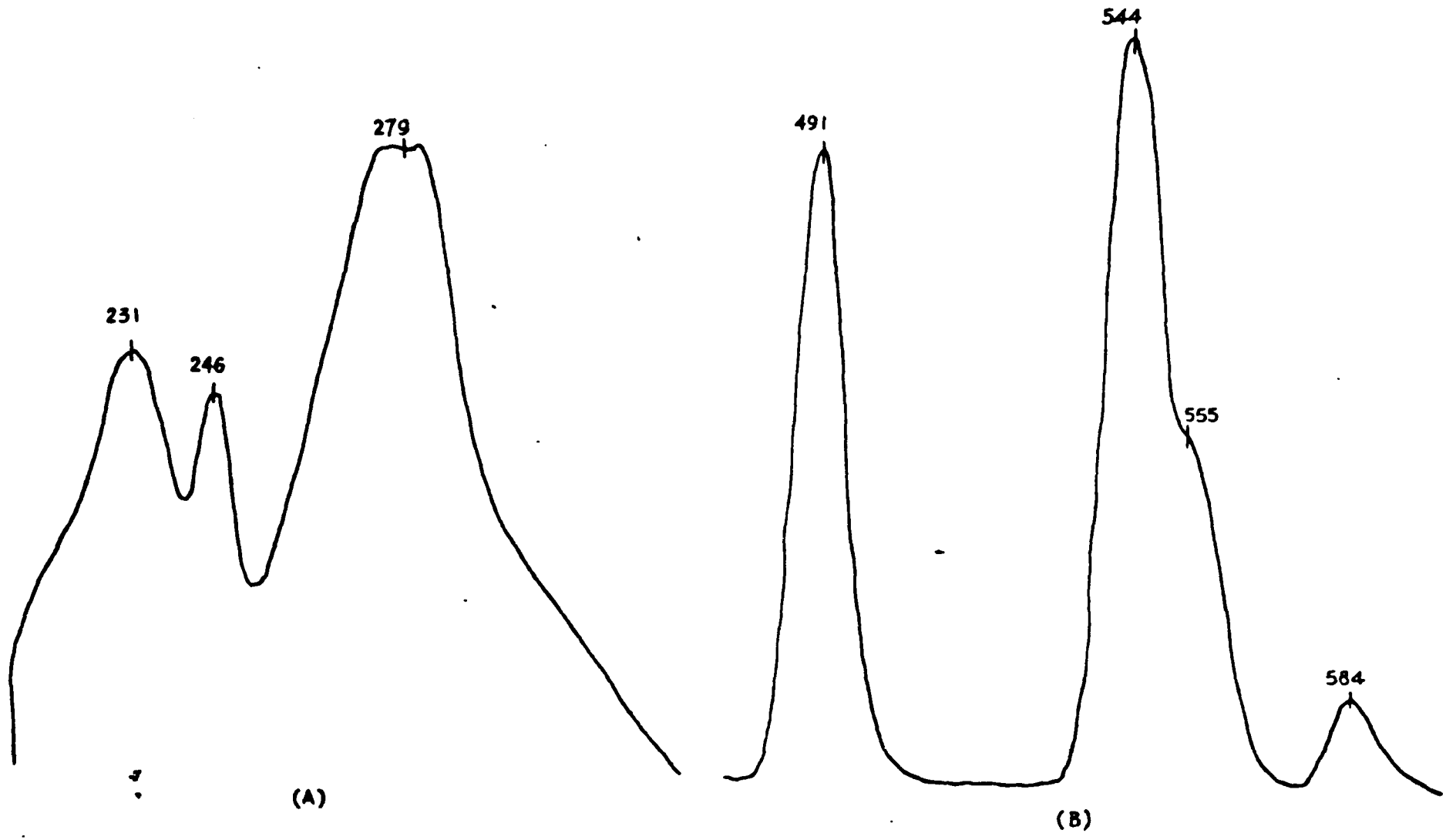
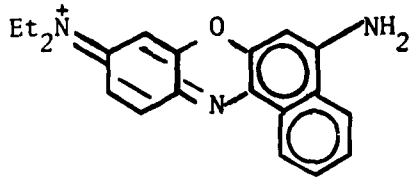
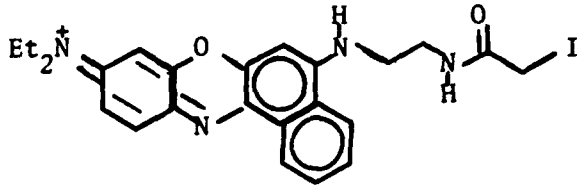


Fig. 6. Fluorescence excitation (A) and emission (B) spectra of 2.6×10^{-6} M aqueous p-aminodipicolinic acid terbium complex. The peaks at 246 and 555 nm are due to scattered light.



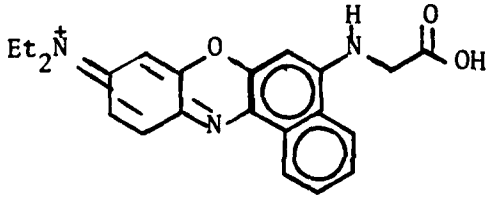
Nile Blue A

A



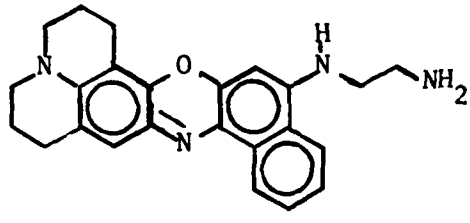
Nile Blue Iodoamide

B



Nile Blue Acetic Acid

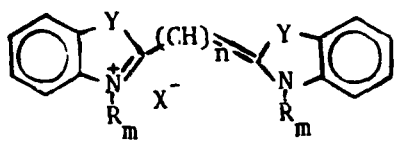
C



Juloidine Nile Blue

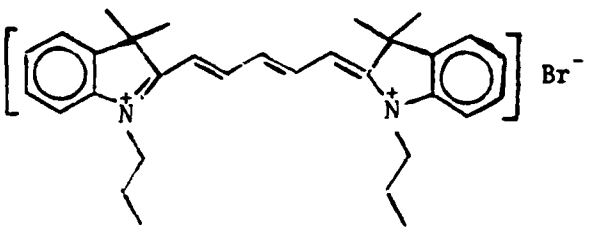
D

Fig. 7. Nile Blue Dyes (phenoxazines)



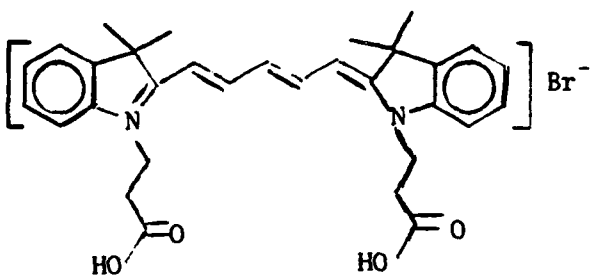
prototype carbocyanine

A



$DiIC_3(5)$

B



$DiIC_3(5)COOH$

C

Fig. 8 . Carbocyanine Dyes.

Polarization
0.10
0.15
0.20
0.25
1.00
0.95
0.90
0.85
0.80
Relative concentration analyte in mixture of gentamycin and antibody

Fig. 9

Std. (ug/ml)	Amt. Std. Used	Buffer[a]	Tracer[b]	Ab	Polarization[c]
0	5 ul	1.495 ml	100 ul	400 ul	.238 ± .003
1	"	"	"	"	.225 ± .003
2	"	"	"	"	.221 ± .007
4	"	"	"	"	.192 ± .004
8	"	"	"	"	.151 ± .004
16	"	"	"	"	.120 ± .006

a) .1M Phosphate/.1% NaN_3; pH=7.5

b) 4.4×10^{-8}M FITC labeled gentamicin

c) average of 10 readings

Table Gentamicin Assay - Conventional Method

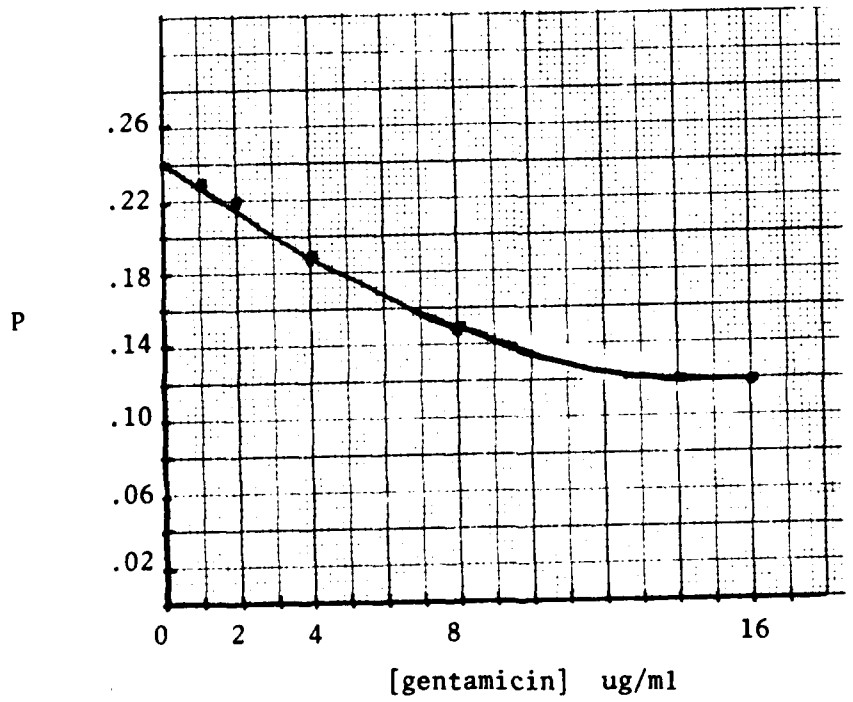


Fig. 10. Data from Table Plotted.

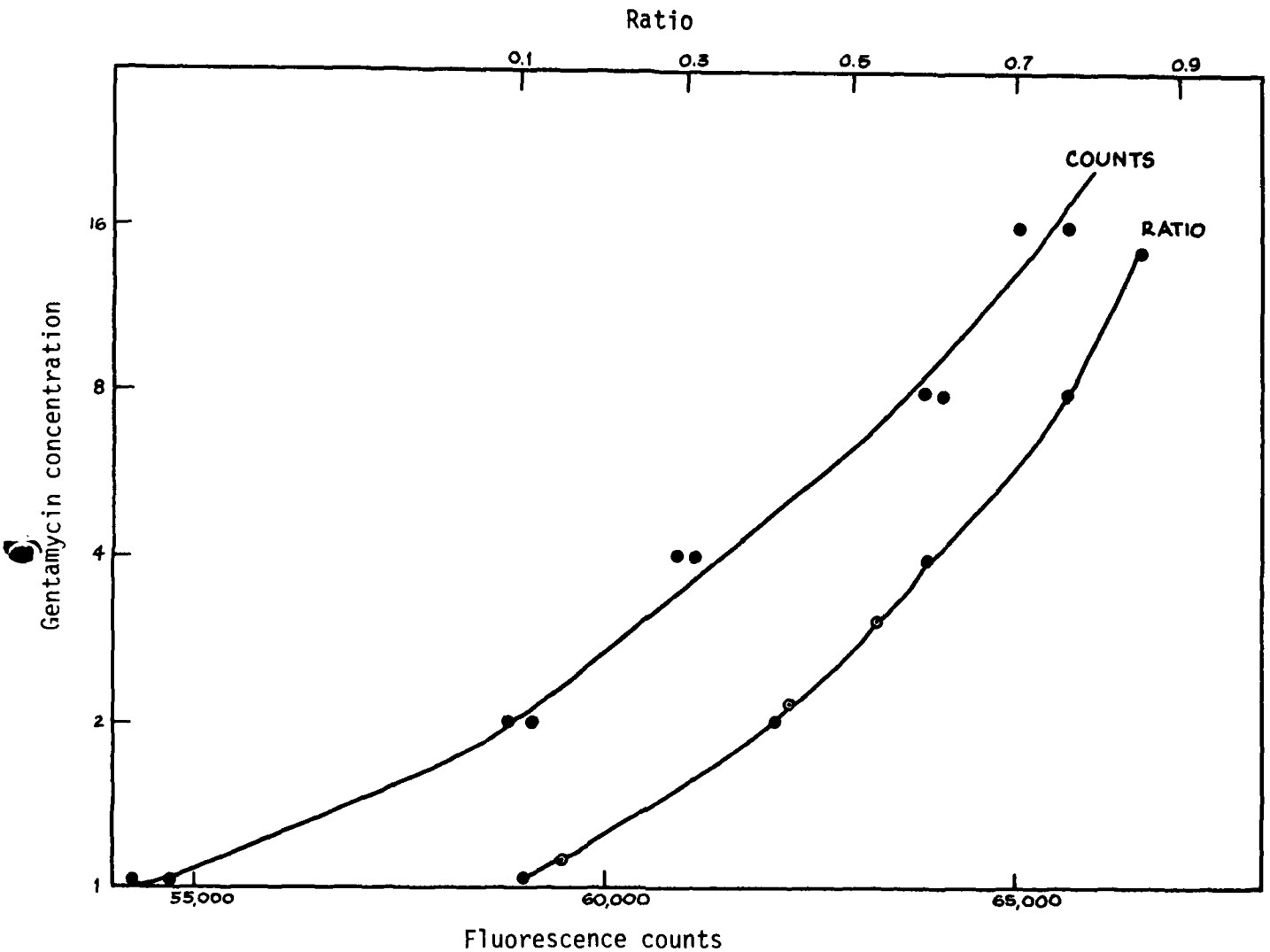


Fig. 11. Plots of gentamycin concentrations against fluorescence counts, or, ratio Rx (see text) against fluorescence counts in a solid phase heterogeneous assay using rhodamine 101 as the fluorescence label.

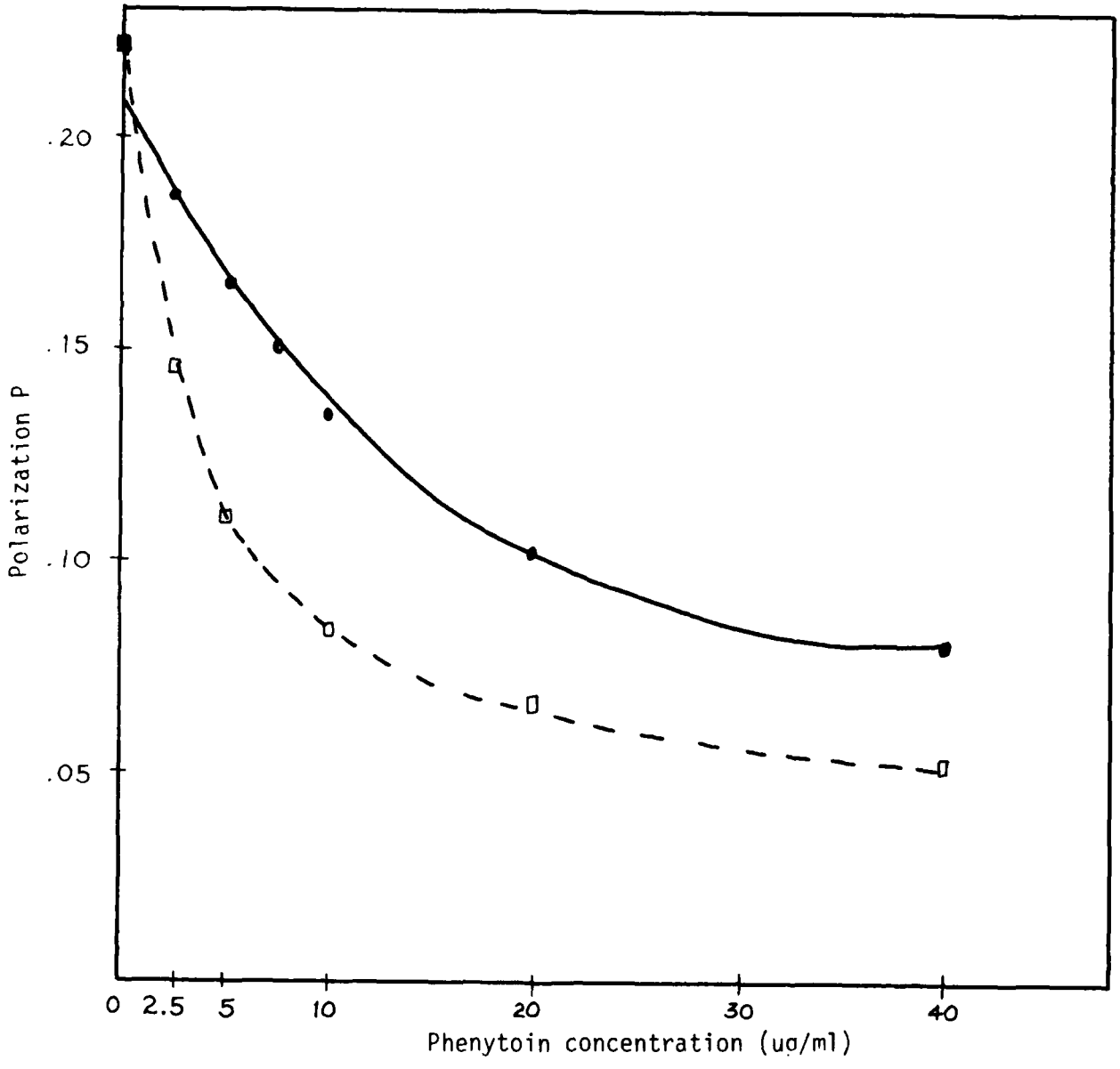


Fig. 12. Standard curve for a phenytoin assay using fluorescein succinic acid labeled phenytoin (●) and rhodamine-101 sulfonyl chloride labeled phenytoin (■).

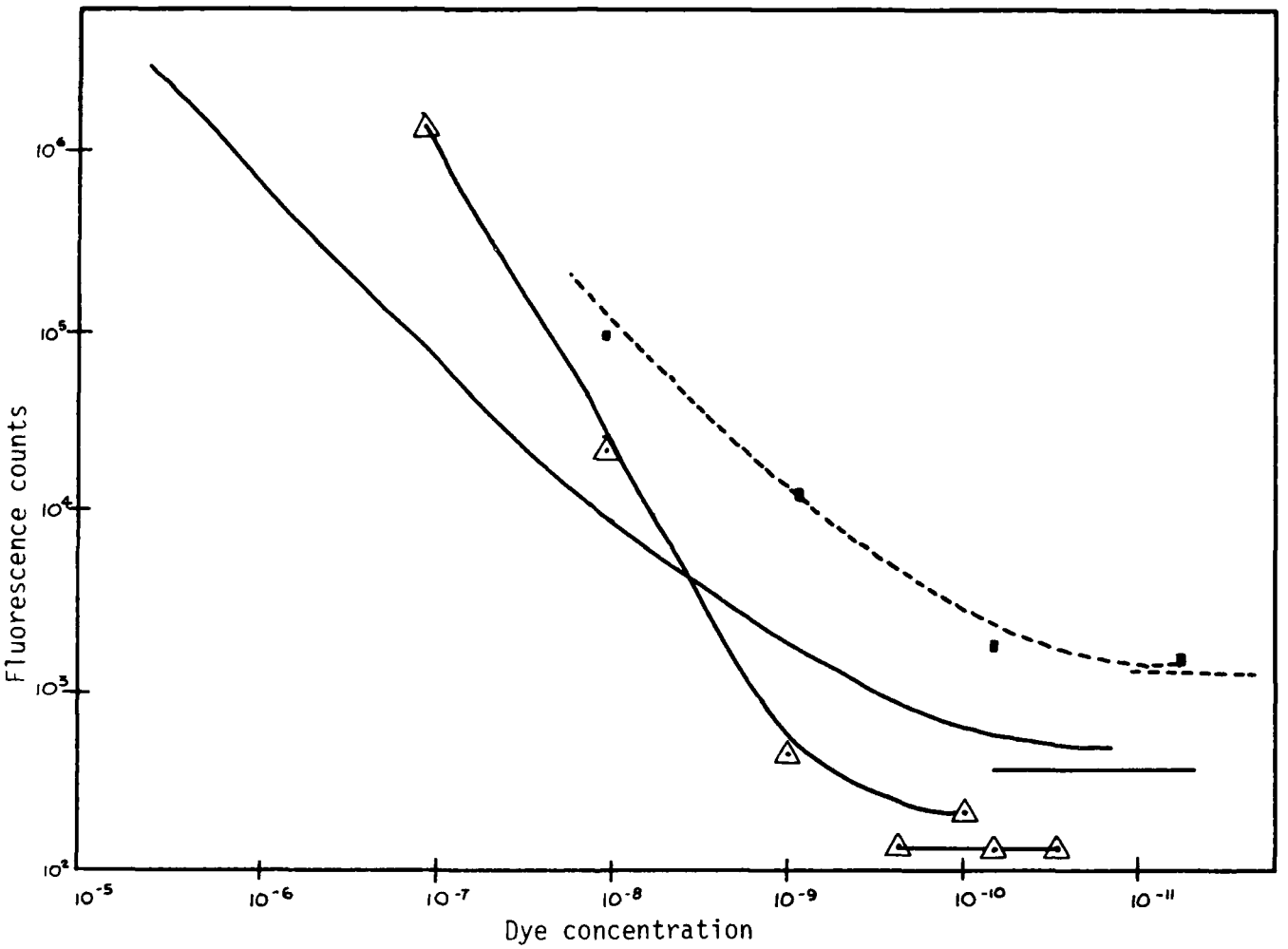


Fig. 13. Plots of fluorescence counts against dye concentrations for fluorescein (---), acetoxypyrenetrisulfonic acid (——), and DiIC3(5)COOH (Δ—Δ) from which the detection sensitivity can be appreciated.

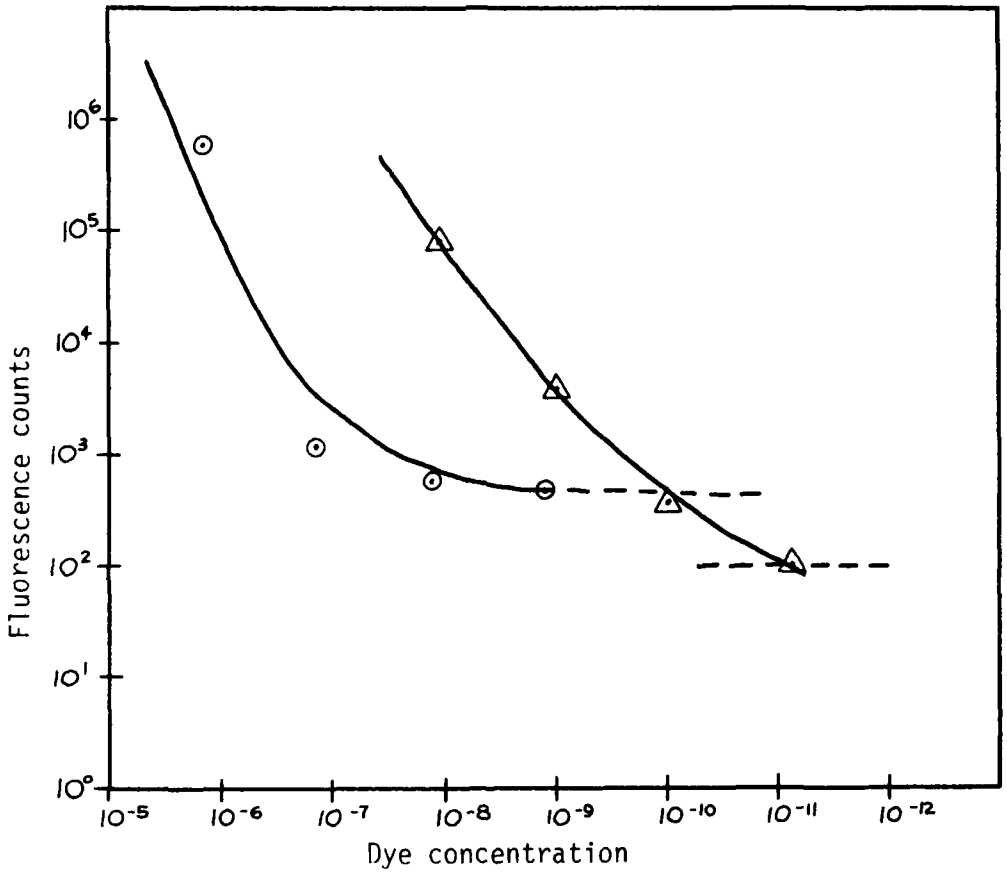
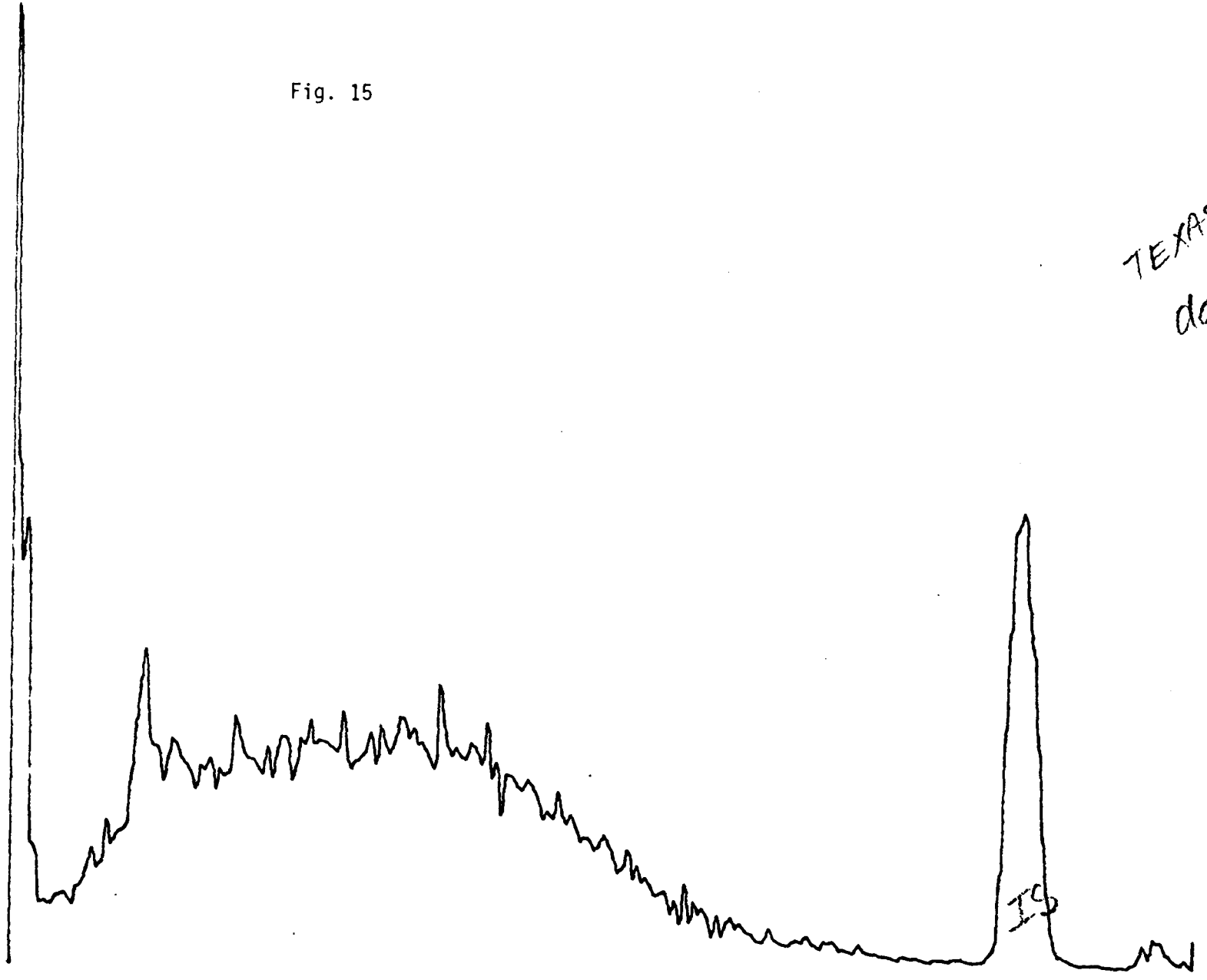


Fig. 14. Plots of fluorescence counts against dye concentrations for fluorescein ( ) and terbium p-amino-dipicolinic acid (Δ).

Fig. 15



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