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Phosphorimetry*

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Phosphorimetry in the past has received limited use because the precision of reproducibility was inadequate, there were solvent limitations, and preparation of test specimens was difficult and time consuming. Detection limits have now been lowered by more than two orders of magnitude by using a rotating capillary sample cell, a more stable excitation-source power supply, and aqueous solvents. These steps have also increased precision by more than an order of magnitude. Considerable reduction in time and effort of sampling and measurement has been effected compared to phosphorimetric measurements made with standard procedures and commercial equipment. Twenty microliters of aqueous solution is all that is required to fill a quartz capillary cell by capillary action. Capillary cells filled with aqueous solutions do not crack when cooled to 77 K or when returned to room temperature. Rotation of the sample cell minimizes effects due to cell orientation and thus improves precision. Reduction of background phosphorescence results in improved accuracy of analysis. A study was made of the influence of methanol-water mixtures and of sodium-halide aqueous solutions on the magnitude of phosphorescence signals from several substances and of the effect on signal-to-noise ratios. The optimum solvent system for many phosphorimetric measurements is discussed. Analytical results are given for several organic substances measured by phosphorimetry at 77 K. These results are compared with those from previous studies by older methods.

Key words: Aqueous solvents in phosphorimetry; phosphorimetry; rotating capillary cell; solvents for phosphorimetry.

I. Introduction

Phosphoresence is a type of photoluminescence in which radiation is emitted by a molecule following radiational excitation of the molecule by means of radiation of higher energy than the emitted radiation. Phosphorescence involves two singlet-triplet transitions and as a result has a longer lifetime than fluorescence which involves two singlet-singlet transitions. Because the first triplet level of a molecule with an even number of electrons has lower energy than the first excited singlet level of the same molecule, phosphorescence also involves lower energy transitions than fluorescence, and so the phosphorescence spectrum of a given molecule occurs at longer wavelengths than the fluorescence spectrum of the same molecule. The mechanism of the production of phosphorescence is well known [1–3][1] and so will not be discussed. This manuscript is concerned with the analytical uses of phosphorescence for quantitative analyses of organic molecules—called phosphorimetry. Zander [4], Winefordner, McCarthy, and St. John [5], Winefordner, St. John, and McCarthy [6], McCarthy [7], and Winefordner, Schulman, and O'Haver [8] have reviewed and described the instrumentation, the methodology, and the uses of phosphorimetry.

Phosphorimetry has been used during the past decade for a limited number of quantitative analyses, e.g., the analysis of impurities in polycyclic aromatic hydrocarbons [9–11], in coal tar fractions [12], in air [13–15], and in petroleum fractions [16–18], the analysis of inhibitors in petroleum products [19], the analysis of pesticides, fungicides, oils, etc., in foods [20–25], and the analysis of amino acids and pharmaceuticals in biological fluids [27–35]. It is evident that up to now, phosphorimetry has been utilized only sparingly as an analytical tool for quantitative analysis and has been confined primarily to those cases where other analytical methods, such as fluorimetry and absorption spectrophotometry, either cannot be used due to lack of sensitivity or presence of interferences or give ambiguous results, and so additional analytical information is desirable. In fact, fluorimetry and absorption spectrophotometry have often been used for quantitative analyses which could quite well have been performed with greater precision, accuracy, and speed by means of phosphorimetry. The lack of use of phosphorimetry (with commercial spectrofluorimeters with phosphoroscope attachments) for quantitative analysis has been a result of the following factors: (i) the greater complexity and time to carry out an analysis due to the use of small sample cells which are difficult

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[1] Figures in brackets indicate the literature references at the end of this paper.

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to clean, fill, align, and empty, the need for solvents which form clear, rigid glasses at liquid nitrogen temperatures, and the use of liquid nitrogen for sample cell cooling; (ii) the poor precision and accuracy of measurements due to the difficulty of alignment of the sample cell in the Dewar flask containing the coolant, due to the poor source stability—particularly long term drift, and due to the ease of contamination of glassware, cells, and solvents; and (iii) the marginal sensitivity (limits of detection) for many molecules due to light losses resulting from the excessive number of optical surfaces, the small sample cells, and the use of the phosphoroscope (mechanical chopper) which cuts out more than 75 percent of the measured signal as compared to continuous excitation-measurement. In this manuscript, the instrumental methods utilized in the author's laboratory to eliminate or minimize the above limitations will be described and typical analytical data indicating the improvement in results will be given.

II. Typical Commercial Phosphorimeter

In order to compare the improvements made in phosphorimetric instrumentation, it is necessary to describe the typical commercial phosphorimeter. A block diagram of a typical commercial spectrophotofluorimeter with rotating can phosphoroscope which has been used by the author (e.g., AMINCO SPF[2] with phosphoroscope assembly) is shown in figure 1. The source of excitation energy is generally a continuously-operated xenon arc lamp driven from a well-regulated dc power supply so as to minimize long-term drift. A portion of the source radiation is introduced into the excitation monochromator to produce a small spectral band of excitation radiation which is focused upon the sample cell, usually a quartz tube (containing sample) which is placed in a quartz Dewar flask which contains a coolant (usually liquid nitrogen) for freezing the sample (see fig. 2). A fraction of the phosphorescence emission is observed by the emission monochromator, and the wavelength region of interest is thus presented to the transducer, usually a multiplier phototube with an S–4 or S–5 response. In the averaging dc measurement systems, frequently employed in commercial spectrophotofluorimeters, the current (usually 10^{-6} to 10^{-9} A) is amplified and either read out directly from a meter face or on a time base or x–y recorder (the x–y recorder is most frequently employed when utilizing one of the monochromators in the scanning mode for recording of spectra, and the time-base recorder is often used for quantitative measurements at one set of excitation and emission wavelengths).

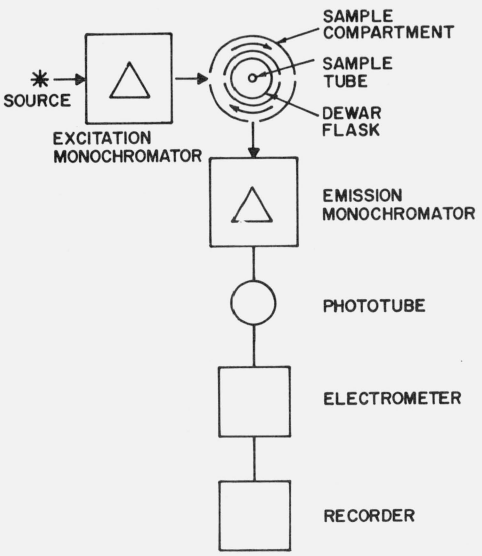

FIGURE 1. Block diagram of typical commercial analytical spectrophotofluorimeter with phosphoroscope attachment.

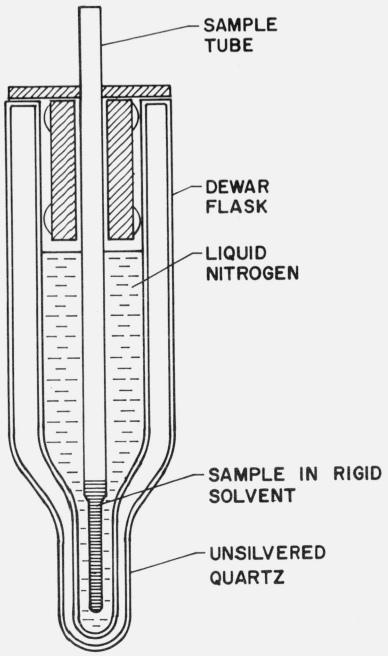

FIGURE 2. Schematic diagram of typical commercial sampling system for phosphorimetry.

A means for terminating exciting light and allowing measurement of a portion of the phosphorescence radiation without interference from the intense, scattered, incident radiation and the faster decaying fluorescence radiation must be included in the phosphorimeter design. The instrument diagrammed in figure 1 utilizes a typical rotating-can phosphoroscope, shown in greater detail in figure 3. In operation, the sample is excited for a short time through a window in the can; the can rotates to terminate excitation, after

[2] In order to adequately describe materials and experimental procedures, it was occasionally necessary to identify commercial products by manufacturer's name or label. In no instances does such identification imply endorsement by the National Bureau of Standards, nor does it imply that the particular product or equipment is necessarily the best available for that purpose.

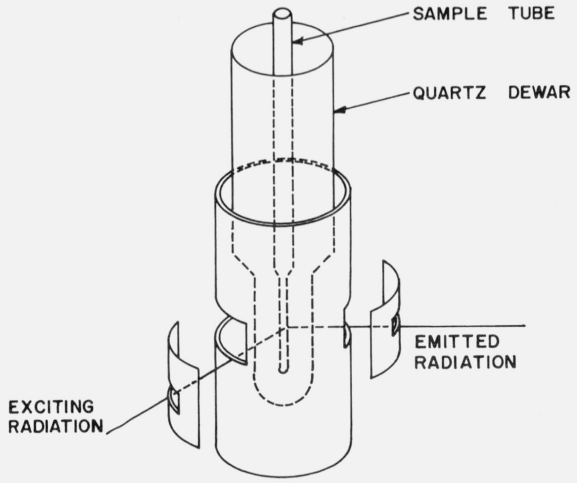

FIGURE 3. Schematic diagram of a typical rotating can phosphoroscope.

which another window is presented to the detection axis. The phosphorescence signal is observed through this window until the window moves off-axis, then the process is repeated. The instrumental response time constant employed in the dc measurement systems used with such phosphorimeters is usually made sufficiently large so as to provide a steady phosphorescence signal at all but the slowest phosphoroscope speeds.

Other means of modulation, such as the Becquerel disc phosphoroscope and pulsed source-gated detector systems, are not used in commercially available phosphorimeters. The pulsed source-gated detector system is by far the most versatile and potentially the most useful analytical system for phosphorimetry. All improvements discussed in this manuscript will be with respect to the system described above and shown in figure 1.

III. Instrumental Improvements in Phosphorimetry

A. Solvent Selection and Sampling Technique Improvements

Phosphorescence is generally not observed in liquid solutions at room temperature because radiationless deactivation due to collisions which occur within the lifetime of the excited molecule result in low phosphorescence quantum yields [36] producing phosphorescence signals buried within the phosphorescence background noise [37]. Therefore, all analytical phosphorimetric studies have been carried out in rigid media [4–8]. The criteria previously used to select a solvent for phosphorimetry have been: solubility of the analyte; low phosphorescence background (easy to purify); and formation of a clear, rigid glass at 77 K (boiling point of liquid nitrogen which is a safe, inexpensive, optically transparent coolant).

Unfortunately few solvents, particularly very polar ones, form transparent glasses at 77 K [38–40]. Ethanol is an excellent polar solvent for phosphorimetry. Small volume percents of water, mineral acids, and strong bases can also be combined with ethanol to produce clear rigid media at 77 K. The most popular mixed solvent has been EPA (a 5:5:2 volume ratio of diethyl ether, isopentane, and ethanol). Unfortunately, these solvents either do not have good solubility characteristics or result in irreversible changes of many organic species, particularly those of biological importance. Solvents, which are crystalline or severely-cracked at 77 K, have been used for some qualitative studies [41–44] but have generally been ignored or discounted as solvents for quantitative measurements. Scattering [45] and surface reflections [46] have been cited as reasons for not using snowed media [4–8] in analytical studies. The cracks or crystals create optical inhomogeneities in the medium which result in nonreproducible phosphorescence signals. Freed and Vise [29], as well as Parker and Hatchard [47], have suggested the use of an internal standard (spectrally removed from the molecule of interest) to be used as a reference to compensate for variations in sample positioning and cracking of the medium. The use of an internal standard offers a reasonable solution to the problem of sample positioning and occasional cracking; however, the choice of an internal standard satisfying the above criteria and not undergoing chemical or physical interaction with the compound being studied is extremely difficult and time consuming. In addition, the use of an internal standard is not applicable to the case of snowed or cracked media because the phosphorescence signal may be influenced by the extent of scattering, which can be wavelength dependent as long as the effective particle size is small [48, 49].

An alternative to the internal standard technique was suggested first by Hollifield and Winefordner [50] and later improved upon by Zweidinger and Winefordner [51] and still later by Lukasiewicz, Rozynes, Sanders, and Winefordner [52], and by Lukasiewicz, Mousa, and Winefordner [53, 54] all of whom used a rotating sample cell assembly to average all orientations of the sample cell, thus resulting in greater precision and accuracy of phosphorometric measurements in clear, rigid glasses [50], in rigid organic snows [51], and in rigid, aqueous snows [52–54]. In addition, there are no difficulties encountered with sample positioning and alignment.

Lukasiewicz, et al. [52–54] have added another great improvement to allow the measurement of aqueous or predominately aqueous solutions at 77 K by means of phosphorimetry. These workers used an open quartz capillary cell as the sample cell. Sample solutions ($\sim 20\ \mu l$) fill the cell by capillary action, and the sample cell is then rotated as described above. As a result of the open capillary cell, these cells with predominately, or pure, aqueous solutions do not crack when rapidly cooled to liquid N_2 temperatures or when warmed back to room temperature by use of a heat gun. Of course, the major advantage of using aqueous or aqueous-organic solutions in phos-

phorimetry are: most biochemical species are more soluble in aqueous solutions than in the solvents needed to produce clear, rigid glasses at liquid nitrogen temperatures; the solution conditions, e.g., pH, ionic strength, etc., can be varied for optimal analytical results; water is easier to obtain in higher phosphorimetric purity than most organic solvents; and contamination problems are less when water can be used to clean glassware, cells, and equipment.

The rotating sample cell assembly currently being used is a modification of the one described by Hollofield and Winefordner [50]; the present system, described in detail by Zweidinger and Winefordner [51] and modified by Lukasiewicz, et al. [52–54], is shown in figure 4. The rotating assembly consists primarily of a Varian A60–A high resolution NMR spinner assembly (Varian Associates, Palo Alto, Calif.) mounted on an AMINCO phosphoroscope sample compartment in place of the usual lid. The pressure cap of the spinner assembly is covered with black tape to ensure a completely light-tight sample compartment. The rotating sample cell is driven by a flow of nitrogen gas or air taken from a compressed gas cylinder. The sample cell must have a 6 mm o.d. and be about 25 cm long. The sample rotation is maintained at some constant speed between 450 and 1400 rpm; the actual speed is unimportant as long as it is constant during a series of measurements and can be assured by means of a normal two-stage regulator and a rotameter flow meter to monitor the gas flow rate. The normal quartz sample cells used by Zweidinger and Winefordner [51] were 5 mm o.d. and 3 mm i.d., whereas the quartz capillary cells used by Lukasiewicz, et al. [52–54] were 5.0 mm o.d. and 0.90 mm i.d. The cells were made of synthetic, high-purity, optical-grade quartz (Quartz Scientific Co., Eastlake, Ohio for the capillary tubing and Amersil, Inc., Hillside, New Jersey for the normal tubing).

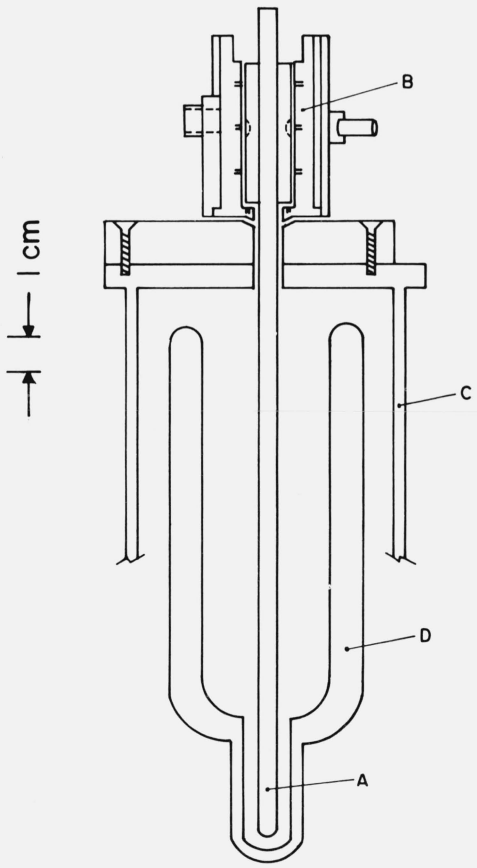

FIGURE 4. Schematic diagram of rotating sample cell assembly [51].
A. Quartz sample cell
B. Varian (909614–04) spinner assembly for NMR
C. Aminco light cover mount
D. Aminco quartz Dewar flask

The inherent photoluminescence of the quartz resulted in an appreciable "phosphorescence" background, especially with the capillary cells. However, the long-lived luminescence of the quartz cells could be minimized by mounting an excitation polarizer oriented perpendicular to the sample cell length between the sample cell housing and excitation monochromator entrance slit. Luminescence excitation spectra of the long-lived component of the quartz capillary sample cells are shown in figures 5 and 6. It can be seen from the results in figure 6 that the lowest background was obtained when both polarizers were used. For quantitative work, however, use of both polarizers is undesirable because each polarizer has a low transmittance and the loss of sensitivity is compounded. The background resulting when only one polarizer was used in the excitation beam was slightly higher than that obtained with two polarizers but still approximately 100 times lower than the background obtained without the use of the excitation polarizer.

The improvement in precision of measurement when the normal sample cell was rotated as opposed to not rotated is given in table 1. It should be stressed that the rotating sample cell holder, even when used in the stationary aligned mode (no rotation), can provide and increase in precision of as much as tenfold over the same cell in random orientation. When the normal sample cell is rotated, a further improvement in precision of about twofold is obtained (see table 1). It is interesting to note that the precision for snowed and cracked matrices is not significantly different from that for an ethanolic, clear matrix. The stationary, randomly oriented sample cell measurements were considerably more precise than those obtained by Hollifield and Winefordner [50] because of the close tolerances of the present rotating sample cell system. One great advantage of using the rotating sample cell is the simplicity of positioning the cell. After the normal sample cell is cleaned and filled (requires about five minutes), the cell with the Teflon (du Pont) turbine is simply dropped into place and spun by flow

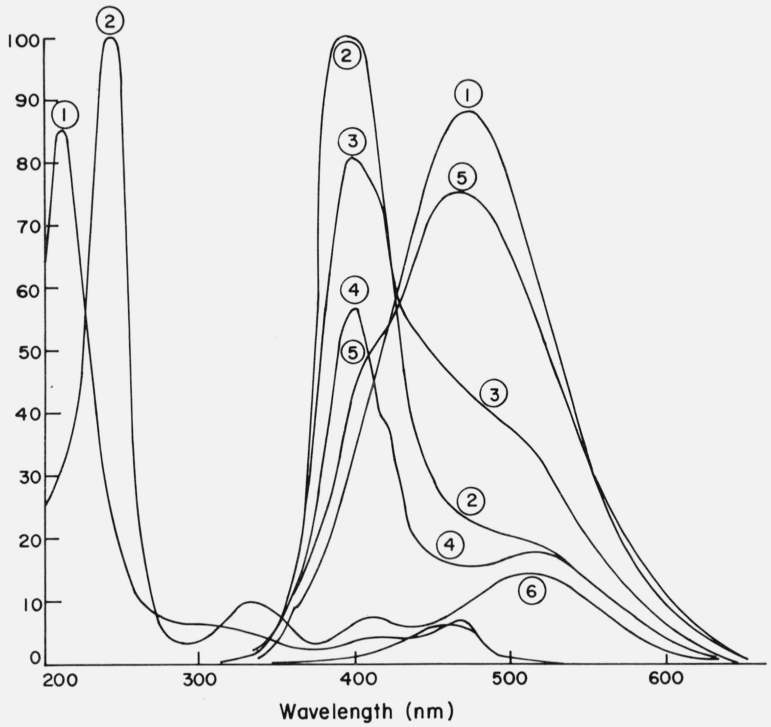

FIGURE 5. Excitation and emission spectra of long-lived component of quartz capillary sample tube (no polarizer used) spectra 1, 2, 3, 4, 5, and 6 corresponding to excitation at wavelengths 210, 240, 230, 250, 220, and 280 nm respectively.

TABLE 1. Precision of phosphorescence measurements [a] for clear glasses and snows using rotating sample cell assembly [51]

Number of determinations	Nature of matrix	Relative percent standard deviation: Stationary [a,d] random orientation	Stationary [a,e] aligned	Rotating [a,f]
4	Clear [b]	8.7	1.3	0.8
6	Clear [b]	6.0	0.5	0.8
5	Cracked [b]	13.7	3.4	1.4
11	Clear [b]	3.1	0.9	0.3
10	Clear [b]	2.9	2.8	1.0
5	Snow [c]	3.6	1.6	0.9
10	Snow [c]	2.8	0.8	0.6
10	Snow [c]	2.7	2.4	0.7

[a] Phosphorescence measurements were made on 1.6×10^{-5} *M* sulfanilamide solutions which give a signal five orders of magnitude above phototube dark current. The normal rotating sample cell was used.

[b] Ethanol solvent.

[c] Isooctane : ethanol, 4 : 1, *v/v* mixture as solvent.

[d] This column is indicative of the reproducibility obtainable with the commercial sampling system.

[e] This column gives results for the rotating normal sample cell assembly but with the normal cell stationary in the assembly.

[f] This column gives results for the rotating normal sample cell assembly but with the normal cell rotating in the assembly.

of compressed air. As a result of spinning the cell, the inner filter effect is also minimized.

A series of 10 replicate determinations of 1 μg/ml 2-thiouracil in 10 percent *v/v* methanol-water were measured to determine the precision obtainable with the rotating quartz capillary cell. The relative standard deviation obtained was 1.5 percent. This is an improvement of nearly an order of magnitude over the stationary quartz capillary tube system used by Lukasiewicz, Rozynes, Sanders, and Winefordner [52], and compares favorably with the standard deviations of results obtained with normal rotating sample cells (see table 1). The major noise contribution in the system with the rotating capillary cell arises from wobble of the cell caused by the loose fit of the turbine sleeve in the spinner assembly. If the capillary cell were held rigidly by the turbine sleeve so that no wobble occurs on rotation the precision would probably be comparable to the best data reported in table 1 for the larger, normal quartz, sample cell.

B. Improvements in Precision and Accuracy of Phosphorimetry

The relative percent standard deviations obtainable with the typical commercial system shown sche-

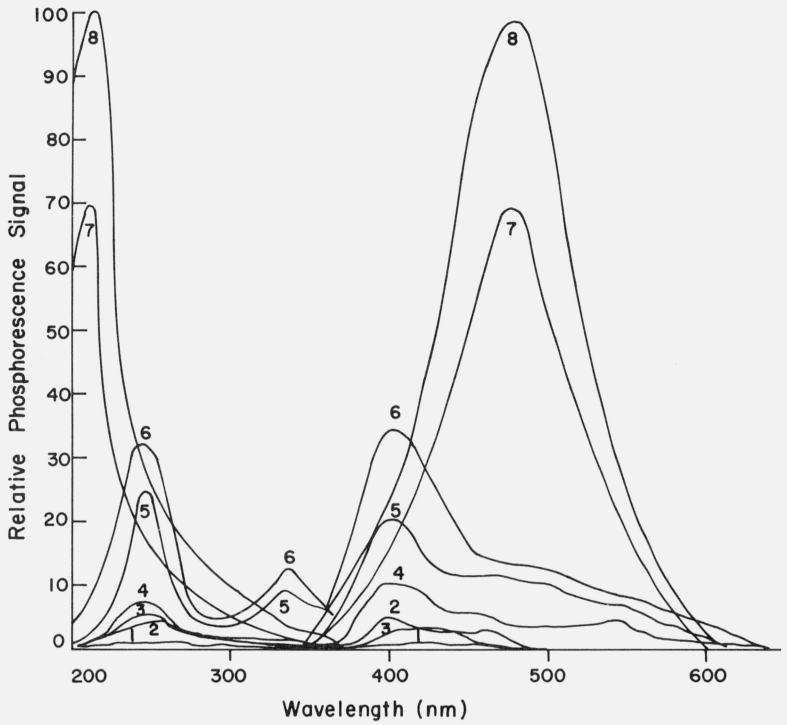

FIGURE 6. Excitation and emission spectra of long-lived component of quartz capillary sample tube, using polarized radiation; orientation of polarizers with respect to vertical axis of cell as follows [52]:

	Excitation	Emission
1.	⊥	∥
2.	∥	∥
3.	⊥	⊥
4.	∥	⊥
5.	∥	no polarizer
6.	⊥	no polarizer
7.	no polarizer	⊥
8.	no polarizer	∥

Spectra 1 thru 6 were obtained with a gain of 5-fold; full-scale approximately equal to twice level of dark current of multiplier phototube. Spectra 7 and 8 were obtained with a gain of unity.

matically in figure 1 are given in table 1. By using the rotating sample cell system as well as a more stable xenon lamp power supply, the precision of phosphorimetric measurements can be increased by as much as tenfold (see data in table 1). In addition, the contamination of glassware, solvents, and sample cells with phosphorescent impurities seriously limits precision of measurements, particularly at low concentrations of analyte. Zweidinger, Sanders, and Winefordner [55] have described techniques for cleanup of sample cells and glassware. These authors recommend cleaning the sample cells and glassware used in solution preparation in an ultrasonic cleaner for about 30 seconds or in concentrated nitric acid if there is serious contamination; when contamination is extremely serious the item can be heated in an oven or placed directly in a flame. Of course, solvents used in phosphorimetry should be purified by distillation, zone freezing, or some other separation method. It should be stressed that distillation of demineralized

water results in a solvent of extremely low phosphorescence background compared to ethanol, EPA, or any other commonly used organic solvent.

By use of an elliptical source condensing system and a more stable xenon lamp power supply, it was possible to reduce drift and noise by about tenfold as compared to those obtained with the standard power supply in the commercial phosphorimeter. The elliptical source condensing system is commercially available (in our case, from American Instrument Co., Inc., Silver Spring, Maryland). The xenon lamp power supply system was assembled using a highly regulated power supply (Harrison Lab Model 6268 A, Hewlett-Packard, Palo Alto, Calif.) operating in the constant current mode at 7.5 A (150 W lamp) with the addition of the starter circuit shown in figure 7.

A comparison of phosphorescence signals and signal-to-noise ratios obtained with the rotating normal sample cell and the Zweidinger, Sanders, and Winefordner [55] sample cell cleanup procedure with the earlier cleanup procedure of Tin and Winefordner [56] is given in table 2.

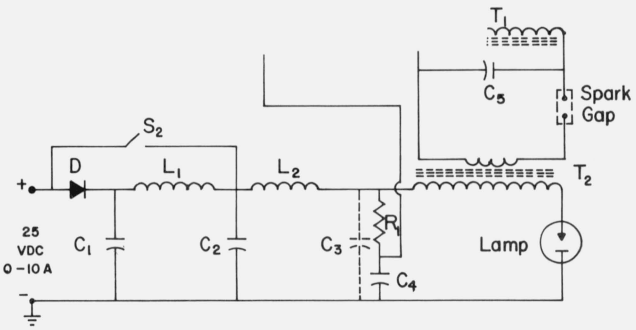

FIGURE 7. Starter circuit for xenon arc lamp (51).

C_1, C_2, C_3—0.5 μF (300 VDC)
C_4 —1300 μF (300 VDC)
C_5 —0.01 μF (30,000 VDC)
C_6 —100 μF (300 VDC)
R_1 —1 Ω
R_2 —1 k Ω
R_3 —1.8 k Ω
S_1 —Momentary contact switch
S_2 —15 Ampere switch—to be closed only after lamp ignites
L_1 —300 mH (10 A, hand wound on ferrite core)
L_2 —30 mH (15 A, hand wound on ferrite core)
D —Diode (15 A, 300 PIV)
T_1 —Autotransformer
T_2 —10 : 1 Ratio transformer

C. Decrease in Limits of Detection in Phosphorimetry

Limits of detection in phosphorimetry are reduced if either the signal level can be increased and/or the noise level can be reduced. By use of the more stable xenon lamp power supply, the elliptical source condensing system, the rotating sample cell assembly, and the improved cleanup procedure as well as the use of aqueous solutions for some molecules, the noise level is reduced and thus detection limits are also reduced. By use of more intense continuously operated or pulsed with gated detector, light sources phosphorescence signals can also be increased and detection limits are decreased even more.

TABLE 2. Comparision of signals and signal-to-noise ratios with rotating normal sample cell and with or without new cleanup procedure [55]

	Signal (nA)[a,b]	Signal-to-background fluctuation[b,c]	Signal-to-noise[b,d]
Conventional cleanup procedure	1.5(0.05)[e,f]	2.5	100[15][e]
New cleanup procedure	0.3[f]	25	45

[a] Signal is due to impurities in ethanol (solvent) background.
[b] All signals, signal-to-background fluctuations, and signal-to-noise ratios are approximate. Excitation is at 262 nm and emission is measured at 380 nm.
[c] Background fluctuation is percent standard deviation of five background readings.
[d] Time constant for all measurements was 0.3 s.
[e] Values in parentheses were obtained without the Aminco elliptical condensing system (American Instrument Co., Inc., Silver Spring, Maryland).
[f] Dark current is approximately 0.02 nA.

The improvement in detection limits, resulting primarily from noise reduction due to the processes described in the above paragraph, is given in table 3 for the measurement of several organic molecules using normal sample cells in the rotating sample cell assembly. Similarily, detection limits obtained for several organic molecules measured using the quartz capillary cells in the rotating sample cell assembly are given in table 4. The range of linearity and limits of detection reported in table 4 compare favorably with those previously obtained in clear, rigid glasses in the normal sample cell (see table 3), even though the sample volume is an order of magnitude smaller with the capillary cell. Because of the small volume of sample solution retained in the capillary tube, the total number of grams of analyte necessary for a determination is in the picogram region, and thus phosphorimetry can be considered both a trace and micro method of analysis. This should be of particular importance in biological and clinical applications where sample size is often limited. The average slopes of the analytical curves (logarithm of phosphorescence signal versus logarithm of analyte concentration) for the molecules in tables 3 and 4 are 0.94 which approaches the ideal slope of unity for phosphorescence signals in snowed matrices [51]. Both positive and negative deviations

TABLE 3. Phosphorescence characteristics of several organic studied molecules [51]

Compound	Solvent	Concentration range (M) of near linearity	Slope of linear portion	Relative percent standard deviation [a]	Linear correlation coefficient	Limit of detection (μg/ml [g])	Limit of detection (μg/ml [h])
Toluene	Ethanol [b]	10^3	0.94	1.5	0.9999	0.03	.....
Toluene	Propylene glycol: [c] water/ (9 : 1, v/v)	10^3	.76	[d] 2.5	.9999	.03	.....
Toluene	Isooctane [c]	10^3	.89	[e] 0.9 [f] (6.1)	.9998	.02	.....
Sulfanilamide	Isooctane: [c] Ethanol/ (4 : 1, v/v)	10^5	.96	[e] .8 [a] (1.1)	.9998	.0004	[i] .012
Oxythiamine HCl	Isooctane: [c] Ethanol/ (4 : 1, v/v)	10^2	1.01	1.9	1.0000	.34	.....
3-Acetylpyridine	Isooctane [c]	10^6	1.03	1.9	1.0000	.012	[i] 3.6

[a] For samples giving signals 5 times the limit of detection.
[b] Formed clear glasses.
[c] Formed snowed samples.
[d] Harrison power supply not used.
[e] Linear portion of the analytical curve.
[f] Nonlinear portion of the analytical curve.
[g] Limits of detection in this study [51].
[h] Best previous limits of detection using standard commercial phosphorimetric equipment.
[i] From Reference 57.

TABLE 4. Phosphorescence characteristics of molecules studied [54]

Compound	Slope of linear portion	Limit of detection (μg/ml)	Number of grams [b] at limit of detection	Concentration range of near linearity
p-Nitrophenol [c]	0.88	7×10^{-3}	1.24×10^{-10}	10^4
Sulfamethazine [a]	1.01	6×10^{-3}	1.07×10^{-10}	10^4
2-Thiouracil [a]	0.81	7×10^{-4}	1.24×10^{-11}	10^4

[a] Solvent 10 percent (v/v) methanol water, all solutions formed snowed samples.
[b] Volume of sample retained in capillary tube calculated as 17.8 μl.
[c] Solvent 10 percent (v/v) methanol water with 1 percent by volume saturated NaOH added.

from a slope of unity have been observed; however, much more data is necessary before a conclusion can be drawn as to whether an individual molecule perturbs the crystalline matrix and alters the slope or whether the deviations can be attributed to instrumental parameters influencing the precision and accuracy of signal measurements. Present theory assumes the scattering coefficient of the sample is not a function of solute molecule or concentration [51, 53].

D. Pulsed-Source Gated-Detector Phosphorimetric System

Fisher and Winefordner [58] have described a pulsed-source gated-detector system for phosphorimetry; such a system was shown to allow an increase in selectivity of analysis due to the use of time resolution [59], which will not be discussed here, and an increase in sensitivity of analysis due to the increased signal-to-noise ratio. The latter increase is a result of an increased signal and a decreased noise. The basis of pulsed-source systems and the system of Fisher and Winefordner [58] will be briefly described in the following paragraphs.

A pulsed-source gated-detector phosphorimetric system consists of much of the same equipment found in the commercial continuously operated source mechanically modulated phosphorimeters; the exception being that no mechanical phosphoroscope is required in the pulsed system. Also, the source of excitation energy is a short-arc high-pressure xenon flashtube rather than a continuously operated xenon arc lamp (usually 150 W); pulsed sources are operated from rather simple electronic circuits and power supplies and have operating characteristics suitable for the excitation of (fast) phosphors, e.g., a blackbody temperature of around 7000 K, a pulse rate as high as several thousand hertz, and most important, a pulse duration on the order of several microseconds (a duty cycle of about 10^{-3}). With too short a pulse, not enough molecules will be excited per excitation-emission cycle to permit the observation of analytically useful phosphorescence signals. With too long a pulse, the early portions of the emission decay curves of fast phosphors will be buried in source scatter noise. Also, with long pulses, the phosphorescence background signal, often a result of slow phosphor species in the sample mixture, may become increasingly significant.

Readout devices for pulsed-source systems are more complex than dc systems employed in commercial phosphorimeters; three principal readout methods have been used. The method proposed by O'Haver and Winefordner [60], involved phototube pulsing (gating) as well as source pulsing and is schematically illustrated in figure 8; it is seen that the first event in the excitation-observation-of-emission cycle for a pulsed-source system is the intense burst of energy from the source with a flash duration half-width, t_f. During this time, the phosphorescence intensity climbs to a value I_0 and begins to decay exponentially. At a delay time, t_d, preferably after the source flash has decayed significantly, the multiplier phototube is turned on (a high-voltage negative pulse is applied to the dynode chain), and the luminescence signal is monitored. The phototube is then turned off by termination of the negative "on" pulse: if an integrating dc measurement system is used to monitor its output, the integrated luminescence intensity I is measured during the on-time t_p of the phototube. If desired, t_d may be varied in such a manner that the gate t_p is scanned along the decay curve, providing the readout device with a display of the phosphorescence decay. This method was more difficult to achieve instrumentally than the other two methods discussed in the following paragraph.

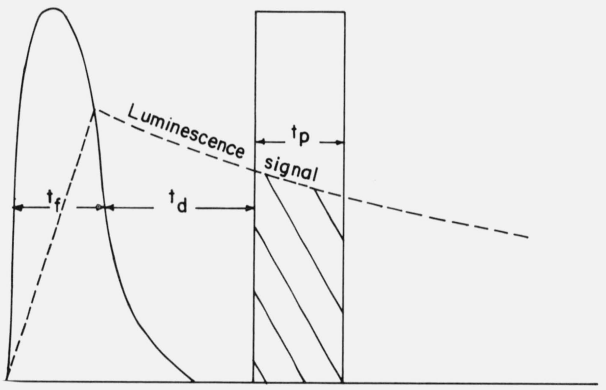

FIGURE 8. Schematic diagram of events occurring during one cycle of sample excitation and observation in a pulsed source phosphorimeter system [60].

Key to Symbols and Lines in Diagram

t_f = half-intensity width of the source flash

t_d = delay time after the end of the excitation pulse (assuming excitation pulse to be a rectangular) to the beginning of observation of the phosphorescence signal ("on" time of the detector or the readout system)

t_p = "on" time of detector or readout system

Dashed line represents buildup and decay of phosphorescence

Solid line tailing peak represents flash intensity temporal distribution

Solid line rectangular peak represents "on" time of detector or readout system

Cross-Hatched area represents measured integrated luminescence signal per source pulse

Two other readout methods for observation of fast phosphor emissions were used by Fisher and Winefordner [58]. In the *first* method, the phototube was operated continuously in the conventional manner, but an electronic gate examined the integrated phosphorescence intensity after a time delay of t_d over the time period t_p; this gate may also be scanned to display the decay curve. This method is termed the *box-car integrator method.* In the *second* method, the phototube was also continuously operated, but the signal for the entire decay curve was presented to a fast multichannel readout device. This method is called the *signal averager method.* The latter method is the simplest one of the three to use for pulsed source studies although the problems of measurement of fast electronic signals accompanied by noise are more important with this method.

The integrated luminescence intensity I (cross-hatched area in fig. 8) achieved with a pulsed-source gated-detector system is given [6] by

$$I = \frac{I_0 f t_f [\exp(-t_d/\tau)]\,[1-\exp(t_p/\tau)]}{[1-\exp(-1/f\tau)]}$$

assuming a dc type readout is used to measure I within the phototube "on" time t_p (terms are defined in fig. 8). If an ac system is used, I will not be directly proportional to f, the source pulse repetition frequency. Figure 9 gives the variation of relative re-

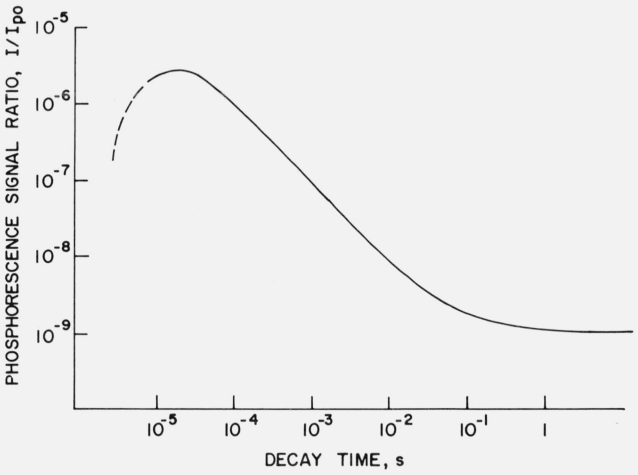

FIGURE 9. Plot of phosphorescence signal ratio (ratio of integrated intensity I to the steady state integrated intensity I_{po}) *versus* sample phosphorescence decay time τ[60] for case of pulsed source gated detector (see text for values of t_f, t_p, and t_d).

sponse (ratio of integrated luminescence intensity I to the steady state integrated intensity I_0) with phosphor decay time τ for parameters typical of an analytically useful pulsed-source gated-detector system ($t_f = 10^{-6}$ s, $t_p = 10^{-5}$ s, $t_d = 10^{-5}$ s). It is evident by the existence of the maxima in figure 9, that the pulsed-source system is capable of providing selective response to short-lived phosphors over long-lived phosphors. This is of considerable importance, because the background phosphorescence signal, which often defines the limits of detection and precision for fast phosphors in alcoholic/hydrocarbon/ether solutions, is usually due to a long-lifetime phosphor impurity. For example, the pulsed system (represented in fig. 9) for a typical fast ($\tau = 1$ ms) sample phosphor in the presence of a slow ($\tau = 1$ s) background phosphor interferent should have a 200-fold greater rejection of background than the commercial mechanical phosphoroscope phosphorimeter.

Therefore, the principle advantages of pulsed-source phosphorimetry over conventional continuous operating sources with mechanical modulation are: the possibility of achieving higher peak source intensities during the measurement period and thus lower detection limits and the possibility of achieving greater selectivity for a given short-τ phosphor over a long-lived interferent. Lesser advantages also exist. For example, since a flashtube pulse may terminate in several microseconds, whereas a typical phosphoroscopic mechanical disk chopper has a termination time (transit time) t_t of 3×10^{-5} and a typical phosphoroscopic mechanical can chopper has a t_t of 1×10^{-4} s, shorter-lived phosphors (τ's as short as 10 μs) can be measured. In addition, since the temporal parameters t_f, t_d, and t_p may be more readily varied with a pulsed-source gated-detector system, larger signals can be measured and greater selectivity achieved. Gating the detector so that it is on for only a predetermined time results in an improvement of the signal-to-noise ratio and a reduction in detection limits, an increase in precision, and an improvement in the accuracy of measurement. Finally, because it is easy to determine the linearity of a log I versus t_d plot, a rapid check on the purity of the phosphor under study can be obtained as well as an estimate of the phosphor decay time, which may be useful for structural (qualitative) studies.

A block diagram of the pulsed-source gated-detector phosphorimeter used by Fisher and Winefordner [58] is given in figure 10; a schematic diagram of the flashtube trigger circuit is shown in figure 11. To compare the standard commercial (AMINCO in our case) spectrophotofluorimeter with rotating can phosphoroscope with the pulsed-source gated-detector system, it was decided to measure the limit of detection of a fast phosphor (4-bromoacetophenone, $\tau = 6.8$ ms) in purified ethanol which was known to contain a phosphorescence background due to a slow phosphor (unknown material, $\tau = 0.8$ s) having phosphorescence excitation and emission spectra which overlapped considerably the corresponding spectra of the fast phosphor. The limit of detection of 4-bromoacetophenone was 10^{-6} M when measured using the standard commercial spectrophotofluorimeter with rotating can phosphoroscope. However, with the pulsed-source gated-detector system of Fisher and Winefordner [58], the detection limit was found to be 10^{-8} M, even though

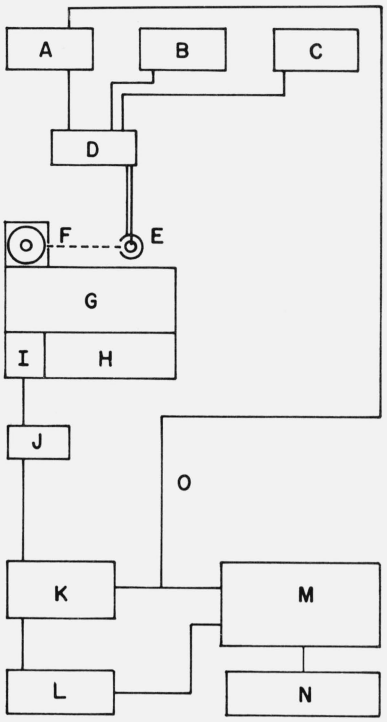

FIGURE 10. Block Diagram of Pulsed Source Phosphorimeter [58].

Key to Symbols

A. Pulse generator (Model 100, Datapulse Inc., Culver City, Calif.)
B. 300 V power supply (Model C–281, Lambda Electronics, Melville, N.Y.)
C. 1400 V power supply (Model 403 M, John Fluke Mfg., Co., Seattle, Washington)
D. Trigger circuit (see fig. 11)
E. Xenon flashtube compartment (Laboratory Constructed)
F. Sample cell compartment (identical to Aminco phosphoroscope accessory minus rotating phosphoroscope can)
G. Emission monochromator (Model EU–700, Heath Co., Benton Harbor, Michigan)
H. Phototube power supply (Model EU–701–30, Heath Co., Benton Harbor, Michigan)
I. RCA 1P28 photomultiplier tube
J. Load resistors (500 Ω to 3M Ω) and fast switching diodes
K. Oscilloscope (Model 545,Tektronix Inc., Orlando, Florida)
L. Preamplifier (Model 465A, Hewlett-Packard, Orlando, Florida)
M. Signal averager (Biomac Model 1000, Data Laboratories, Ltd., London, England), or Boxcar Integrator (Model 160, Princeton Applied Research, Princeton, New Jersey)
N. *x-y* Recorder

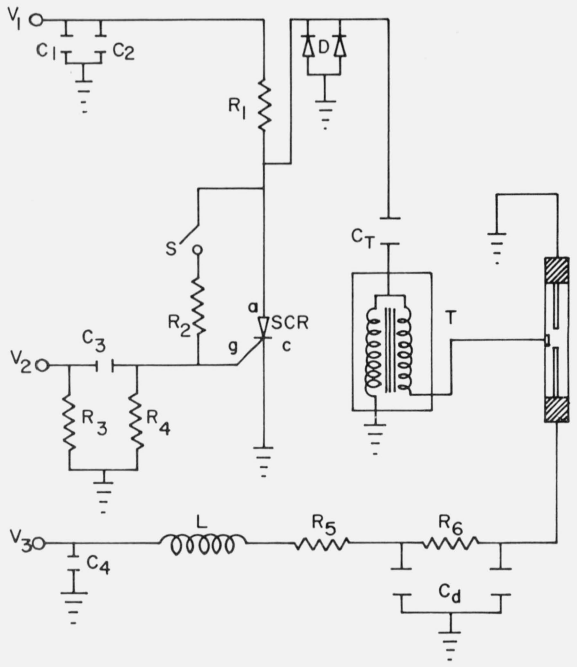

FIGURE 11. Schematic diagram of flashtube trigger circuit [58].

Value of Components

C_1 0.001 μF	R_1 100 kΩ	L ~100 μh
C_2 0.1 μF	R_2 33 kΩ	S SPST switch
C_3 0.022 μF	R_3 56 Ω	T EG & G TR 132 Trigger Transformer
C_4 0.002 μF	R_4 1.1 kΩ	V_1 300 V dc power supply
C_t 1.0 μF (600 V)	R_5 6.8 kΩ	V_2 15 V dc power supply
C_d 0.1 μF (5000 V)	R_6 190 kΩ	V_3 1400 V pulse power supply

the average power of the continuously operated xenon source in the commercial system (150 W) was 75 times as great as the average power of the pulsed xenon flashlamp (2 W). The main reason for the 100-fold reduction in the limit of detection with the pulsed system is the reduction in noise, i.e., essentially no phosphorescence signal due to the long-lived background component was observed with the pulsed system, and so the noise level was only about twice the dark current shot noise level.

One other factor which contributes to phosphorescence background should be mentioned. The diethyl ether used in the Fisher and Winefordner [58] studies was Baker and Adamson anhydrous reagent grade. Listed as impurities in this solvent are "0.001 percent carbonyl (as $>C=0$)." As expected, this also produced a fast decaying phosphorescence background to which the pulsed instrumentation was responsive. Fortunately, the ether background was *very* short-lived, and this interferent decayed before introducing error into the phosphorescence signal of the analyte with the pulsed-source system.

IV. Noninstrumental Improvements in Phosphorimetry

Lukasiewicz, Rozynes, Sanders, and Winefordner [52] showed that phosphorescence signals remain nearly constant for a variety of analytes in snowed matrices of mixtures of ethanol/water (methanol/water) at 77 K over the range of 10 to 50 percent *v/v*; as the volume percent of ethanol (or methanol) increased from 0 percent to about 10 percent, the phosphorescence signals of 2-thiouracil, *p*-nitrophenol, or sulfamethazine increased by about 3 orders of magnitude and simultaneously the physical appearance of the frozen matrix changed from a translucent severly cracked medium to an opaque completely snowed matrix. The phosphorescence signal levels were nearly the same in either methanol/water or ethanol/water (10–50) percent *v/v* of alcohol/water), but the phosphorescence background level was considerably lower in the former than the latter solvent mixture (spectrograde methanol versus 95 percent ethanol). The increased phosphorescence signals of analytes in 10 to 50 percent *v/v* of alcohol/water mixtures at 77 K as compared to those in pure aqueous solutions was correlated with the physical nature (snow versus cracked glass) rather than with chemical properties of the analyte and/or the solvent as has been further verified by Lukasiewicz, Mousa, and Winefordner [53] (see below).

Lukasiewicz, Mousa, and Winefordner [53], in a more recent study, have made careful phosphorescence measurements of the influence of solvent (methanol/water and alkali-halide salt solutions) composition upon the phosphorescence of 3-indole acetic acid, hippuric acid, and sulfacetamide at 77 K. Typical results for the methanol/water mixtures at 77 K are shown in figure 12. The three analytes all show the same solvent dependency, i.e., a region where there is an abrupt increase in phosphorescence signal between 0 and 3 percent *v/v* methanol/water, followed by a plateau region between about 5–30 percent *v/v*, and a region where the phosphorescence signal decreases slightly for solutions with more than 30 percent methanol. Little or no spectral shift was observed for the phosphorescence excitation and emission spectra as a function of solvent composition. Perhaps the most significant observation was that each region corresponded to a visible change in the nature of the matrix. Pure water solutions were severely cracked, but nearly transparent. Addition of small amounts of methanol to water produced an opaque matrix which corresponded to the region of greatest slope in the solvent dependency curves. Between approximately 5 and 30 percent *v/v* methanol/water, the frozen matrix became completely snowed, and the signal remained nearly constant. Increasing the percentage of methanol even more resulted in a signal decrease which corresponded to the reappearance of a cracked, translucent,

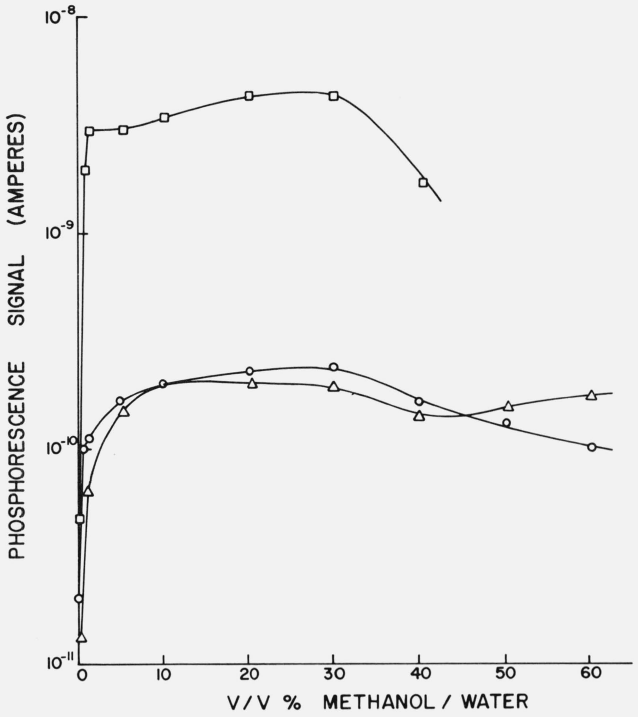

FIGURE 12. Phosphorescence Signal as Function of Solvent Composition (methanol-water) [53].

○ $2.0 \times 10^{-6}M$ 3-indole acetic acid
△ $4.7 \times 10^{-6}M$ hippuric acid
□ $1.7 \times 10^{-6}M$ sulfacetamide

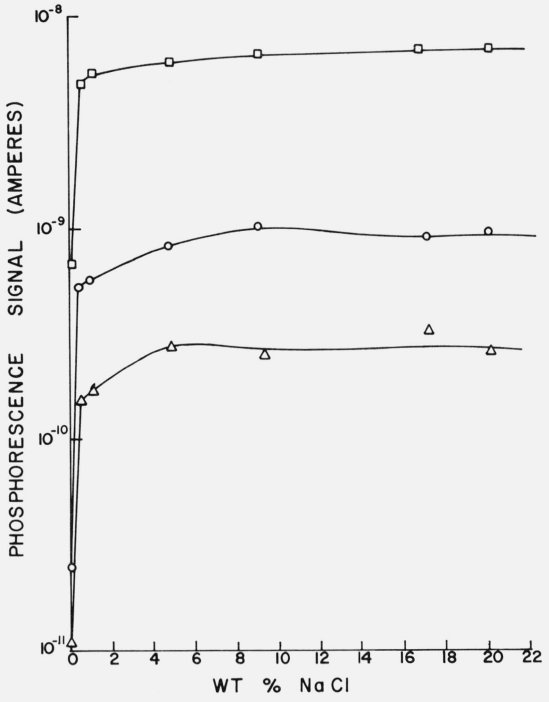

FIGURE 13. Phosphorescence Signal as Function of Solvent Composition (sodium chloride-water) [53].

○ $3.7 \times 10^{-6}M$ 3-indole acetic acid
△ $1.8 \times 10^{-6}M$ hippuric acid
□ $3.9 \times 10^{-6}M$ sulfacetamide

rigid matrix; the precision of measurement of phosphorescence signals for methanol/water solutions with 40 percent *v/v* or more methanol is poor because of the nonrandom cracking of the frozen solvent.

The shapes of the phosphorescence signal versus solvent composition curves when sodium chloride is added to water rather than methanol (or ethanol) are identical to those for the alcoholic solutions (fig. 13), and the previous conclusions regarding the physical nature of the matrix seem to be justified. It should be stressed, however, that the maximum phosphorescence signal for the NaCl solutions is about 3 times greater than the methanol/water solutions (the salt solutions at 77 K result in a rigid, snowed matrix with more finely divided particles than the snowed matrix for the methanolic solutions).

The use of the external heavy-atom effect to increase the transition probability from the first excited-singlet to the first excited-triplet state and therefore, to increase the sensitivity of phosphorescence, has also been reported [62, 63]. However, in the *past*, results obtained using the external heavy-atom effect have been of limited analytical value because of the following restrictions. First of all, the heavy-atom perturbant had to be appreciably soluble in the solvents used for phosphorimetry and also had to form clear, rigid glasses with the solvent. Second, the perturbant should not absorb in the same spectral regions as the compounds being studied. Third, the perturbant had to be available in extremely pure form, since impurities could cause a large background signal. Ethyl iodide has been most commonly used as an external perturbant; however, results obtained were limited because ethyl iodide did not fulfill all the requirements stated above, especially the last two. In aqueous solutions, however, the use of simple perturbants, such as alkali halide salts is possible. Solubility and purity of these salts make them desirable for phosphorimetric studies of aqueous solutions.

The effect of adding the alkali halide salts, NaI, NaBr, and NaCl to pure water solutions of 3-indole acetic acid, is shown in figure 14. The combined physical matrix and heavy-atom effect result in the greatest phosphorescence signals with added NaI. All three salts (see background spectra in fig. 14) result in significant phosphorescence background—NaBr giving the largest. Also, both NaBr and NaI solutions result in the same general shaped composition curve as for NaCl and alcohol with water, except that the NaI-water curve in figure 14 is somewhat sharper than the others. An increase in phosophorescence

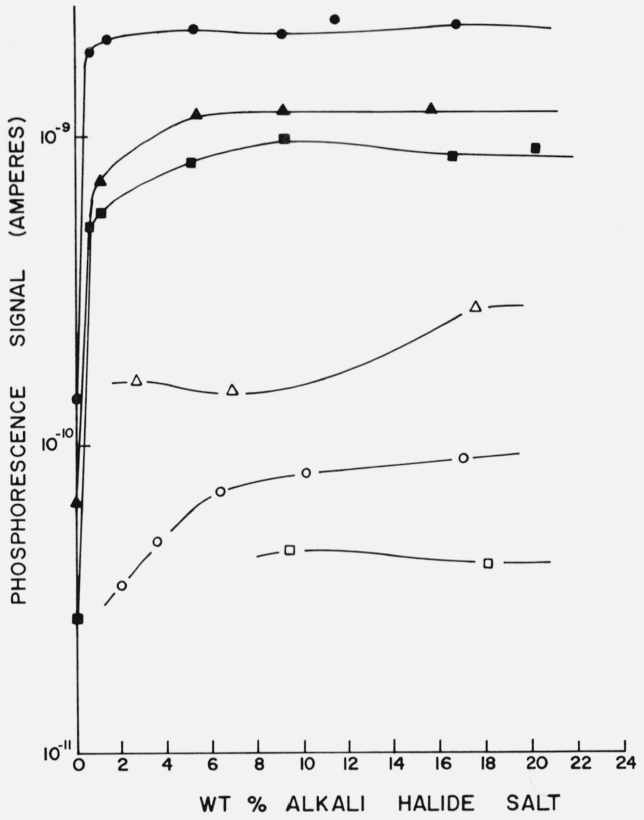

FIGURE 14. Phosphorescence Signal of $3.7 \times 10^{-6} M$, 3-indole Acetic Acid in Alkali Halide Salt Solution:

● aqueous NaI solution as solvent

▲ aqueous NaBr solution as solvent

■ aqueous NaCl solution as solvent

Phosphorescence background of alkali halide salt crystals:

○ NaI crystals

△ NaBr crystals

□ NaCl crystals

Excitation Wavelength 285 nm, emission wavelength 435 nm [53]. Reprinted from Anal. Chem. **44**, 965 (1972). Copyright (1972) by the American Chemical Society. Reprinted by permission of the copyright owner.

signal occurred for concentrations of NaI up to 1 *M*.

Therefore, the use of alkali-halide/water solutions as solvents for phosphorimetric analysis of polar organic molecules is advantageous for a number of reasons: alkali-halide salts may be obtained in ultrapure form, and thus phosphorescence background may be reduced to extremely low levels; salt solutions have the same matrix effect as alcohol/water solutions, while producing a much lower background signal; and, in the case of sodium iodide, an additional factor, namely the heavy-atom effect, enhances the phosphorescence signal. In some cases, however, use of aqueous sodium iodide solvents may be less desirable than other alkali halide salts. Phosphorescence decay time measurements for the compounds studied in this study by Lukasiewicz, Mousa, and Winefordner [53] indicated that decay times in aqueous sodium iodide solutions were considerably shorter than in methanol/water solutions. The changes in decay times for the compounds studied in aqueous sodium iodide and alcohol/water solutions are summarized in table 5. These observations are consistent with the heavy-atom perturbation theory [61]. Phosphorescence signals, however, did not increase for all three compounds listed in figure 13. A sixfold increase in phosphorescence signal was observed for 3-indole acetic acid, and a threefold increase for hippuric acid in 1 *M* sodium iodide. Sulfacetamide, on the other hand, showed a threefold decrease in signal even though the phosphorescence decay time was lower.

TABLE 5. Phosphorescence decay times in aqueous sodium iodide and aqueous alcohol solutions

Compound	Lifetime(s)	
	Solvent 10 percent *v/v* methanol-water	Solvent 1 *M* aqueous sodium iodide
3-Indole Acetic Acid	7.0	0.90
Sulfacetamide	1.7	0.83
Hippuric Acid	3.0	0.97

It is therefore quite evident that proper selection of the solvent for phosphorimetry can lead to an appreciable increase in phosphorescence signals of some species and in some cases an increase in selectivity of analysis. The advantages of using alkali-halide salt solutions over alcohol/water solutions as phosphorimetric solvents include: the availability and low cost of high purity salts; increased signal levels; and the high solubility of many biochemically related species.

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