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An instrument for determination of the polarization of fluorescence

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APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

Stephen W. Flax, Chairman

Jack C. Riley

Wendelin H. Mueller

Robert L. Morris

   This thesis is concerned with the development of a compact, sen-
sitive and reliable instrument for determination of the polarization of
fluorescence of fluorescein tagged molecules in solution. The instru-
ment as designed and constructed differs from others (2,3,4,5,6,8,9).
The difference is that all of its optical system is aligned on a single
axis. This was accomplished by the use of an ellipsoidal mirror posi-
tioned between the excitation source and the phototube fluorescence

signal receiver. In addition, the excitation source, a 45 watt tung-sten-iodide light was electronically regulated to control the intensity.

Two types of determinations were made to verify the performance of the instrument. The first was the absolute sensitivity to varying fluorescein concentrations. Concentrations of fluorescein in 0.1 molar sodium phosphate buffer were prepared ranging from 10^{-6} molar to 10^{-11} molar. After the minimum detectable concentration was determined, five runs on samples of each concentration from the minimum detectable to 10^{-6} were made. The second check was for the sensitivity and linearity of the system in the measurement of polarization. Solutions of glycerol ranging from 62% to 90% were prepared with 10^{-6} molar fluorescein con-centration.

The data from the fluorescein sensitivity and polarization runs were averaged and the averages plotted on linear paper. In each case the instrument exhibited excellent linear response and reasonable stan-dard deviation.

AN INSTRUMENT FOR DETERMINATION

OF THE POLARIZATION OF FLUORESCENCE

by

GEORGE IRWIN JOHNSTON

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
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TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the thesis of

George Irwin Johnston presented December 12, 1978.

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Fred M. Young, Head, Engineering & Applied Sciences

Stanley E. Rauch, Dean of Graduate Studies and Research

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Then there was Jack Riley, also of the PSU Engineering and Applied Science Department, who came to my rescue when I had difficulty sorting out some of the theory of polarization.

Finally, and certainly not least, there is the person who helps you communicate all this information to the outside world. The excellent typing of the manuscript was performed by my office manager, Jeanne Bunch.

To all of you may I say "Many Thanks" for making this a wonderful and rewarding year.

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INTRODUCTION AND HISTORY

Interest in the partial polarization of the fluorescence of macromolecules goes back to Perrin (1) who first published on the subject in 1926. Perrin's theory, based on the assumption that Einstein's equations describing Brownian motion are applicable, focused on the measurement of the rotational relaxation time of molecules as compared to the lifetime of the excited state of the fluorescence. Several workers (11,12,13) in the late 20's through the mid 30's showed the lifetime of the excited state of the fluorescence of many dyes in water solution to be of the order of 10^{-8} seconds, while the relaxation time of the rotation of these molecules in water is much shorter. Thus almost completely depolarized fluorescence was expected and was observed. Weber (2) in 1952 comments that "On the other hand, fluorescent macromolecules, i.e., proteins, should emit partially polarized fluorescence in water solution as their rotational relaxation times are of much larger orders of magnitude. Measurements of the degree of polarization of the fluorescence should in consequence afford a convenient means of determining the relaxation times of macromolecules in dilute solution under a variety of circumstances." Weber further extended the work to include particles of more than one size carrying the same fluorescent tag.

About this same time papers describing applications of Perrin's and Weber's technique began to appear in the literature, e.g., Laurence (3) "A Study of the Adsorption of Dyes on Bovine Serum

Albumin by the Method of Polarization of Fluorescence", McKay (6) "Effect of Various Environments on the Intrinsic Fluorescence Polarization Spectra of Horse Liver Alcohol Dehydrogenase" (1969) and Watson, et.al. (10) "Polarization Fluoroimmunoassay of Gentamicin" (1976) span the period of the 50's to the present day. Coincident with these application papers additional manuscripts highlighting evolutionary instrument development can be found. Weber (4) published another paper in 1956 on a "Photo-electric Method for the Measurement of the Polarization of the Fluorescence of Solutions". This was followed by others, e.g., Johnson and Richards (5), Rosen (7), Lavorel and Vernotte (8), and Kelly and Dandliker (9). These later reports represented considerable improvement on earlier reported instrument techniques. Further refinement of these instrumentation techniques in terms of simplification and improved sensitivity is the subject of this thesis.

THEORY

Perrin (1) developed the theory of the polarization of fluorescence and published it in 1926. The technique is based on the principle that a molecule tagged with a fluorescent dye, when excited by polarized light, will emit fluorescent light having the same plane of polarization as that of the incident light. However, due to Brownian motion, these tagged molecules, when present in solution, are constantly moving and rotating. Since the fluorescence does not occur until the excited electron decays back into its original energy level and the molecule has continued to rotate during this time, the plane of polarization of the incident and emitted light will differ.

Although typical excitation lifetimes of many fluorescent dyes in
water solution are of the order of 10^{-8} seconds, the rotational relaxa-
tion times for small molecules are much shorter than this (2). Thus
the emitted light from small molecules becomes nearly randomly polar-
ized and may be likened to "electronic noise" or depolarized fluores-
cence. This is sometimes referred to as "partial" polarization. As
the molecules become larger and/or the liquid medium becomes more vis-
cous the rotational relaxation time becomes longer. When it becomes of
the order of the fluorescence lifetime, the angular deviation of the
plane of polarization becomes small and the measured fluorescence be-
gins to show a preferential polarization - that of the exciting light.
Since Brownian motion is directly related to temperature, this too must
be considered as a parameter affecting the observed degree of polari-
zation.

An explanation of the technique of measuring polarization is nec-
essary to understand the underlying principle of the apparatus developed.
Partial polarization (p) is defined as:

$$p = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$$

Parallel and perpendicular refer to the alignment between the excita-
tion polarizer and the fluorescence polarizers. $I_{||}$ and $I_{\perp}$ are the
light intensities of the fluorescence measured through polarization
filters oriented both parallel and perpendicular to the excitation fil-
ter. The system is shown in Figure 1. The excitation beam I_1 is ran-
domly polarized as indicated by the numerous electric vectors (E) exist-
ing throughout all 360 degrees normal to the axis of the beam. After
passing through the interference filter (to establish the preferred
excitation wavelength) and the polarizer, the beam is vertically polar-
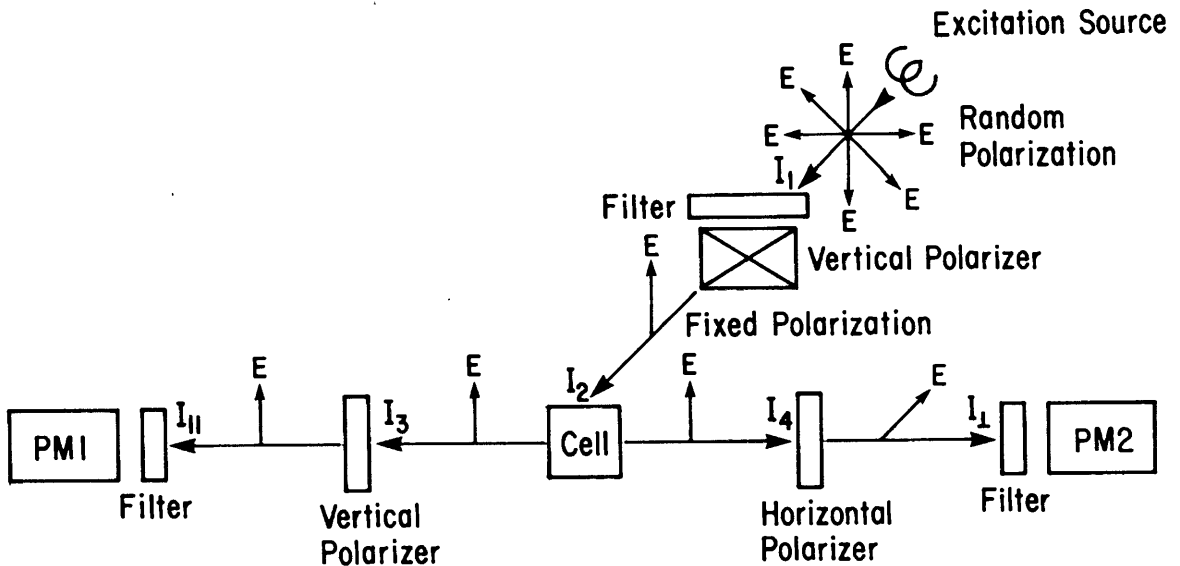
Figure 1. *Block diagram showing generalized system for measurement of polarization of fluorescence. Excitation source (upper right) starts out depolarized; is then filtered for appropriate excitation wavelength and vertically polarized before illuminating sample cell. At right angles to the excitation path are two signal photomultipliers; one looking at the vertically polarized fluorescence signal and the other at the horizontally polarized fluorescence signal. Both fluorescence signals pass through light filters establishing the appropriate fluorescence wavelength.*

ized as shown by the electric vector being normal to the beam and normal to the axis of the fluorescence beams. The two photomultipliers reading the vertical and horizontal components of fluorescence polarization are positioned on an axis perpendicular to the excitation beam. Not only does this minimize any excitation signal getting to the photomultipliers but it establishes the correct relationship between the fluorescence and excitation polarizers. As shown in Figure 1 the fluorescence signals emanating from the left and right of the cell (I_3 and I_4) will be vertically polarized, as indicated by their electric vectors, except for a deviation caused by Brownian motion. I_3 passes through a vertical polarizer and a fluorescence filter (selected for

optimum transmission of the fluorescence wavelength) before impinging

on PM1 as $I_{||}$.

I_4 passes through a horizontal polarizer and an identical fluores-

cence filter before impinging on PM2 as $I_\perp$. If there were no Brownian

motion, $I_{||}$ would be maximum and $I_\perp$ would be zero, yielding a p = 1.

It should also be noted that if the excitation source were horizontally

polarized the fluorescence polarization would be along the axis of the

photodetectors, and no light would be seen except for that arising

from the depolarizing effects of Brownian motion.

PREVIOUS INSTRUMENTAL TECHNIQUES

The system that Weber describes in his 1956 paper (4) measures

the polarization directly (Figure 2). It consists of a high pressure

mercury arc lamp for the excitation source collimated through a series

of lenses. The collimated light passes through an excitation filter

to set the excitation wavelength and then through a polarizer, which

can be rotated between horizontal and vertical polarization, before

finally impinging on the sample cell. At right angles to the excita-

tion source are two photomultiplier receivers on a line with each

other through the sample cell. On one side of the cell is a reference

photomultiplier, fluorescence filter, fixed horizontal polarizer and

variable slit. On the other side of the cell, 180 degrees opposed,

is the signal photomultiplier, fluorescence filter, fixed vertical

polarizer, a variable polarizer and the sample cell. The outputs of

the two photomultipliers go to a null-detector galvanometer for cur-

rent comparison. The measurement technique is as follows:

1. The excitation polarizer is set for horizontal polarization.
    The variable polarizer is aligned with the fixed polarizer on

the signal side. The slit on the reference side is adjusted

until the signals from the two photomultipliers are nulled.
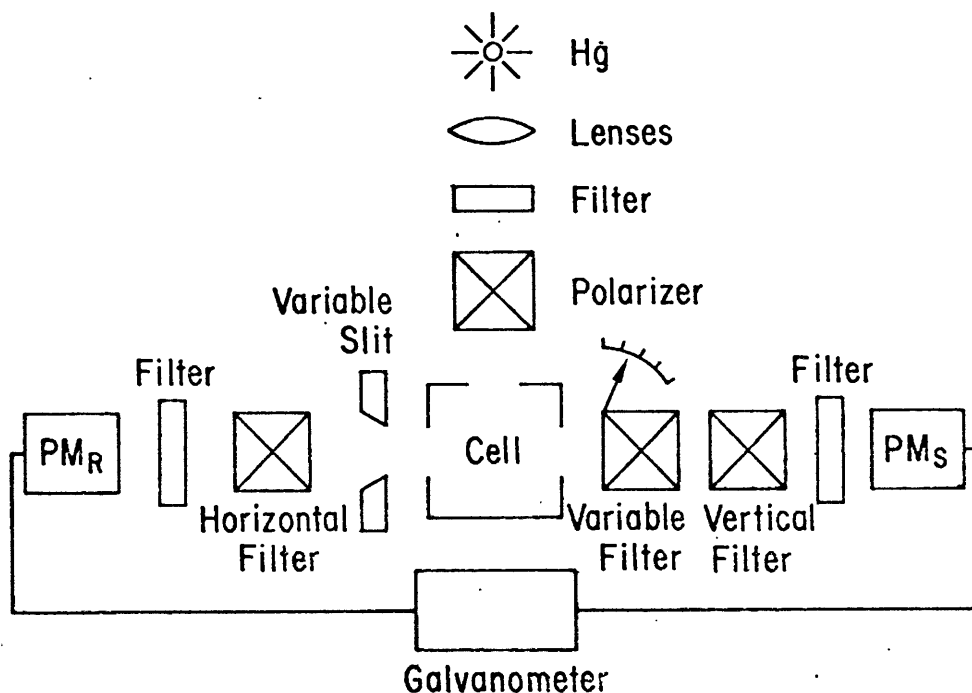
Figure 2. *Block diagram of Weber polarization of fluorescence measuring system which reads the polarization angle directly. The excitation path consists of the mercury source, collimating lens, filter and adjustable (vertical and horizontal) polarizer. The reference signal path to the left consists of a variable slit, horizontal polarizer, filter and photodetector. The polarization signal path to the right consists of a continuously variable polarizer, vertical polarizer, filter and photodetector. The outputs of the two photodetectors are compared by a null galvanometer.*

2. The excitation polarizer is rotated ninety degrees. This

   has the effect of switching the signal beam from horizontal

   polarization to vertical polarization. The reference beam,

   however, consists of either the horizontal polarization sig-

   nal, which is a minimum, or the vertical polarization signal

   attenuated by the horizontal polarizer, also a minimum and

presumably differing from zero only because of the Brownian
motion of the fluorescing molecules, but in either case
approximately of the same magnitude.

3. The variable polarizer is now adjusted to restore the null.

Since the angle of polarization is trigonometrically related to
intensity, one can rebalance the two intensities and directly read the
angle of polarization from the variable polarizer which is calibrated
in minutes of arc. The polarization value is then computed from:

$$p = \frac{\sin^2\theta}{1+\cos^2\theta\cos 2\theta}$$

In 1961 Johnson and Richards (5) reported on a simple instrument
using a single photomultiplier and considerably simplified optics. The
light source was a mercury lamp with a condensing quartz lens. Between
the lens and the sample cell was an excitation filter, polarizer and
slit. At right angles to the sample cell was a fluorescence signal
light path consisting of the cell with mirrored back walls for increased
sensitivity, a fluorescence filter, iris diaphragm, adjustable (zero to
ninety degree positions) polarizer, lens, limiting stop, quartz de-
polarizer and photomultiplier tube. The technique here was to make
separate intensity measurements at the zero and ninety degree positions
of the polarizer in the signal path. Degree of polarization was then
formula determined with the quartz depolarizer eliminating any prefer-
ential polarization sensitivity of the photomultiplier tube.

Two-photomultiplier tube techniques were reported on by Rosen
(7) in 1970 and advertised as a commercial instrument by SLM Instru-
ments of Urbana, Illinois. Here the two ninety degree to excitation
co-axial light paths are used to simultaneously determine vertical
and horizontal polarization intensities. The excitation path as in

previous instruments uses a mercury or xenon light source followed by an excitation filter and fixed polarizer. The two signal paths on a straight line opposed to each other have a fluorescence filter followed by a polarization filter followed in turn by a photomultiplier. In one path the polarizer was parallel to the excitation polarizer and in the other perpendicular to it. Each photomultiplier signal was recorded and degree of polarization calculated by the formula mentioned above. In the SLM Instrument, electronic ratio processing after 10 or 100 (selectable by the operator) samples having been taken provides the polarization factor as a digital display.

McKay (6) in 1969 and Kelly and Dandliker (9) in 1976 added another innovation - that of the rotating polarizer. Here the excitation source was polarized by a fixed polarizer and the fluorescence signal was processed through a rotating polarizer to a single photomultiplier tube. The maximum and minimum signals were taken as $I_{||}$ and $I_{\perp}$ and the polarization value determined by the previously mentioned equation.

Lavorel (8) in 1972 reported a system using a rotating polarizer. However, his rotating polarizer like the system described in this thesis was placed in the excitation path.

DESCRIPTION OF INSTRUMENT

Figure 3 depicts the overall organization of the instrument described in this thesis. Figure 4 is a picture of the actual system. All components of the system are mounted on 5" square aluminum plates keyed on a brass tongue attached to a steel base plate for alignment purposes. Critical portions are further held in alignment by two ¼" diameter drill rods passing through two corners of the plates. The
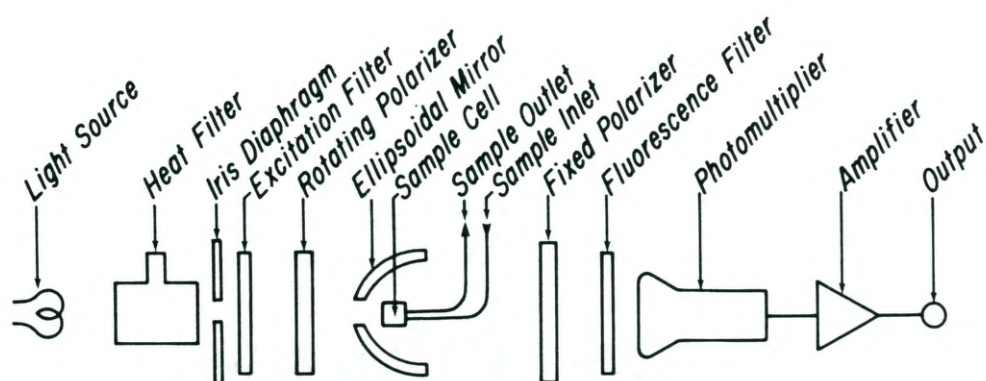
Figure 3. Block diagram of instrument. Complete optical path is on a single axis.
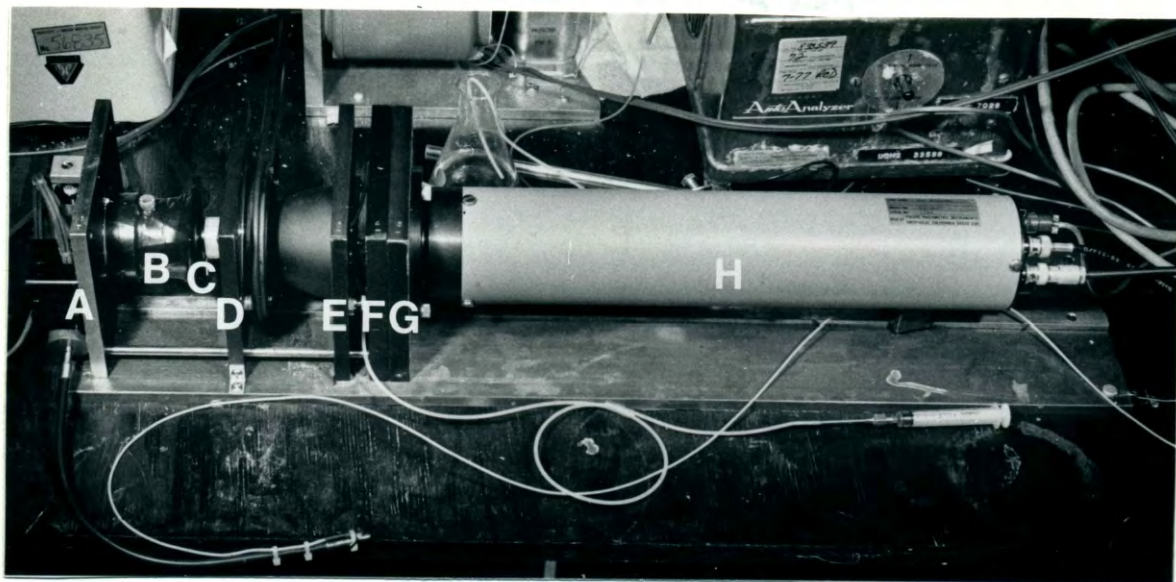

Figure 4. Picture of instrument system. Excitation source (A) is at left and photodetector (H) is at right. Just below the letter (A) is the fiber optic bundle used to isolate the silicon solar cell from the lamp's heat. On the end proximal to the lamp is the 490 nanometer interference filter.

entire assembly is mounted in a four-foot by two-foot by two-foot
lightproof plywood box with an access door on one of the large sides.
The entire inside of the box is painted flat black to minimize reflec-
tions from the light source as is the metal structure of the instrument
itself. Painting of the metal structure was necessary when it was noted
that reflections were contributing to polarization of the light source.

At the left end of the system shown in Figure 4 is the light
source (A) - a 45 watt quartz-iodide lamp (GE Q6.6AT2½/CL) rated at
6.6 amperes. The lamp's intensity is controlled by an electronic
servo loop consisting of a solar cell "looking" at the lamp intensity
and an operational amplifier comparing that intensity to a regulated
voltage. This "looking" provides regulation not only against line
and power supply variations but also against lamp aging characteris-
tics. The solar cell is a Hoffman Electronics custom unit consisting
of a standard boron-doped silicon solar cell of 1/8" diameter and
1/16" active area. To prevent the cell from heating, by being too
close to the lamp source, an eight inch piece of 1/8" fiber optics
was interposed between the lamp and the solar cell. Between the fiber
optic bundle and the lamp another 490 nanometer interference filter
is interposed to assure that the solar cell sees the same wavelength
as the sample cell. Thus regulation is centered on 490 nanometers
rather than the much more abundant infrared.

Figure 5 shows the regulator circuit. A CA3140 FET input opera-
tional amplifier is used in a follower-with-gain configuration to
boost the solar cell signal from approximately 50 mv. to 5 volts.
The high output impedance of the solar cell is effectively buffered
by this amplifier. The 10K potentiometer between pins 1 and 5 of the

LIGHT CONTROL CIRCUIT
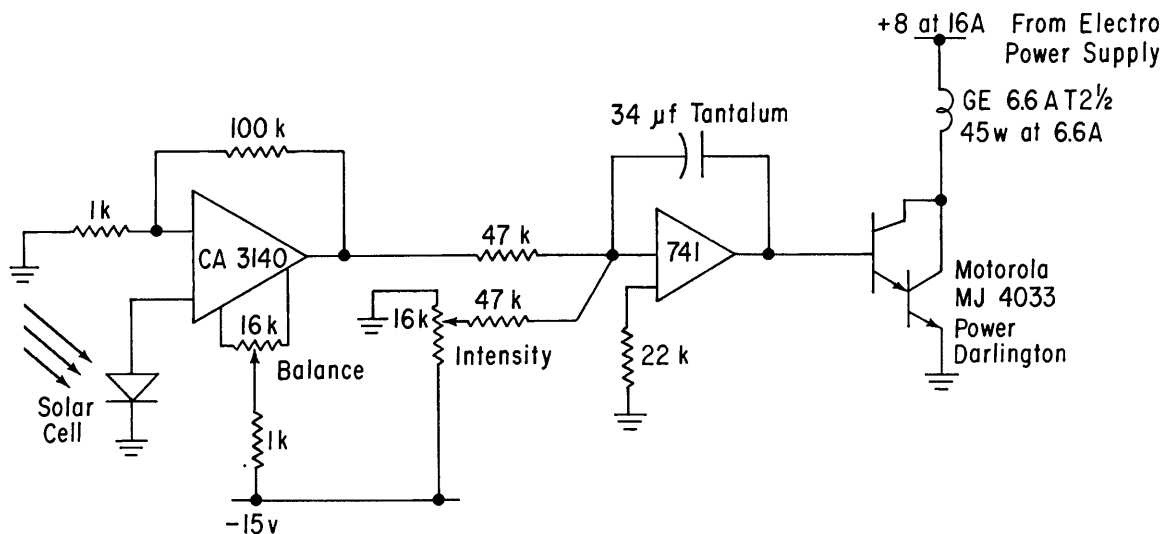
Figure 5. Circuit diagram of lamp intensity control.

CA3140 allows dark current zeroing of the solar cell. Lamp intensity

is controlled by the 741 operational amplifier which is wired as an

integrating comparator. The amplified solar cell signal seen through

the 47K resistor is compared with the reference signal from the 10K

"Intensity" potentiometer seen through another 47K resistor. Any

difference is integrated on the 34 mfd. tantalum capacitor to prevent

changing the light control voltage more rapidly than the lamp can re-

spond - thus preventing oscillation of the lamp control signal. The

741's output is amplified by the Motorola MJ4033 Darlington power

transistor to the appropriate driving current for the lamp (6.6 to

7 amperes). The power supply for the Darlington lamp circuit is a

separate heavy-duty Electro Power Supply designed for battery charging

but possessing filter capacitors for smoothing.

The CA3140 and 741 circuitry are breadboarded on an E&L Instru-

ments Adam System breadboard which is complete with required ±15 volt

power supply. The MJ4033 power Darlington is mounted on a heat sink attached to the rear of the Electro Power Supply cabinet.

Directly in front of the lamp and attached to the opposite side of the lamp plate is a cell with quartz end plates [Figure 4, (B)] filled with a one-molar copper sulphate solution acting as a heat filter. The cell path length is 50 mm and the diameter 46 mm giving a solution volume of 83 ml. Originally a smaller cell was tried with a solution volume of only 6.3 ml. The heat of the lamp quickly brought this small volume to a boil. Attached to the right front of the heat filter (as seen in Figure 4) is an adjustable iris diaphragm (C) which allows for stopping down the light to only that needed to fill the mirror aperture, thus minimizing stray light in the system. Tests showed that there was an excess of stray light from the source getting into the photomultiplier and to reduce this light a hood was installed over the light source and the heat filter cell was wrapped with black tape.

The next 5" plate to the right (D) houses the excitation filter and the rotating polarizer. The excitation filter is a Corian multilayer oxide interference filter (SS4900) transmitting at 490 nanometers. Peak transmission is 50% with a 10 nanometer half bandwidth. Upper and lower blocking regions have a transmission of $1x10^{-4}$. The polarizer is a 2"x2" piece of plastic film available from the Polaroid Division as type HNP'B Linear Polarizer for ultraviolet radiation. At 490 nanometers it has a transmittance of approximately 0.6 and at 550 nanometers a transmittance of approximately 0.4. In the blocking orientation these transmittances are approximately $3x10^{-5}$ and $2x20^{-5}$ respectively. Rotation is accomplished by a Superior Electric Slo-

Syn motor driving the rotating member of the 5" plate via an O-ring belt. The 72 rpm speed of the motor is stepped down to approximately 0.5 rpm for the rotating polarizer.

The third plate to the right has the ellipsoidal mirror (E) attached to it. This is a Melles Griot product number 02 REM 001 mirror. It has the property of reflecting light originating at the primary focus within the mirror and refocusing it at the secondary focus without altering the polarization. There was some concern about the metallized ellipsoidal surface of the mirror altering the polarization angle. According to Strong (14) the reflection of polarized light from plane dielectric reflecting surfaces is only altered by a 180 degree phase shift. In the case of metallized reflecting surfaces such as the ellipsoidal mirror, which is Rhodium coated, Jenkins and White (15) point out that such surfaces produce an ellipsoidal polarization consisting of p and s components. Because of the symmetry of the ellipsoidal mirror, however, the s components cancel out leaving only the p component with a 180 degree phase shift much as in dielectric reflectors. The mirror has an overall length of 58 mm and a 12.7 mm aperture at its rear to admit the excitation light. The front face of the sample cell is mounted at the primary focus which is 12.7 mm back from the aperture. Details of the mirror are given in Figure 6, a reproduction of the data sheet.

Details of the sample cell are given in Figure 7 and Figure 8. It consists of a 304 stainless steel cylinder with O-rings around its body to hold a glass cup in place. The end of the glass cup is a quartz window cemented to the cup body which is made from glass tubing. With the cup in place there is a 1 mm gap between the front of the stainless steel body and the rear of the cup window forming a

ELLIPSOIDAL REFLECTORS
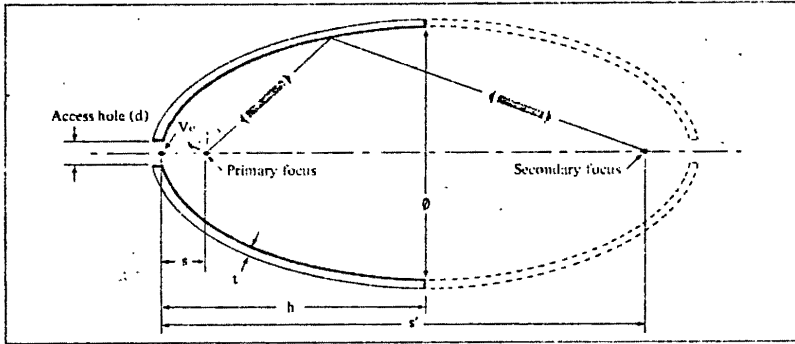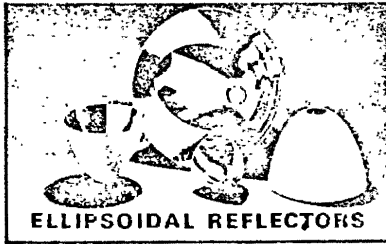
ELLIPSOIDAL REFLECTOR. Rays passing through either focus must, after reflection, also pass through the other. The foci are conjugate points, and the imaging is stigmatic. Stigmatic conjugate distances s and s' are tabulated (without regard to any sign convention) below. These are often called focal lengths. The positions of the foci are best determined experimentally.

SPECIFICATIONS: ELLIPSOIDAL REFLECTORS

s, t, and d: ± 0.13mm   θ: ± .25mm

h, (θ + 2t): ± 0.40mm   s: ± 0.80mm

Maximum separation of optical and mechanical axes at vertex: ± 0.13mm

Maximum angular deviation of reflected ray from ideal path: 2 minutes of arc

Maximum Operating Temperature: 260°C (500°F)

Reflectance: See table for rhodium earlier in this section for nominal values. Electroformed coating reflectances may be a few percent lower than these published values, which were determined under ideal conditions for vacuum-deposited films.

Coating durability: Bright rhodium meets or exceeds all applicable military and DIN specifications.

ELLIPSOIDAL REFLECTORS

s (mm)	θ (mm)	h (mm)	s' (mm)	s'-s (mm)	d (mm)	t (mm)	Ω (spheres)	PRODUCT NUMBER
12.7	75	58.0	102	88.9	12.7	.51	.85	02 REM 001
12.7	75	57.2	102	88.9	25.4	.51	.68	02 REM 005
*20.6	143	105.0	241	221.0	31.8	.58	.76	02 REM 011
33.8	254	184.0	508	474.0	50.8	.89	.76	02 REM 016
33.8	254	183.0	508	474.0	57.2	.89	.74	02 REM 023
33.8	254	181.0	508	474.0	63.5	.89	.71	02 REM 028

* Note: This is not a true ellipsoid. Its design has been deliberately altered to achieve uniform illumination of Super 8mm (5.31mm x 4.01mm) film gate.

MELLES GRIOT   77

Figure 6. Copy of data sheet showing details of ellipsoidal mirror. Mirror used is product number 02 REM 001.
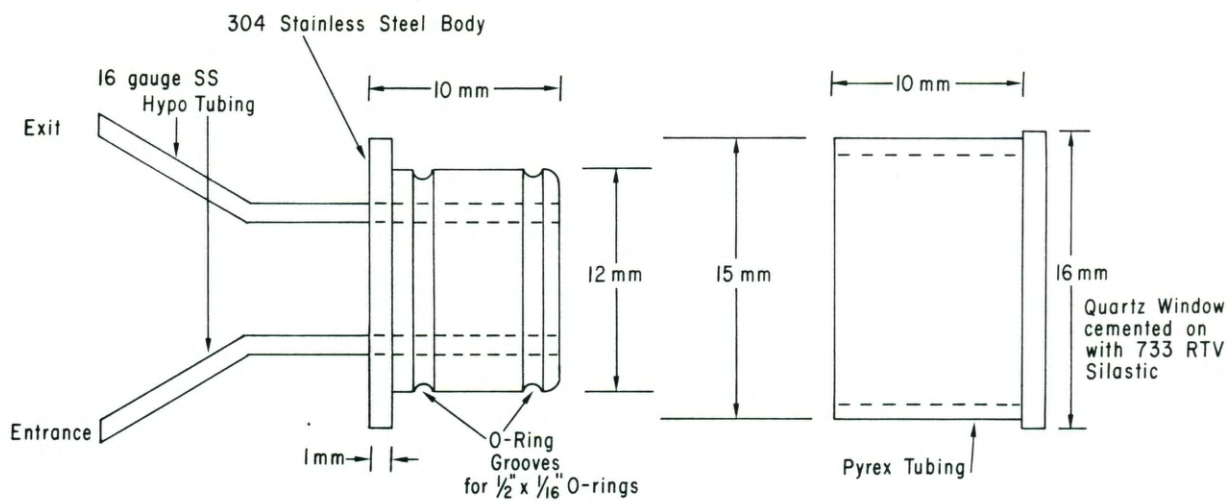
Figure 7. Block diagram of sample cell.

Figure 8. Picture of sample cell.

250 µl. sample space. The sample is injected through a port at the bottom and removed through a port at the top. Connection to these two ports is by 16 gage hypodermic tubing projecting from the rear of the stainless body. A 6-32 brass stud projects from the center of the stainless body and is attached to a plastic bar. This bar is then attached by thumbscrews to the 5" plate to mount the cell in position. The 6-32 screw allows positioning of the cup face at the primary focus.

The fourth (F) and fifth (G) plates are bolted together as an assembly. The fourth plate contains the fixed polarizer of the same size and material as the rotating polarizer. The fifth plate has the photomultiplier and fluorescence filter (H) attached to it. The fluorescence filter is also a Corian interference filter (SS5500) transmitting at 550 nanometers and having blocking and transmitting characteristics similar to the SS4900. It is installed in the photo-multiplier housing. The photomultiplier detector is a Pacific Photo-metric Instruments Model 62/3A27 with eleven current ranges from 10^{-6} through 10^{-11} amperes in a 10-3-1 sequence and a built-in amplifier. The PM tube itself is an EMI 9635.

The built-in photomultiplier amplifier is an operational ampli-fier in a current-to-voltage conversion circuit. It also has five ranges of offset capability to allow cancellation of dark current and ambient light, thus allowing full dynamic signal range. A Pacific Photometric Instruments Model 225 zero to two kilovolt power supply provides high voltage to the photomultiplier tube and an existing dual low voltage laboratory supply provides the ±15 volts for the tube's amplifier section.

DISTINGUISHING FEATURES

Previously reported systems (4,5,6,8,9) were reviewed before the development of the present system. It was felt that a simpler, more sensitive, more reliable and more easily used system could be developed utilizing modern technology. The several objectives to be achieved were: simplicity, reliability, ease of use and suitability for packaging into a compact, efficient laboratory instrument. Sensitivity is still limited and ease of use can be enhanced even further with the addition of a microprocessor for signal processing. The distinguishing features of the present system are:

1. A 45 watt quartz-iodide tungsten light source.

2. Electronic regulation of the light source by direct monitoring of the light through an interference filter and solar cell combination.

3. Polarization shift by rotation of the excitation polarizer.

4. Enhancement of sensitivity by utilizing an ellipsoidal mirror to reflect the fluorescence signal to the photomultiplier.

5. Use of a fixed fluorescence polarizer in front of the photomultiplier which eliminates the effect of preferential polarization of the photomultiplier.

6. The use of film type UV polarizers utilizing much less space than more expensive crystal polarizers.

7. The use of a small, flow-through sample cell assembly requiring less than 250 μl. of sample. Samples are injected directly by syringe.

8. The instrument is a small (less than four feet long)

assembly with a colinear light path. The basic system is 5" x 5" square. Even with the required power supplies and light regulator it can easily be packaged into an efficient assembly requiring less laboratory space than many conventional instruments.

9. The system is designed to have maximum light bandwidth and at the same time maximum optical simplicity. The light source has reasonable output down to 320 nanometers and of course extends into the infrared region. A copper sulphate heat filter with quartz end windows eliminates much of the infrared while passing light into the UV region. Collimating and focusing optics which would have to be quartz for passing UV signals are eliminated. Only the iris diaphragm is included to eliminate excess stray light and hence "noise" in the system. The polarization filters are specified to transmit down to 275 nanometers. Also the ellipsoidal mirror, which is a Rhodium plated front surface mirror, is specified to have an absolutely flat 80% reflectance from 375 nanometers to 700 nanometers.

Taking the distinguishing features of the system by number and in order, comments on the system are:

1. The 45 watt quartz-iodide tungsten light source has the advantage of minimizing the problems of size and heat. It is also easier to regulate than mercury-arc and xenon pressure sources. However, the relatively low intensity compared to reported systems with 150 watt and 450 watt mercury and xenon light sources inhibits sensitivty despite

the gains made by the use of the ellipsoidal mirror. Later development to improve sensitivity may require a higher wattage light source if existing signal to noise ratio cannot be improved.

2. Electronic regulation of a low voltage tungsten lamp is quite easy. However, if it becomes necessary to utilize higher wattage mercury or xenon sources the electronic regulation and the power supply will become more sophisticated and bulky.

4. The ellipsoidal mirror utilized in a colinear optical path improves the sensitivity of the system by capturing more fluorescence signal. However, it will be shown in the test results that surface reflections of the excitation light from the front face of the sample cell do contribute to a poor signal to noise ratio. An anti-reflective coating for the quartz window will be sought to reduce this problem.

3,5. Placing the rotating polarizer in front of the excitation source and the fixed polarizer in front of the photomultiplier eliminates any polarization preference of the photomultiplier. However, it also assumes that polarization of the light source is zero or minimal - not an entirely valid assumption. Some polarization of the light source exists (see TEST RESULTS). However, the magnitude of the polarization is small compared to the noise signal and its resulting polarization signal which exists in the basis system. Some of the ambient noise signal comes from the 4% surface reflection of the sample cell.

6. The polarization material is Polaroid's HNP'B linear polar-
   izer film suitable for use down to 275 nanometers. This
   material is much less expensive and bulky than Glan-Thomson
   prisms and other crystal polarizers.

7. The sample cell is ideal for volume work such as that of the
   Clinical Pathology Laboratory at the University of Oregon
   Health Sciences Center. It requires minimal volume (less
   than 250 µl.) and the samples clear readily when fresh in-
   jections from a syringe are made (see TEST RESULTS). How-
   ever, sample temperature control is very important - to
   within 0.1 or 0.2 degrees C. Two methods are under consid-
   eration for achieving this: 1) Modification of the back
   of the sample cell to incorporate a water jacket and pumping
   temperature stabilizing water into this jacket or 2) Addi-
   tion of a thermoelectric heat pump to the back of the cell.
   The latter is preferred for simplicity and efficiency but
   the cost of acquiring such a unit specifically designed for
   this configuration might be prohibitive.

8. The original goal was to develop a system which was small
   and efficient; the arrangement of the colinear light path
   employing an ellipsoidal mirror served these purposes. Ad-
   dition of the required motor drive for the rotating polar-
   izer, power supplies for the photomultiplier and electronic
   drive for the lamp do not compromise the original concept.
   Readout, whether it be through oscilloscope (as present) or
   through microprocessor and digital printout (anticipated for
   further development) do not compromise this basic package
   as they can be quite small and efficient auxiliary packages.

9. Elimination of focusing and collimating lenses considerably simplifies the system and allows for maximum light bandwidth. The bandwidth becomes important when excitation wavelengths are required below 400 nanometers. Fluorescence wavelengths are not required in this region, but the focusing characteristics of the ellipsoidal mirror negate the requirements for focusing lenses and simplify the optical system.

METHODS AND TECHNIQUES

Several types of tests were conducted to verify the operating characteristics of the instrument. The first test was to measure the instrument's sensitivity to fluorescein concentration and its linearity of response to varying concentrations. Solutions of fluorescein in 0.1 molar sodium phosphate buffer were prepared. Concentrations were prepared in decade steps from 10^{-6} molar to 10^{-11} molar. These samples were run with the rear polarizer removed and the front polarizer stationary but rotated for maximum response (an indication that some polarization existed in the light source). The photodetector signal was displayed on a Tektronix 7633 storage oscilloscope using a 7A22 preamplifier. Pictures of the signal response, stored on the oscilloscope screen, were taken with a Tektronix C-12 camera. The vertical sensitivity and horizontal sweep speed appear at the top of each picture. More than one photomultiplier sensitivity setting was used during each run. To accomodate this the vertical sensitivity of the oscilloscope was multiplied by a factor of one at the highest photomultiplier setting. When the photomultiplier sensitivity was decreased it was decreased by a factor of ten. To accomodate this the vertical oscilloscope sensitivity was multiplied by a factor of ten for proper scaling.

This gave voltage readings ranging up to 45 volts even though the output swing of the photodetector system was limited to 15 volts. However, the scaling of the actual light intensity was correct. During each oscilloscope trace the photomultiplier high voltage was turned off momentarily half-way through the sweep. This allowed verification of zero during each trace and made later reading easier because the scale markings in the center of the screen were not obliterated by the trace.

The first sample of each series was a 0.1 normal hydrochloric acid wash. This was to wash out any previously left contamination. Each sample consisted of an injection of approximately 6 milliters of fluid to guarantee adequate clearance of the previous sample in the 250 microliter chamber plus the volume of the fill and exhaust tubing. Since acid inhibits fluorescence it also gave a system reading on the basic ambient "noise" level. Each acid injection was followed by an injection of buffer consisting of 0.1 molar sodium phosphate with no fluorescein. This washed out the acid and eliminated the possibility of fluorescein inhibition by the acid. Then the range of samples were injected in increasing order of concentration to minimize possible errors from "tailings" of the previous solution, even though earlier tests with injected food dyes did indicate total clearance with 6 milliters of solution.

Since the basic ambient light level of the system (comparable to electronic "noise" level) was so high it restricted the minimum detectable fluorescent level to a concentration of 10^{-8} molar. An attempt was made to determine the major source of the stray light. One source was reflections from the front surface of the sample cell window. These would again be reflected by the ellipsoidal mirror to the photodetector. Black velour paper was used to cover the front surface of the cell and

the ambient light level measured. The signal under these conditions was compared with that from the uncovered cell filled with an acid solution possessing no fluorescence. Figure 9 shows the results. The top trace is with the cell uncovered and a non-fluorescing acid solution in the chamber. The bottom trace is with black velour paper covering the front window of the cell. As seen from the figure the black paper provided
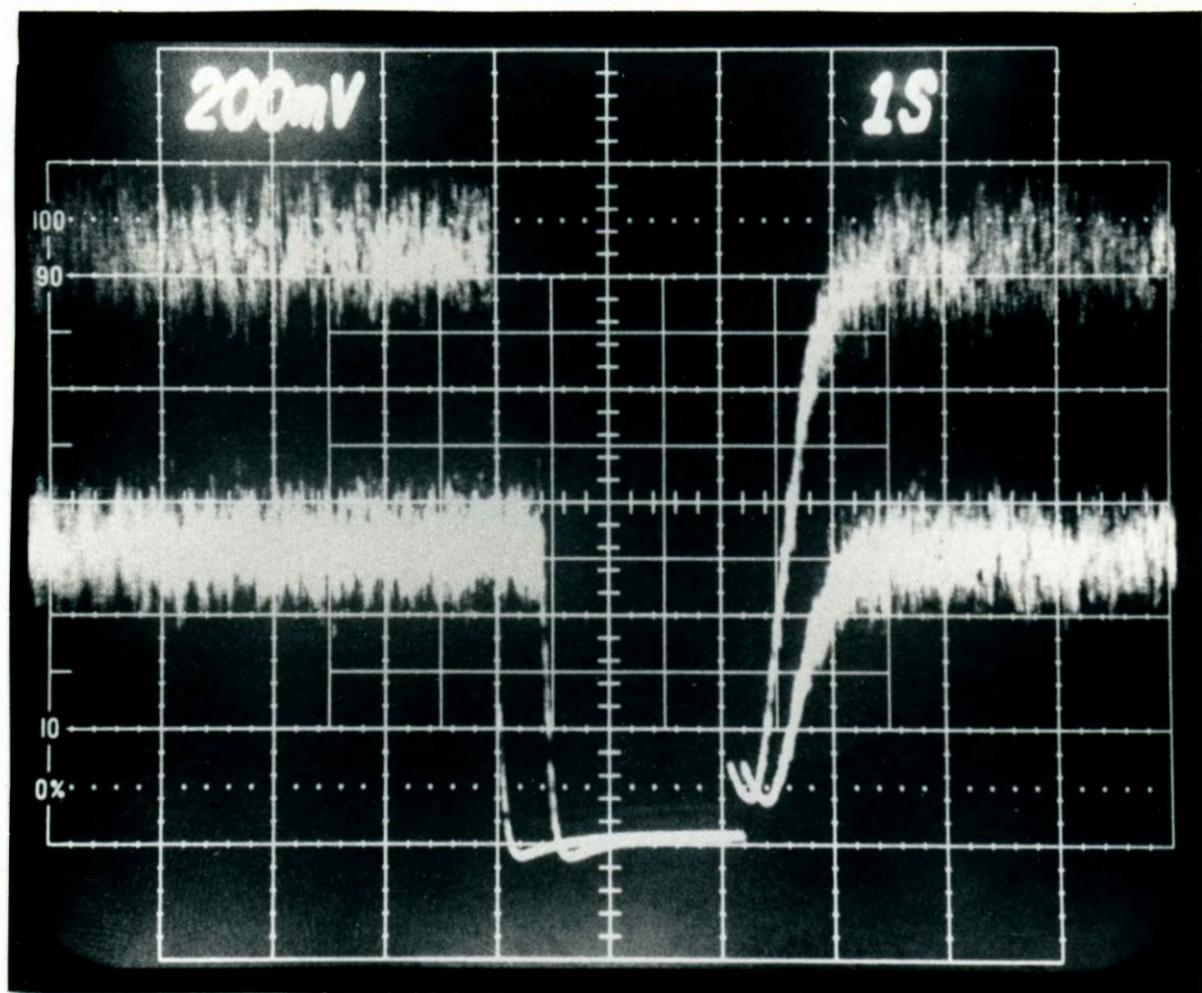
Figure 9. Oscilloscope tracing of system ambient light levels. Upper trace is with cell in normal operation filled with 0.1 molar hydrochloric acid to inhibit fluorescence. Lower trace is with front face of cell covered with black velour paper. Difference between two indicates magnitude of cell front surface reflection of excitation light. Signal zero is 1 cm. from bottom of screen and verified by momentarily turning off photomultiplier high voltage half-way through sweep.

an almost 50% reduction in ambient light. These tests were made with a PM sensitivity setting of 10^{-7}A. The excitation lamp current was 7A., a value used throughout the experiments.

After the sensitivity and linearity of the system to varying fluorescein concentrations were determined, the system was checked for its ability to measure polarization. Solutions of glycerol ranging from 62% glycerol to 90% glycerol were prepared with 1×10^{-6} molar fluorescein concentration. This high a fluorescein concentration guaranteed a good signal to noise ratio. The rear polarizer was reinstalled and the motor rotating the front polarizer was turned on. The previously described sequence of an acid wash followed by a buffer wash followed by the samples in ascending order of viscosity was followed for each run. Pictures were taken of the oscilloscope signal for each sample point for later analysis. The rotating polarizer produced a sinusoidal signal in time rather than the constant signal obtained in the straight fluorescein sensitivity runs. Values for polarization were calculated from:

$$\frac{I_{max} - I_{min}}{I_{max} + I_{min}}.$$

Scaling was done as before with the highest photomultiplier sensitivity used giving a factor of unity to the oscilloscope's vertical amplifier and each x10 decrease in photomultiplier sensitivity giving a x10 increase to the vertical amplifier sensitivity. Only two photomultiplier ranges were used, 10^{-8}A and 10^{-7}A.

To reproduce the curve of 1/p vs T_K^0/η as in Lavorel (8) it was necessary to calculate the viscosity, η, in poise of the standard solution used. This required the use of a viscometer to determine the time of a calibrated volume to run through a calibrated orifice. Then a pycnometer was used to determine the solution density by weighing a

calibrated volume. These two readings allowed the calculation of η by:

$$\frac{\eta_1}{\eta_2} = \frac{\rho_1 t_1}{\rho_2 t_2}.$$

The t's are the viscometer times and the ρ's are the pycnometer den-
sities. Subscript (1) is for the glycerols and subscript (2) is for
water.

Temperature instability was a problem throughout the experiments.
No provision was made to control the temperature of the sample cell
at this stage of development because of the mechanical complexity in-
volved. All work was done at room temperature as rapidly as possible
to minimize any temperature change during a run. The temperature was
recorded at the beginning and end of each run and typically varied only
two or three degrees centigrade. Nevertheless it is certain that this
affected the scatter of the data somewhat as even water has a change in
viscosity of the order of $1\%/^{\circ}C$. Also the fluorescence in fluorescein
is temperature dependent.

TEST RESULTS

Several tests were performed to assess the performance charac-
teristics and sensitivity of the system. The first was a fluorescein
sensitivity test to determine the linearity of the response and the
ultimate sensitivity of the system. Concentrations of fluorescein in
phosphate buffer were made ranging from 10^{-11} molar to 10^{-5} molar in
decade steps. The voltage output of the photomultiplier was read on
a Tektronix 7633 storage oscilloscope using a 7A22 plug-in amplifier.
The sweep speed used was 1 sec./cm. throughout the tests. The first
detectable response was at a concentration of 10^{-8} molar. It was

found that system sensitivity is not limited by instrument sensitivity but rather by system "noise" - stray light and reflections in the system. The lamp was maintained at 7 amperes. The photomultiplier was operated with 1.5 kV on the dynodes at a sensitivity setting of 10^{-8}A. Thus three more decades of photomultiplier sensitivity were available and the dynodes could still be raised to 2 kV. The other part of this test was for linearity of response to varying concentrations of fluorescein. Concentrations of fluorescein in buffer were prepared ranging from 0.1×10^{-6} to 0.9×10^{-6}. Five runs were made and indeed the system did exhibit excellent linearity. The average of these runs are shown in the plot of Figure 10 and a table of the results including the average and standard deviation for each point is shown in Figure 11. All runs were done with the rear polarizer removed and the front polarizer set for maximum response. The fact that there was a variation in response with the angular position of the front polarizer indicated that there was a slight polarization of the light source. This fact was also evident by the value of the (p) numbers when the fluorescein in glycerol concentrations were run in later tests. Each run was preceded and ended by an 0.1N HCl wash to establish a "noise" baseline and system reproducibility as well as cleaning out the system. This was followed by a buffer wash to eliminate the acid residue which would inhibit fluorescence. The system baseline signal ranged between 500 and 600 mV. whereas the fluorescein signal ranged between 4.0 and 45 volts. Rotation of the front polarizer produced no discernable polarization of the acid "noise" signal. Occasionally there were obvious "bad" data points characterized by low response. These were determined to arise from bubbles in
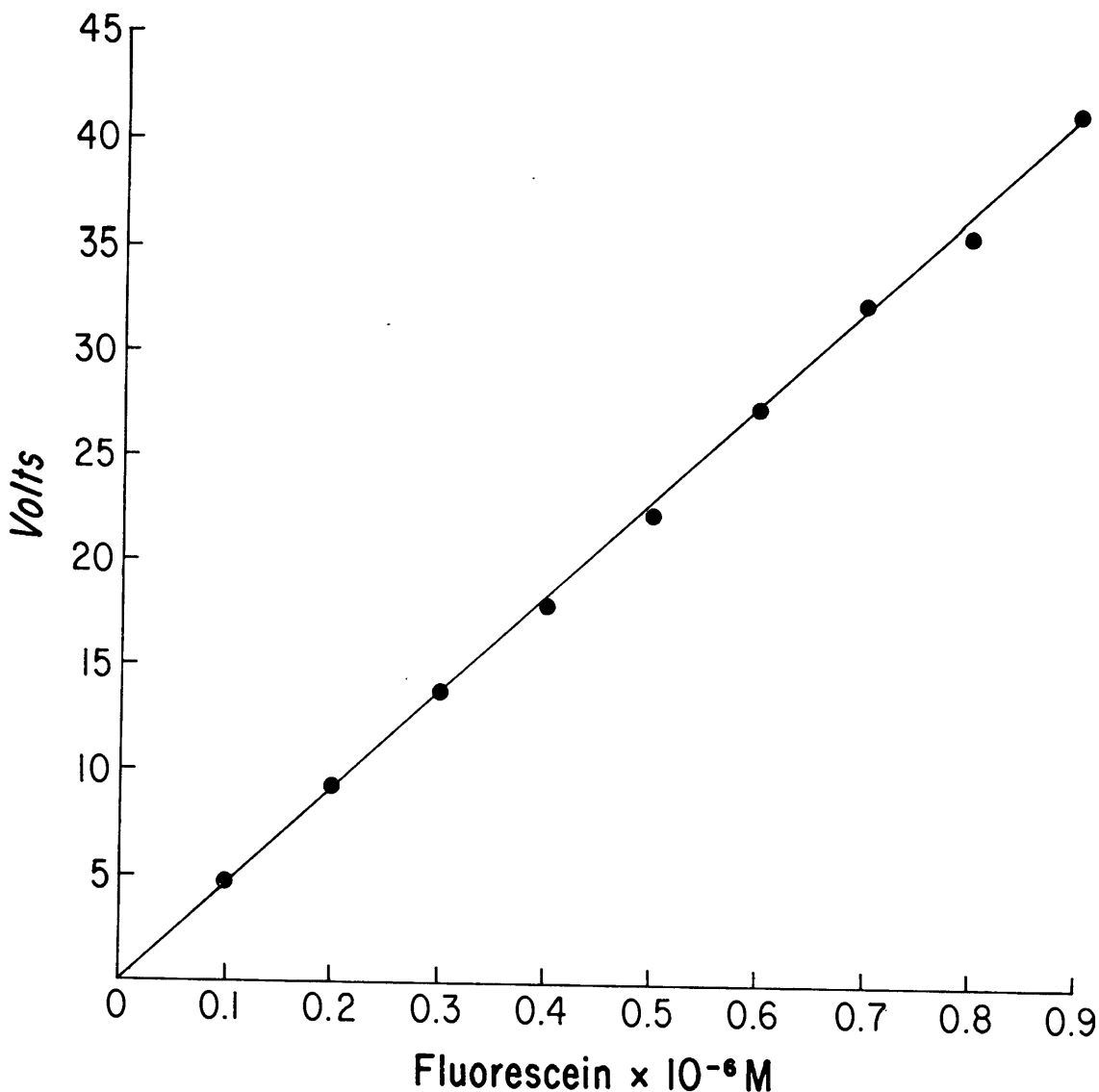
Figure 10. Plot of the averages of five runs of fluorescein concentration versus fluorescence signal response in volts.

the sample cell. Reinjection of the sample solution always cleared the bubble and gave an appropriate result.

Tests were made of the linearity of the polarization (p) response and compared with results of a similar experiment by Lavorel (8). A 10^{-6} molar fluorescein concentration in various concentrations of glycerol in buffer were prepared. Eight different concentrations ranging from 60% glycerol to 90% glycerol were prepared covering the

CONCENTRATION	F_1	F_2	F_3	F_4	F_5	$\bar{x}$	s
0.1×10^{-6}	4.0	4.5	5.4	4.3	5.7	4.78	0.73
0.2×10^{-6}	9.6	9.6	10.0	7.6	10.4	9.44	1.08
0.3×10^{-6}	13.4	15.0	14.0	11.2	15.5	13.82	1.68
0.4×10^{-6}	15.0	19.0	18.0	16.5	21.0	17.90	2.30
0.5×10^{-6}	21.5	23.5	22.5	20.5	23.5	22.30	1.30
0.6×10^{-6}	24.5	29.0	26.5	28.0	29.0	27.40	1.92
0.7×10^{-6}	30.0	33.5	31.0	34.0	34.0	32.50	1.87
0.8×10^{-6}	34.0	37.0	35.0	40.0	39.0	37.00	2.55
0.9×10^{-6}	38.0	43.0	40.0	45.0	41.0	41.40	2.70

Figure 11. Table of fluorescence signal responses (in volts) versus concentration (molar) for five runs. The average and standard deviations are shown in the last two columns to the right.

range indicated by Lavorel. Again five runs were made and again the system did exhibit excellent linearity of response. Figure 12 shows a typical oscilloscope tracing of the signal with the polarizer rotating. $I_{||}$ is the signal maximum and $I_{\perp}$ is the signal minimum. Zero is one division above the bottom of the screen. 1/p was plotted against T/η and the average of the responses shown in Figure 13. T is in degrees Kelvin and η is poise. Figure 14 is a table of the data collected with the average and standard deviations shown.

A 10^{-6} molar fluorescein concentration was picked to agree with the samples used by Lavorel. This also provides a healthy photomultiplier signal with good signal-to-noise ratio. The values of (p) were somewhat smaller than those reported by Lavorel. Thus where Lavorel's graph of (1/p) runs from 0 to 5, Figure 13's abscissa runs from 0 to 18. This arises from the presence of horizontal polarization of the light source mentioned before. This polarization yields a higher than normal $I_{\perp}$ making (p) somewhat smaller than it should be. Nevertheless this constant offset of $I_{\perp}$ does not destroy the linearity of the system response.

Early in the data collection stage attempts were made to determine the effect of temperature on fluorescein sensitivity and polarization values. Within the limits of variability of room temperature (22 to 25 degrees C.) there was no observable effect on readings. Data runs were made quickly enough that system temperature did not vary more than 0.5 degrees C.
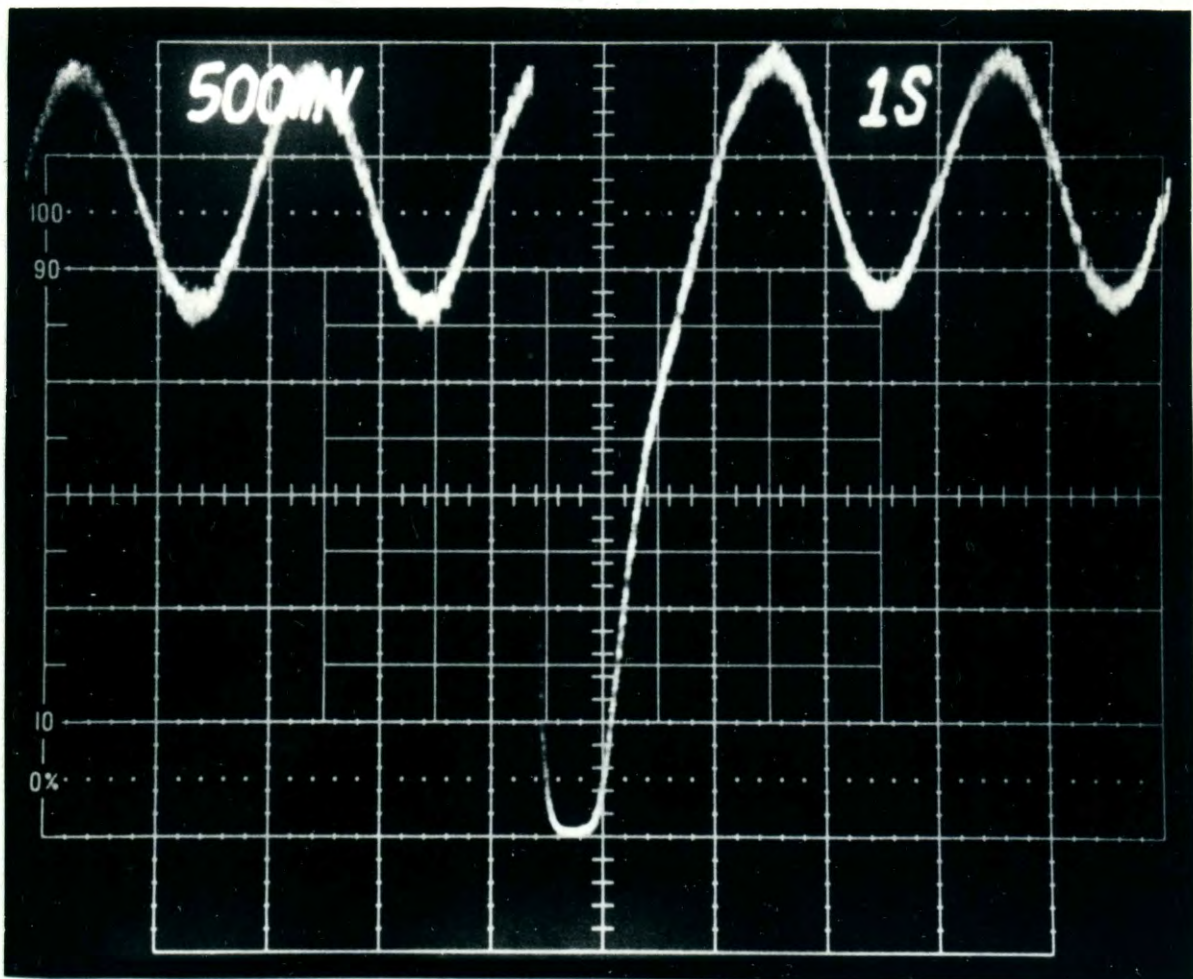

Figure 12. Typical oscilloscope tracing of the polarization signal. $I_{||}$ is taken as the maximum of the sinusoidal signal and $I_{\perp}$ as the minimum. Signal zero is 1 cm. from the bottom of the screen and verified by momentarily turning off photomultiplier high voltage half-way through sweep.
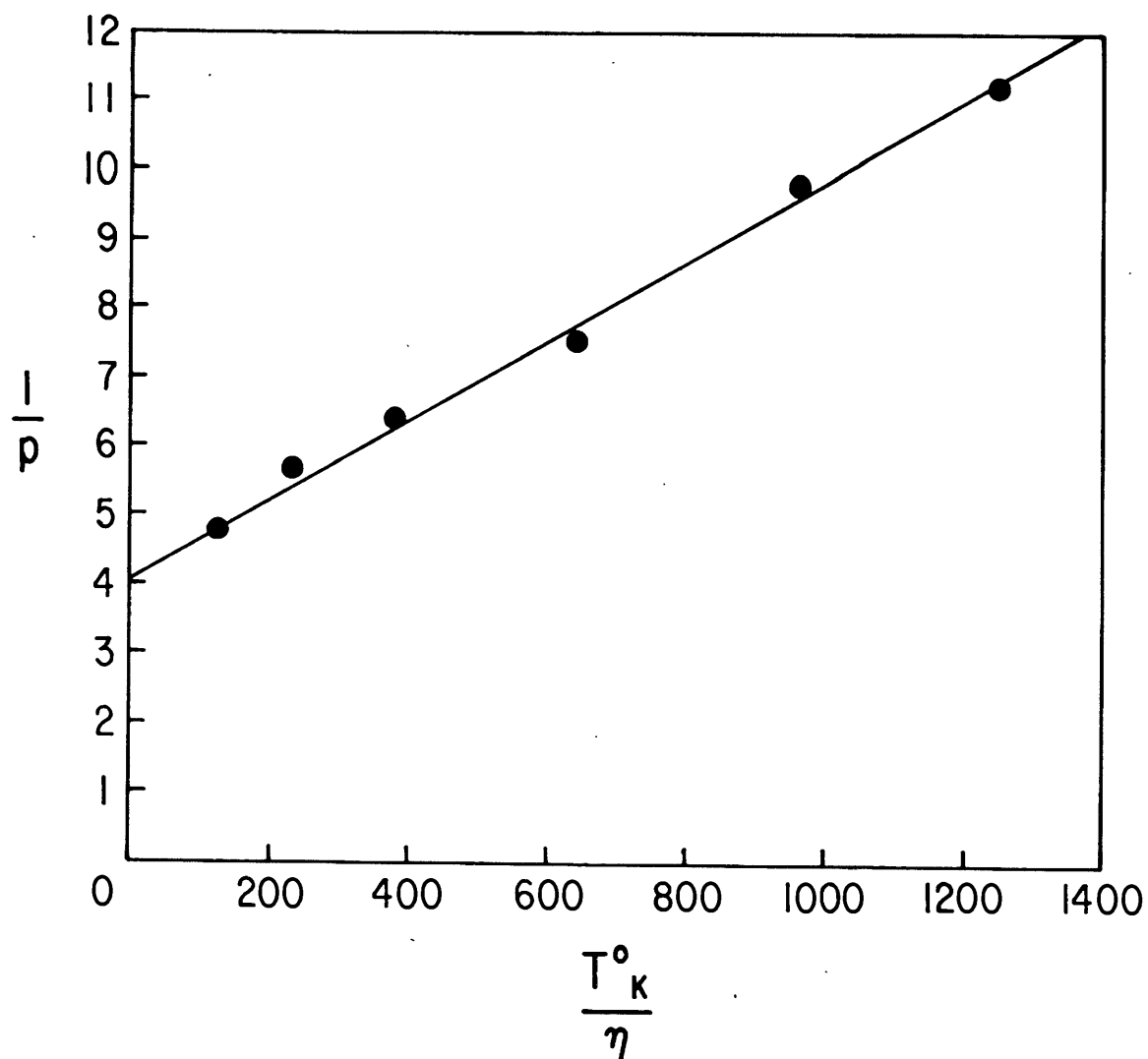
Figure 13. *Plot for fluorescein (10⁻⁶M) in various concentrations of glycerol at 25.5°C. T/η is in °K per poise.*

% GLYCEROL	P_1	P_2	P_3	P_4	P_5	$\bar{x}$	s	$1/\bar{x}$	η	$T^o_{K/\eta}$
67%	.0909	.0909	.0833	.0957	.0826	.0887	.0056	11.2740	.2390	1249.62
70%	.1053	.1071	.0909	.1008	.1057	.1020	.0066	9.8039	.3096	964.66
75%	.1321	.1346	.1287	.1333	.1304	.1318	.0023	7.5873	.4700	635.45
80%	.1624	.1453	.1607	.1567	.1473	.1565	.0096	6.3898	.7869	379.54
85%	.1736	.1667	.1826	.1765	.1729	.1745	.0058	5.7307	1.2950	230.63
90%	.2069	.2069	.2182	.200	.2097	.2083	.0066	4.8008	2.4143	123.68

$T = 25.5 \ ^oC; \quad (T_K = 273.16 + 25.5 = 298.66)$

Figure 14. *Table of polarization responses for fluorescein ($10^{-6}M$) in glycerol concentrations ranging from 67% to 90%. Five runs were made and the average and standard deviation calculated. The reciprocal of the average for each run was determined as $1/p$ for the ordinate values. η was calculated for each glycerol concentration and $T^o_{K/\eta}$ was calculated for the abscissa values.*

CONCLUSIONS

The results show that the system has good linearity for fluorescence response and polarization measurement. It is, however, limited in its sensitivity, not by the lack of photomultiplier sensitivity, but by the high light "noise" level. Considering the remaining three decades of photomultiplier sensitivity and an additional 500 volts which could be applied to the photomultiplier dynodes it is quite likely that the system sensitivity can be increased from $10^{-8}M$ to $10^{-11}M$ or even $10^{-12}M$ if the ambient light level can be reduced sufficiently.

The use of the ellipsoidal mirror does seem to compensate for any loss in sensitivity arising from the lack of collimating optics and the relatively low-power excitation source. However, this axial optical system involving the mirror with the sample cell at the focal point may be responsible for the high ambient light level. Reflections from the front surface of the sample cell are also directed by

the mirror to the photomultiplier. However, the axial optical system offers as compensation compactness and simplicity.

The artifically low values of (p) arising from horizontal polarization of the excitation source can be corrected by introducing a depolarizer in series with the light source. This can be either a short length of non-coherent fiber optics or a series of glass plates stacked until depolarization has been accomplished. In either case the optical characteristics of the depolarizer should assure passage of light wavelengths well into the UV region.

Although at the fluorescein concentrations and sensitivities studied, temperature fluctuations of a few degrees centigrade did not seem to effect the data, it is certain that at higher sensitivities effects would be seen. At such time it is paramount that the sample cell be temperature stabilized to within 0.1 or 0.2 degrees C.

The results of this study are encouraging. It appears that with further work to improve the ambient light problem and provide temperature regulation of the sample cell, adequate sensitivity and stability can be achieved to satisfy the needs of a laboratory instrument. The basic design goals of compactness and simplicity have already been achieved. The addition of a microprocessor to read and interpret the signal will further enhance this device and it is believed that at that time it will be a marketable instrument.

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