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CHEMILUMINESCENCE: A NEW METHOD FOR DETECTING FLUORESCENT COMPOUNDS SEPARATED BY THIN-LAYER CHROMATOGRAPHY

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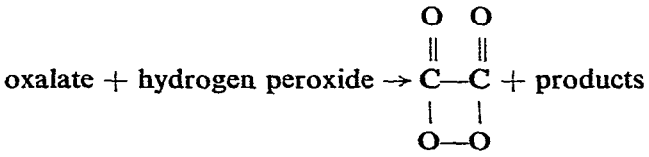
SUMMARY

Dansyl derivatives separated by thin-layer chromatography can be detected by successive spraying with solutions of bis-2,4,6-trichlorophenyloxalate (TCPO) and hydrogen peroxide in dioxane. The TCPO and peroxide react to produce a dioxetanedione intermediate which transfers its energy to the dansyl compound causing it to luminesce. This detection method is generally comparable to conventional fluorescence detection, offering as advantages that it does not require excitation radiation and can be used to uniformly excite the plate.

INTRODUCTION

Fluorescence is widely used for the qualitative and quantitative analysis of compounds separated by thin-layer chromatography (TLC). In conventional fluorescence TLC detection, luminescence is observed from electronically excited molecules that are generated photolytically, *i.e.* by absorbing light from an independent source. In this study the possibility of generating the luminescing molecules chemically rather than photolytically was explored. The chemical reaction used for excitation was of the peroxyoxalate type:



(1)

1,4-dioxetanedione

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$$+ \text{flr} \rightarrow \text{flr}^* + 2\,CO_2 \qquad (2)$$

$$\text{flr}^* \rightarrow \text{flr (flr = fluorophor)} \qquad (3)$$

Since the only function of the fluorophor is to accept energy from the 1,4-dioxetanedione intermediate, many different fluorophors can be used. In the absence of added fluorophor no emission is observed[1,2]. At low fluorophor levels, luminiscence intensity is proportional to fluorophor concentration. Thus, it should be feasible to spray developed TLC plates successively with oxalate and peroxide solutions and quantitatively relate the resulting luminiscence intensity to concentration. Since this luminescence is produced chemically, it is known as chemiluminescence (CL) even though the emission process is the same as in conventional fluorescence.

Peroxyoxalate CL has been observed for a wide variety of oxalate compounds[2–5]. Of these, electronegatively substituted aromatic oxalate esters give the greatest efficiency as well as having a long lifetime. For this study, bis-2,4,6-trichlorophenyl oxalate (TCPO) was chosen because it is stable and easy to prepare[6]. Greatest CL efficiency is observed in ester and ether solvents, although peroxyoxalate CL reactions are compatible with many other solvents including water. With most oxalate esters, including TCPO, small base concentrations lead to an intensification of luminescence, although there are also systems that are catalyzed by the addition of acid. With oxalate esters, luminescence can last for several hours. High hydrogen peroxide concentrations intensify CL but reduce its duration. Increases in temperature also reduce the CL lifetime as do high concentrations of base.

The fluorophors chosen for most of the study reported here were dansyl derivatives of amino acids. These were chosen because dansylation is widely used to prepare fluorescent derivatives of many different types of biologically important compounds[7].

There are a number of potential advantages to be gained by using chemiluminescence instead of fluorescence detection for TLC. The need for a light source to excite luminescence is eliminated. Scattered excitation radiation is no longer a source of potential error. Since only a detector is required, there are fewer geometry problems.

EXPERIMENTAL

Instrumentation

A block diagram of the instrumentation is shown in Fig. 1. An X-Y recorder (Houston Instrument, Model HR-100) was modified by removing the pen and replacing it with a small wooden block holding one end of a 2 ft. long × 1/8 in. diameter Corning fiber optic, numerical aperture 0.63, acceptance angle 39°. The surface of the fiber optic is approximately 1/8 in. from the plate surface. At 500 nm, the fiber optic has a transmittance of 63%. The optic consists of many fibers which are scrambled. The other end of the optic is positioned in front of a stationary RCA 1P21

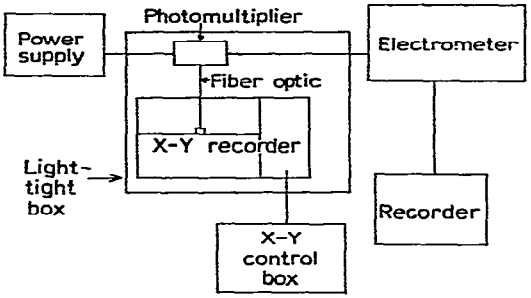


Fig. 1. Block diagram of detection system for measuring CL from a TLC plate.

photomultiplier tube. A broad band filter with maximum transmittance at 525 nm is interposed between the optic and the photomultiplier.

The photomultiplier was maintained at 800 V by a Kepco ABC regulated power supply. The photomultiplier current was amplified by a Keithley 610A electrometer and recorded on either a Health-Schlumberger SR255-A1B recorder or a Hewlett-Packard 7100B recorder.

Fig. 2 shows a schematic of the electronics used to control the position of the fiber optic on the plate. The recorder sensitivity and zero positions for both X and Y axes were adjusted so that the fiber optic would be positioned over one edge of the plate when the appropriate ten-turn pot was turned fully clockwise and over the opposite edge of the plate when the ten-turn pot was fully counter-clockwise. When moving the fiber optic over a series of zones, the optic position was adjusted to scan over the center of the zone. If the zones were visible, this could be done by adjusting the appropriate ten-turn pot while observing the fiber optic position. It could also be done by manually adjusting the appropriate ten-turn pot to get maximum intensity from one of the zones.

The X-Y recorder and photomultiplier are enclosed in a large light-tight box.

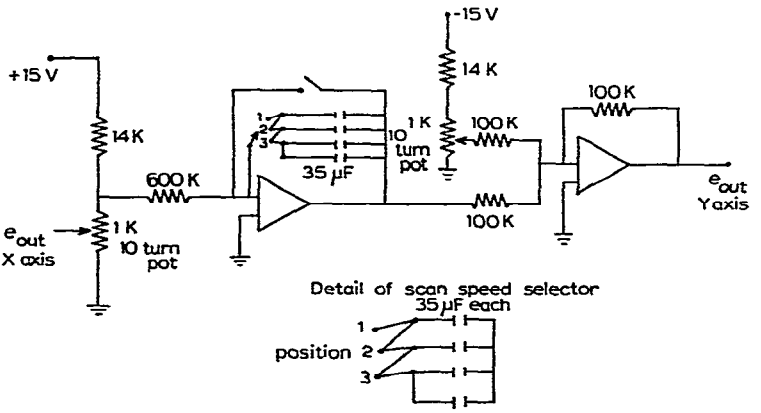


Fig. 2. Circuit diagram of control box used to scan the fiber optic across the TLC plate. The two ten-turn pots control position of the fiber optic on the TLC plate. The scan is initiated along the Y-axis by opening the switch across the input and the output of the operational amplifier on the left, which then functions as an integrator.

Unfortunately, the X-Y recorder available for this study scanned jerkily in both directions, making it impossible to accurately quantitate peak areas. Hence all the data here are reported in terms of peak heights.

Materials

The substances to be separated were applied using a 10-μl precision sampling syringe (Fisher No. 11-124-35B).

Most observations reported here were made on 250 μ thick Analtech silica gel G plates (Cat. No. 1011). A few were made on 250 μ thick Analtech Avicel (microcrystalline cellulose) plates (Cat. No. 5011) (Analtech, Newark, Del., U.S.A.).

Reagents were applied to the plates with either a Sprayon Jet-Pack Sprayer with interchangeable plastic jars (Analtech, Cat. No. A-50) or an all-glass aspirator system using compressed air.

Plates were developed in a glass tank with a glass cover (Analtech, Cat. No. N250).

Chemicals

TCPO was prepared by a published procedure[6]. Dansyl-glycine was purchased from Nutritional Biochemicals (Cleveland, Ohio, U.S.A.). Dansyl-L-arginine and dansyl-L-leucine were from Sigma (St. Louis, Mo., U.S.A.).

Glycine was purchased from Matheson, Coleman and Bell (East Rutherford, N.J., U.S.A.) and Fluram (fluorescamine) was obtained from Roche (Welwyn Garden City, Great Britain). The fluorescamine derivative of glycine was prepared according to the procedure in the literature accompanying the product.

Procedures

The fluorophor was either applied to a thin-layer plate and evaluated directly or developed in a solvent system prior to evaluation. Development was carried out in either an acid solvent system of chloroform–ethyl acetate–methanol–acetic acid (9:15:4.5:0.2)[7] or a basic solvent system of methyl acetate–2-propanol–aqueous ammonia (9:7:2)[7].

The plates were sprayed first with TCPO solution and then with hydrogen peroxide. At least ten passes were made with each solution to completely wet the plate surface. After having been sprayed with peroxide, the plate was placed on the X-Y recorder, and CL was measured by scanning the fiber optic over the zones of interest. The scan rate was 20 cm/min, along the Y-axis.

Stock solutions of 0.5 mg/ml dansyl-glycine and dansyl-leucine in ethyl acetate and 0.2 mg/ml dansyl-arginine in 40% ethyl acetate in methanol were prepared. Less concentrated solutions were prepared by dilution.

RESULTS

Choice of solvent for TCPO and H_2O_2

When dansyl compounds in ethyl acetate were applied directly to a silica gel TLC plate, maximum CL intensity was observed when both TCPO and hydrogen peroxide were in dioxane. No further increase in intensity was observed if the plate was exposed to triethylamine or ammonia vapors and/or heated to 100° before

spraying with TCPO and peroxide. Ethyl acetate, dimethyl phthalate, and isobutanol were also evaluated as solvents but they generated very low intensities either with or without the above plate pretreatments. It should be noted that TCPO gradually decomposes in dioxane over the course of several days by reacting with the peroxides that tend to form in dioxane. This is less of a problem in ester solvents.

For plates developed in the acidic solvent system (chloroform–ethyl acetate–methanol–acetic acid) efficient CL was observed without pretreatment when the plates were sprayed with TCPO and hydrogen peroxide in dioxane. Plates developed in the basic solvent system (methyl acetate–2-propanol–aqueous ammonia) had to be heated for 5 min at 90° prior to spraying with both reagents in dioxane. Heating the plate for a short period reduces catalysis of the reaction by traces of base remaining on the plate after development, but leaves enough base so that intensification of the CL when compared to the acid system is generally seen.

Effect of TCPO concentration

Fig. 3 shows the effect of varying TCPO concentrations on the intensity of CL on silica gel: Above 10^{-3} *M* TCPO, further increases in oxalate concentration have little effect on the intensity–time curves. This is an important observation since it means that small variations in the amount of TCPO reaching different parts of the plate should not significantly affect quantitation. In all cases, intensity is reasonably constant for about 12 min, followed by a rapid decrease to zero. The rapid decrease corresponds to the point where the dioxane solvent completely evaporates off the plate.

The recorder used in this study generated considerable heat, which was trapped

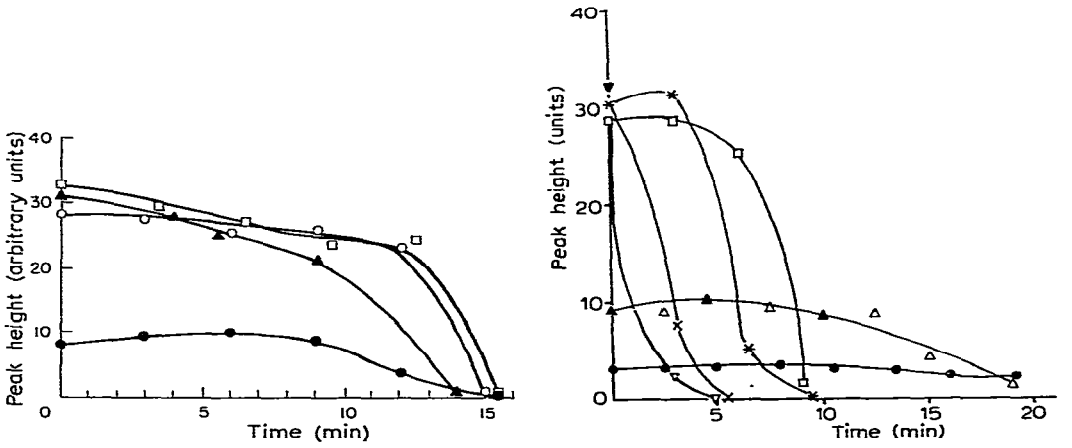


Fig. 3. Effect of varying TCPO concentrations on CL intensity *vs.* time curves observed on silica gel plates. ●, 4×10^{-4} *M* TCPO; ○, 1×10^{-3} *M* TCPO; ▲, 4×10^{-3} *M* TCPO; □, 8×10^{-3} *M* TCPO. Each zone contained 0.5 μg dansyl-glycine. The peroxide concentration was 0.08 *M*. Both peroxide and TCPO were dissolved in dioxane.

Fig. 4. Effect of varying hydrogen peroxide concentration on CL intensity *vs.* time curves observed on silica gel plates. ●, 4×10^{-2} *M* H_2O_2; △, 8×10^{-2} *M* H_2O_2; □, 1×10^{-1} *M* H_2O_2; ✳, 4×10^{-1} *M* H_2O_2; ×, 8×10^{-1} *M* H_2O_2; ▽, 1.2 *M* H_2O_2. Each zone contained 0.5 μg dansyl-glycine. The TCPO concentration was 4×10^{-2} *M*. Both peroxide and TCPO were dissolved in dioxane.

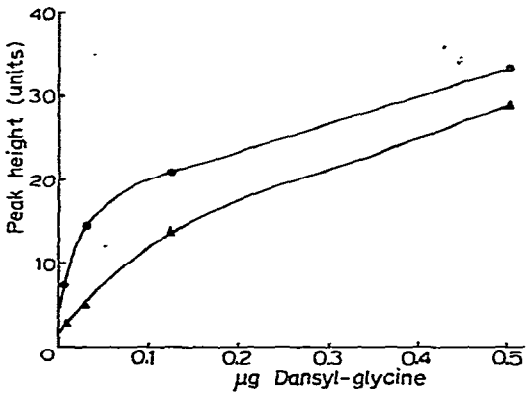
in the light-tight box, causing a significant increase in the rate of dioxane evaporation. In another system using some type of ventillation and/or a recorder generating less heat, this effect could be reduced, resulting in a longer-lived signal.

Effect of peroxide concentration

Fig. 4 shows the effect of varying peroxide level on CL intensity–time curves. The trade-off between intensity and life-time is clearly seen. At low peroxide concentrations, intensity is low but lifetime is long, while at high concentrations intensity is much greater but the CL does not last as long. For the highest peroxide concentrations, 0.8 and 1.2 *M*, most of the CL is emitted between the time the plate is sprayed with peroxide and is measured in the recorder. For visualizing spots by CL in a dark room, these higher peroxides concentrations would be better because of the greater intensity observed with them.

Response to concentration

Fig. 5 shows peak height as a function of concentration on two different days for dansyl-glycine in ethyl acetate spotted directly on the plate. The slope of this



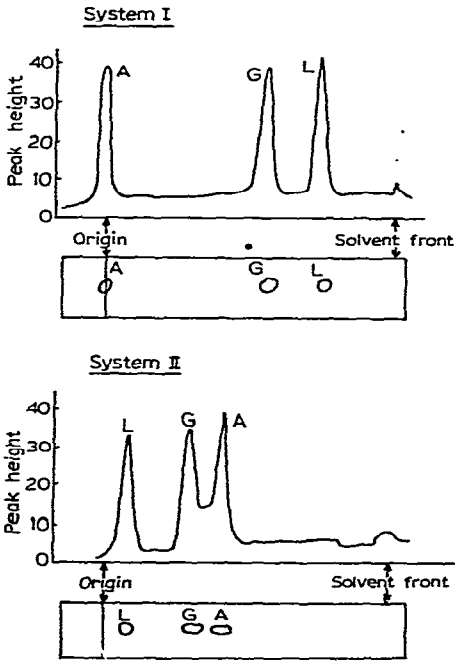


Fig. 5. Calibration curves of peak height vs. amount of dansyl-glycine on the TLC plate. The two curves were observed on different days. The TCPO concentration was 8×10^{-3} *M and the peroxide concentration* 8×10^{-2} *M. Both reagents were in dioxane. The data were taken on the first scan after spraying the plate, i.e., about 1 min after spraying. All four concentration levels were applied to the same plate in a row with about 1 in. between zones.*

Fig. 6. Separation and CL detection of dansyl-amino acids separated by TLC, showing recorder traces of CL intensity *vs.* plate position and the position of the spots as visualized under UV light. System I was developed in the acidic solvent system and System II in the basic solvent system. A = dansyl-arginine; G = dansyl-glycine; L = dansyl-leucine. 0.5 μg dansyl compound per spot; [TCPO] = 8×10^{-3} *M*; [H_2O_2] = 8×10^{-2} *M*.

curve varies with concentration, falling off at high concentrations. Small blank peaks were observed for pure ethyl acetate. The variation in these peaks established the detection limit of 0.007 μg dansyl-glycine. The presence of these peaks accounts for the non-zero intercept on the calibration curves. Presumably, the detection limit could be reduced by tracking down and eliminating the source of these background peaks.

There is measurable background luminescence from the plate itself so that a more efficient detection system would not lead to an improvement in signal-to-noise ratio. However, if the background emission occurs at a different wavelength than the dansyl-glycine emission, then using a filter with a narrower bandpass might reduce the background intensity more than it would reduce the signal-of-interest. This would lead to a net increase in the signal-to-noise ratio, thereby improving the detection limit. Clearly, the signal throughput must still be sufficient so that detector noise does not become a significant factor.

Separations

Fig. 6 shows the smoothed recorder tracings observed for actual separations of dansyl-glycine, dansyl-argine, and dansyl-leucine in both acidic and basic solvent systems. Below each recorder tracing the position of the spots as visualized by fluorescence is indicated.

Studies on Avicel

Dansyl compounds spotted on Avicel luminesce when sprayed with TCPO and peroxide in ethyl acetate if the plate is first exposed to ammonia or triethylamine vapors and heated to 100° for 15 min. Spots on Avicel also luminesce without pretreatment when sprayed with TCPO and peroxide, both in dioxane. This system was not further investigated since dansyl compounds do not separate satisfactorily on Avicel.

Fluorescamine

Fluorescamine has recently been developed for making fluorescent derivatives of compounds containing primary amine functional groups[8]. It was demonstrated that it was possible to observe the fluorescamine derivative of glycine on a silica gel plate by spraying with TCPO and hydrogen peroxide. The intensity was somewhat less than for dansyl compounds, and the duration of CL was about the same.

DISCUSSION

It has been demonstrated that peroxyoxalate reactions are suitable for detection of dansyl compounds separated by TLC. Most characteristics of the TCPO–peroxide reaction on a TLC plate are the same as previously reported in solution[3–5]. This includes the effect of TCPO and peroxide concentrations on CL intensity–time curves as well as the observed increase in intensity in the presence of small base concentrations. The observed intensity–time curves for TLC are also affected by solvent evaporation from the plate. It is also felt that the degree of association between the TCPO and peroxide solvents and the surface of the plate is an important factor. The failure to achieve efficient luminescence with ester solvents on silica gel is thought to be due to the failure of the relatively non-polar esters to "wet" the silica surface. It should be noted that esters work better on the less polar Avicel.

Although only dansyl and fluorescamine derivatives were examined in this study, CL detection should be applicable to a variety of fluorescers. In solution the nature of the fluorophor does not significantly influence the kinetics of peroxyoxalate reactions[2–5]. Most studies involving peroxyoxalate CL have involved hydrocarbon fluorophors[2–6,9]. Unpublished work at the University of Georgia has shown the Ru(II)-tris(bipyridyl) complex can serve as the emitter in peroxyoxalate reactions[10]. The effect of other fluorophor structures on CL efficiencies is not known although to the best of the authors' present knowledge there are no examples of fluorophors in the visible that cannot be used with peroxyoxalate reactions. Peroxyoxalate reactions have been shown to excite fluorophors down to 280 nm although efficiency falls off substantially in the ultraviolet[9]. The nature of peroxyoxalate CL is such that kinetics and the effects of peroxide and TCPO concentration should not vary much for different fluorescers.

The fact that luminescence was observed for both acidic and basic solvents suggests that CL detection is compatible with a wide variety of solvent systems. The heating step required in the basic solvent system probably removes excess base which otherwise causes the duration of CL to be too short.

Since peak height varies with concentration, CL can be used for quantitating compounds separated by TLC. As with other methods of TLC detection, it will be imperative that both samples and standards are run on the same plate. When CL is used for quantitative TLC detection, the plate should be scanned perpendicular to the development direction, so that both samples and standards for the same compound will be measured in the same scan. This will require that all zones move the same distance from the point where they are initially applied. Also, if peak height is used as a measure of concentration, the zone geometries must be similar.

In conclusion, CL offers an alternative technique for detecting fluorescent compounds separated by TLC. The main advantage of the system described here is that the instrumentation is simpler than required for conventional fluorescence. The paper immediately following this one describes the coupling of CL to detection with a Vidicon rapid scanning spectrometer to produce a system for rapid determination of fluorescent compounds separated by TLC.

The CL detection system is also particularly well suited for photographic detection. In this case a plate including both samples and standards would be sprayed and then exposed to a photographic plate for some fixed time interval. It is easy to spray an entire plate with reagent relatively evenly while with conventional fluorescence, it would require that the plate be illuminated uniformly. A potentially useful feature of this system is the absence of any power requirements. Hence, it might be useful for doing chemical analysis in remote places where no power is available.

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