results of the complete quantitative analysis of the seven-component PAH mixture. The data obtained by RTP analysis are in good agreement with the amounts of PNA compounds present in the mixtures.

CONCLUSION

The SEHAP technique opens up new avenues for the analysis of complex samples by phosphorimetry in general and RTP in particular. One of the unique features offered by this method is the ability to "pick out" specific target PNA compounds of interest in an environmental sample using appropriate heavy atoms. Present applications of the SEHAP technique include the analyses of coal-derived products (Synthoil) and aerosol sample extracts from the Source Assessment Sampling Systems (SASS). The results of these analyses will be published elsewhere (*19*).

ACKNOWLEDGMENT

The technical assistance of P. R. Martinez is acknowledged.

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Room Temperature Phosphorescence of Selected Pteridines

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The phosphorescence properties of a number of pteridines were investigated at room temperature and 77 K. Most of the pteridines exhibit intense phosphorescence at 77 K, and several pteridines were found to exhibit weak phosphorescence at room temperature when alkaline solutions were spotted on filter paper and rigorously dried. The room temperature phosphorescence (RTP) emission from the pteridines was greatly enhanced when the compounds were adsorbed on sodium acetate impregnated paper. In fact, all of the pteridines which exhibit phosphorescence at 77 K show RTP when adsorbed on sodium acetate impregnated paper. Many of the pteridines show both RTP and an E-type delayed fluorescence emission. The external heavy atom effect was also investigated with several compounds. The results of our studies provide additional insight into the RTP phenomenon and indicate that the highly selective and sensitive RTP technique should prove quite useful for the analysis of pteridines in biological systems.

Until recently, strong phosphorescence of organic molecules has been a phenomenon whose observation usually was confined to systems in the gas phase, in rigid media such as plastic films and boric acid glasses, or at cryogenic temperatures (*1*, *2*). Such conditions were required to prevent the nonradiative decay of the excited triplet state molecules by processes such as collisional deactivation and quenching by ground state triplet oxygen. As such, only a limited number of cases of phosphorescence from fluid solution have been observed, and these solutions usually require thorough degassing in order to prevent quenching by oxygen (*3*–*7*). Because of the considerable experimental difficulty involved in these techniques or the limited number of compounds to which some of the techniques have applied, the use of phosphorescence has generally been discouraged as a routine analytical tool. However, recent reports by Roth (*8*) and by Schulman and Walling (*9*) revealed that a variety of polar or ionic organic molecules exhibit phosphorescence at room temperature upon rigorous drying of solutions adsorbed on a suitable support such as silica, alumina, or cellulose. The development of this novel technique is interesting not only in a physical sense, but, owing to its relative simplicity, from an analytical standpoint as well.

Schulman and Walling noted the necessity of thorough drying in order to observe room temperature phosphorescence (RTP) from compounds adsorbed on paper and other supports, and they suggested that surface adsorption of the phosphor molecules to the support held them rigid enough to restrict collisional deactivation and to inhibit quenching by oxygen (*9*). Subsequent investigation into the RTP phenomenon by Schulman and Parker demonstrated that a support capable of hydrogen bonding to the phosphor molecule was necessary in order to observe RTP (*10*). They also found that moisture in the atmosphere acted as a powerful quencher of RTP and greatly enhanced the ability of oxygen to act as a quencher. This study led to the conclusion that hydrogen bonding of the phosphor molecules to the support is the predominant mechanism in preventing collisional deactivation. The moisture serves to disrupt the hydrogen bonding network, thus allowing collisional deactivation while increasing the ability of oxygen to penetrate and quench the

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sample (*10*). In a recent study by von Wandruszka and Hurtubise on the RTP of *p*-aminobenzoic acid adsorbed on sodium acetate, it was shown by infrared and reflectance spectra that hydrogen-bonding was occurring between the phosphor and the support (*11*). Additional evidence of hydrogen bonding has been presented in a study of RTP from phthalic acid isomers and other compounds adsorbed on silica gel (*12*).

The analytical applicability of RTP to quantitative analysis has been demonstrated by a number of workers. Winefordner et al. first illustrated this for a variety of compounds adsorbed on paper, including many compounds of biological importance (*13, 14*). Later von Wandruszka and Hurtubise developed a rapid and selective RTP analysis for *p*-aminobenzoic acid which employs sodium acetate as the adsorbant (*15*). These workers also reported that a number of other compounds display RTP when adsorbed on sodium acetate.

The phosphorescence emission from RTP systems has been enhanced or maximized by several techniques. The external heavy atom effect was first applied to RTP systems by Seybold and White (*16*). Sodium iodide was employed by these workers to greatly enhance the RTP emission from 2-naphthalene sulfonate and several related compounds adsorbed on paper. Similar techniques have since been employed to enhance the emission and lower the detection limit for a number of compounds of biological and pharmaceutical interest (*17, 18*). The restriction of moisture and oxygen from samples is also an important factor in obtaining strong RTP emission (*10*). Winefordner et al. have described an automated RTP system employing a "continuous filter paper device" which offers the potential of protecting RTP samples from extraneous moisture and oxygen. This device offers many potential applications in areas such as clinical and environmental chemistry.

Since room temperature phosphorescence is a relatively simple and economical technique which offers exceptional selectivity and excellent sensitivity (*10, 13, 14, 17*), we have investigated its application in the analysis of a series of pteridines. We have focused our attention on the pteridines because they represent a very interesting and unusual class of compounds, many of which exhibit important biological roles as enzymic cofactors and inhibitors or catabolites of the vitamin, folic acid (*20*). Recent reports that elevated levels of 6-hydroxymethylpterin may be indicative of neoplastic diseases have provided strong motivation for us to develop procedures for the determination of pterins in tissues and body fluids (*21*). Studies reported in this manuscript demonstrate that RTP is well suited to the analysis of pteridines. In addition, these investigations provide new insight into the physical nature of the RTP phenomenon.

EXPERIMENTAL

Chemicals. Pterin-6-aldehyde was synthesized by the sodium sulfite cleavage of folic acid using the method of Waller et al. (*22*). The reduction of pterin-6-aldehyde was employed to prepare 6-hydroxymethylpterin by the following procedure. While stirring, 2 mL of 0.13 M sodium borohydride in 0.1 M NaOH (aq.) was added dropwise to 2 mL of 0.12 M pterin-6-aldehyde in 0.1 M NaOH (aq.). Both solutions were cooled in an ice bath before the addition, and the reaction mixture was stirred for 2 h at 0 °C in the dark. Concentrated HCl was next added to bring the reaction mixture to a pH of 1, and the resulting precipitate was collected and washed extensively with 5-mL portions of cold water, ethanol, ethanol:diethyl ether (50:50 v/v) and diethyl ether, respectively. The precipitate was dried under vacuum and 11 mg of product was obtained. Paper chromatography and TLC indicated the presence of unreacted pterin-6-aldehyde in the product (*20, 23*); therefore, the crude product was further purified on an ECTEOLA-Sephadex column (*23*). Fractions were analyzed by paper chromatography, TLC, and UV in order to determine purity (*20, 23*). Pure fractions of 6-hydroxymethylpterin were pooled, lyophilized, and stored at −60 °C prior to use. Basic permanganate oxidation of the column purified product resulted in the expected quantitative conversion of 6-hydroxymethylpterin to pterin-6-carboxylic acid (*24*).

Folic acid (Sigma) was purified on a Sephadex G-10 column as described in a report on the low temperature fluorescence of folic acid (*25*). Pterin was purified by ECTEOLA-Sephadex column chromatography as described by Fukushima and Shiota (*23*). All other chemicals were of the highest available commercial purity and used as received.

The pteridines, many of which are prone to decomposition, were stored in desiccators at −60 °C. Sample purity was inspected using a Water's model ALC/GPC 202/401 high pressure liquid chromatograph equipped with a Water's 400 UV absorbance detector and a Microbondapak/C_{18} column. A mobile phase of 20 mM KH_2PO_4 (aqueous) was used at a flow rate of 1 mL/min, and the absorbance of the eluent was monitored at both 254 and 280 nm. Only minor impurities were found in the samples studied.

Phosphorescence Apparatus. Phosphorescence intensity measurements and excitation/emission spectra were obtained on an Aminco-Bowman spectrophotofluorometer equipped with a phosphoroscope attachment. A 150-W xenon arc light source and a 1P21 photomultiplier tube detector were used. Low temperature phosphorescence spectra were made using the quartz Dewar and sample tubes supplied with the Aminco phosphoroscope assembly, while room temperature phosphorescence (RTP) measurements were carried out using a sample holder previously described by us (*10*). Instrument slits were set at 2 mm throughout this study.

Since RTP samples are quenched by moisture, the instrument cell compartment was flushed with dry argon at a flow rate of 500 mL/min. The argon was dried by passing it through two drying tubes filled with Drierite and one gas wash bottle containing concentrated H_2SO_4. For RTP measurements, the argon was connected to the RTP sample holder which permitted the gas to flush directly over the surface of the sample and into the cell compartment.

Sample Preparation for Phosphorescence Measurements. A 1 mM solution of the various analytes in 0.1 M NaOH (aqueous) was employed unless otherwise noted. Since many pteridines tend to decompose upon standing in solution (*20*), all solutions were made up immediately before use. A 5-μL aliquot of the analyte solution was applied to a 7.1-mm diameter circle of Whatman 1 filter paper or similar Whatman 1 paper which had been previously impregnated with sodium acetate or other agents.

Desiccator Dried RTP Samples. Application of the analyte solution to the paper support, as well as drying of the spotted sample, was accomplished in a 75 × 10 mm test tube. About one third of the test tube was filled with indicating Drierite, and a cotton plug was inserted to constrain the desiccant to the bottom portion of the tube. The desiccant filled tube was held horizontally on a ridged tray during the insertion of the paper support, application of analyte, and drying of the sample. The paper support was placed approximately 1 cm inside the mouth of the test tube with the surface of the support lying in a level horizontal plane. The paper circle was suspended by two opposite points on its perimeter which rested against adjacent sides of the test tube wall. Both the top and bottom surfaces of the paper were free from contact with the test tube. The analyte was applied to the support with a 5-μL disposable pipet by dispensing the solution on the center of the top surface of the paper. With the solution volume employed, virtually no sample drainage was observed at the two points on the test tube wall where the edges of the paper made contact. The pipet tip was used to carefully level the sample if disturbed while spotting, and the sample tube was gently stoppered so that the position of the sample was maintained. The light sensitive samples were placed in the dark.

After drying 24 h in the test tube desiccator, the sample was removed with tweezers and mounted on the RTP sample holder (*10*). Caution was taken to handle the filter paper by the extreme edge only, and mounting was carried out carefully but rapidly to minimize the exposure of the sample in moist air. The mounted sample was placed in the phosphorimeter cell compartment, and dry argon was flushed into the cell. The sample was dried an additional 15 min in the cell to exclude moisture picked up while mounting, after which the RTP excitation and emission spectra were recorded.

Table I. Room Temperature Phosphorescence Characteristics of Selected Pteridines

compound	excitation maxima, nm[a]	emission maxima, nm[a]	relative phosphorescence intensity NaOAc/paper[b,c]	relative phosphorescence intensity paper[b,c]	intensity ratio DF/P[d]
pterin-6-carboxylic acid	375	505	strong (100)	very weak (1.4)	0.25
6-hydroxymethylpterin	375	515	moderate (15)	not measured	0.11
pterin	370	505	strong (57)	very weak (0.8)	0.13
pterin-6-aldehyde	375	515	weak (8.1)	very weak	0.20
lumazine (2,4-Dihydroxypteridine)	370	510	moderate (28)	very weak (1.2)	0.12
4-hydroxypteridine	350	490	weak (6.6)	0	0
folic acid	380	520	weak (7.4)	0	0.12
isoxanthopterin (7-Hydroxypterin)	345	495	very weak (3)	0	0
xanthopterin (6-Hydroxypterin)	-	-	0	0	-

[a] Peak maxima are reported for the compounds adsorbed on sodium acetate treated paper. [b] Whatman #1 filter paper was employed. [c] Wavelengths are rounded off to the nearest 5 nm. Relative intensities are given in parentheses for 5 nmol of adsorbed compound. The emission intensity of pterin-6-carboxylic acid has been arbitrarily set at 100. [d] DF/P = emission intensity of the delayed fluorescence divided by the emission intensity of the phosphorescence.

An analytical calibration plot was obtained for a series of pterin-6-carboxylic acid solutions using the same sample preparation procedure; however, these samples were dried 1 h in the cell before making intensity readings.

Preparation of Impregnated Supports. Whatman 1 filter paper was treated with 1 M aqueous solutions of sodium acetate or potassium iodide, or a combination of both. The 7.1-mm diameter circles were spotted with 5 μL of the agent, using the same spotting procedure described for the desiccator dried RTP samples. In the case of the sodium acetate–potassium iodide treated paper, the paper was first spotted with sodium acetate, dried, and then the potassium iodide was applied. Circles of Whatman GF-C glass fiber (6.4-mm diameter) were also treated with 10 μL of 1 M sodium acetate in a similar manner. A 10-μL Hamilton syringe was used for the application of solutions, and the iodide supports were stored in the dark.

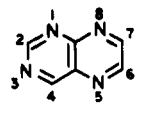
In-Cell Dried RTP Samples. Several samples were dried in the instrument cell compartment while continuously monitoring the intensity of phosphorescence with a strip chart recorder (*10*). In this case, first the paper support was mounted on the RTP sample holder, and then the sample was applied to the premounted support. The wet sample was placed in the instrument cell compartment, and the cell was flushed with dry argon to enact drying.

Samples for Phosphorescence at 77 K. A basic methanol solvent was employed for low temperature phosphorescence to prevent cracking of the quartz sample tubes upon freezing. The solvent was prepared by diluting 100 mL of 1 M NaOH (aq.) to 1 L with methanol. Sample concentration was 1 mM for all the pterins with the exceptions of pterin-6-carboxylic acid and isoxanthopterin, which formed saturated solutions below the 1 mM level. For chromatographically purified pterin, the sample was prepared by diluting 1 mL of the column eluant with 1 mL of the basic methanol solvent. Low temperature phosphorescence spectra (77 K) were recorded immediately after the solution preparation. Negligible background emission was observed from the solvent in the region of the pteridine phosphorescence.

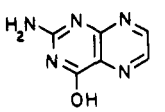
A 77 K spectrum of pterin-6-carboxylic acid was also made on paper. The sample was prepared exactly as described for the desiccator dried RTP samples. After removing the sample from the desiccator, a small strip (approximately 1 mm wide) was cut from the center of the paper circle and placed in the quartz sample tube. The sample was then handled like the other 77 K samples.

RESULTS AND DISCUSSION

Spectral Characteristics. *Phosphorescence.* Exceptionally brilliant fluorescence has long been one of the distinguishing characteristics for many of the pteridines, and analysis of the pteridines by fluorescence has proved to be an extremely sensitive and valuable technique to the clinical and biological chemist (*20*). Our investigations have revealed that a number of pteridines exhibit phosphorescence at room temperature as well. In fact, virtually all of the pteridines at our disposal proved to be phosphorescent when alkaline



PTERIDINE



PTERIN

Figure 1. Structures of pteridine and pterin (2-amino-4-hydroxypteridine)

solutions were spotted on sodium acetate impregnated paper and thoroughly dried (Table I). The only exception was xanthopterin; however, previous studies have shown that xanthopterin fluorescence disappears in strong alkaline media (*26*).

The basic pteridine chromophore can be seen in Figure 1 along with the pterin ring system. The RTP excitation maxima for pteridines are generally located in the area of 370 nm which agrees well with the usual fluorescence excitation maxima of these compounds (*20*). The phosphorescence emission maxima are typically around 500 nm as indicated in Table I. Since the vast majority of the known compounds yielding RTP exhibit excitation maxima at wavelengths shorter than 330 nm (*9, 11, 13, 14*), the RTP technique should afford extraordinary selectivity in the analysis of the pteridines.

Phosphorescence spectra were also measured from supercooled solutions of the pteridines at liquid nitrogen temperatures. Consistent with the RTP result, xanthopterin did not phosphoresce at 77 K. All other pteridines exhibited intense phosphorescence.

The low temperature spectra are qualitatively similar to the RTP spectra, although a few of the pteridine spectra show peak broadening at 77 K. Comparison of the RTP and 77 K excitation spectra of pterin illustrates this broadening in one of the more severe cases (Figure 2A). Low temperature spectra normally show slightly better resolution than RTP spectra (*9, 10, 13*); however, the 77 K pterin spectrum displays significant distortion of the excitation peak. That this is the result of impurities in the sample is suggested by comparison of the 77 K spectra for pterin with that of chromatographically purified pterin (Figure 2B). The RTP spectrum of the impure sample (Figure 2A) shows normal resolution with no indication of emission from contaminants and is virtually superimposable

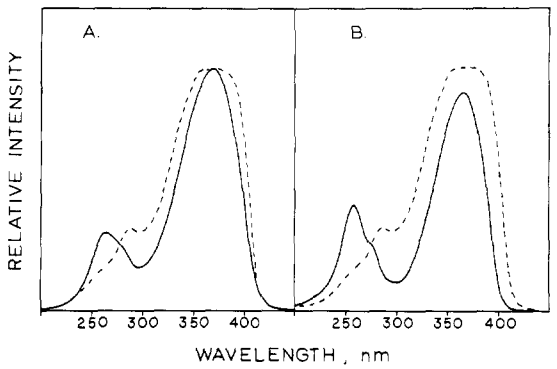


Figure 2. (A) Comparison of the excitation spectra of unpurified pterin at 77 K (---) and 300 K (——). (B) Comparison of the low temperature (77 K) excitation spectra of unpurified pterin (---) and column purified pterin (——). Low temperature (77 K) spectra were obtained from frozen solutions, and the room temperature (300 K) spectrum was made from the sample adsorbed on sodium acetate treated paper. Intensities were arbitrarily adjusted

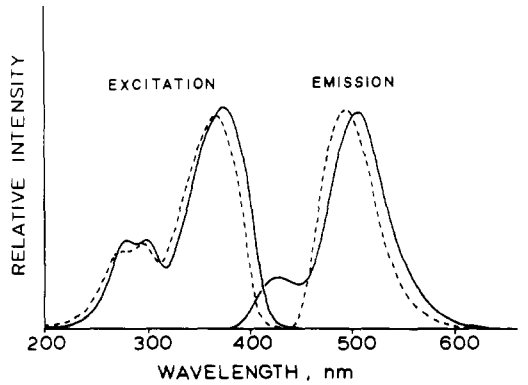


Figure 3. Excitation and emission spectra of pterin-6-carboxylic acid at 77 K (---) and 300 K (——). Both 77 K and 300 K spectra were made from samples adsorbed on sodium acetate treated paper. Intensities were arbitrarily adjusted

with the 77 K spectrum of purified pterin (Figure 2B). Results of high pressure liquid chromatography indicated that only minor impurities were present in the pterin sample; however, low temperature phosphorescence is often plagued with high background emissions resulting from trace contaminants (*2*). The exceptional selectivity of the RTP technique largely eliminated the background problems which occurred with several of the pteridines at 77 K. Also, many pteridines are difficult if not impossible to obtain in highly purified form owing to their tendency to decompose (*20*, *25*). In light of these factors RTP might offer a unique method for analyzing some pteridines in the presence of common contaminants.

Delayed Fluorescence. Delayed fluorescence, a long lived luminescence which occurs at the same wavelengths as fluorescence, was first brought to light in the classic study by G. N. Lewis (*27*). Close spacing (around 10 kcal/mol) of the lowest excited singlet–triplet energy levels (S_1–T_1 energy gap) is a molecular requirement for E-type delayed fluorescence (*28*). Sufficient thermal energy must also be supplied in order to reactivate a portion of the molecules in the lower energy T_1 state back to the S_1 state. Although phosphorescence studies at 77 K do not provide adequate thermal energy to observe E-type delayed fluorescence, RTP permits access to the thermal energy necessary for the occurrence of this interesting phenomenon (*9*).

The emission spectra of most of the pteridines display a partially resolved shoulder at room temperature which is not present in the corresponding low temperature spectrum (Figure 3). The shoulder coincides with the area where fluorescence normally occurs, and the temperature dependence

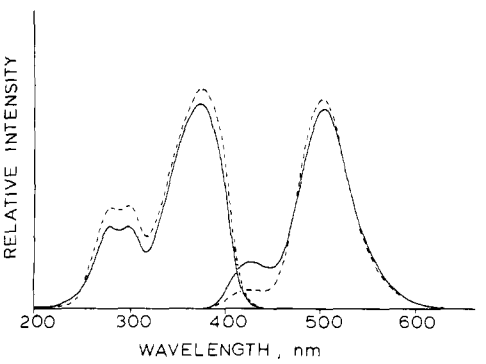


Figure 4. Comparison of the room temperature phosphorescence spectra of pterin-6-carboxylic acid samples treated with KI (---) and without KI treatment (——). Samples were adsorbed on sodium acetate treated paper and the intensities were arbitrarily adjusted

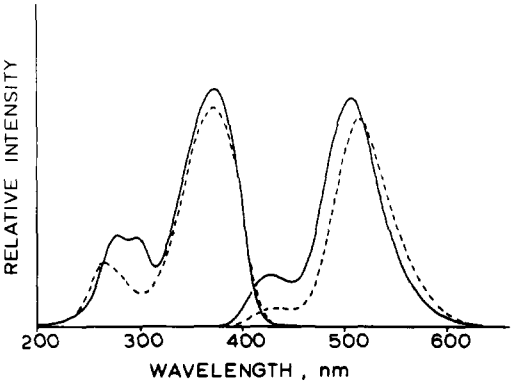


Figure 5. Room temperature phosphorescence spectra of 6-hydroxymethylpterin (---) and pterin-6-carboxylic acid (——) adsorbed on sodium acetate treated paper. Intensities were arbitrarily adjusted

of the phenomenon strongly suggests the presence of an E-type delayed fluorescence in the RTP spectrum. The small difference in the S_1–T_1 energy levels (less than 10 kcal/mol for most pteridines) as roughly approximated from determining 0,0 bands of the RTP spectra supports this conclusion. In addition, potassium iodide treatment of pterin-6-carboxylic acid (PCA) RTP samples results in a considerable reduction in the intensity of delayed fluorescence with respect to phosphorescence (Figure 4). Since the heavy atom effect enhances the rate of intersystem crossing to the T_1 state (usually observed as an enhancement of phosphorescence at the expense of fluorescence) (*29*), this result is consistent with a delayed fluorescence process. Finally, a P-type delayed fluorescence mechanism seems unlikely because the intensity ratio of delayed fluorescence to phosphorescence remains constant with extreme variations in concentration of PCA samples.

The intensity ratio of delayed fluorescence to phosphorescence (DF/P) for the pteridines studied is reported in Table I. An interesting trend can be seen upon visual comparison of the RTP spectra with the DF/P ratio. As the excitation and emission peaks show wider spacing on the wavelength axis (in other words, the S_1–T_1 energy gap increases), the ratio of DF/P decreases. Figure 5 illustrates this comparison between 6-hydroxymethylpterin and PCA. The 6-hydroxymethylpterin S_1–T_1 energy gap is approximately 1 kcal/mol larger than that of PCA, and the DF/P ratio is 45% lower for the 6-hydroxymethyl compound. Theoretical studies have predicted that the S_1–T_1 energy difference should have dramatic effects on the intersystem crossing rate (*30*), and one would expect to see a substantial decline in E-type delayed fluorescence as the S_1–T_1 energy gap increases (*28*). Our study bears out this expectation and indicates that the pteridines might provide useful models in physical studies of the effects

Table II. Effects of Support Treatment on the RTP Emissions of Pterin-6-carboxylic Acid and 4-Biphenylcarboxylic Acid[a]

support condition	relative intensity: pterin-6-carboxylic acid	relative intensity: 4-biphenylcarboxylic acid	intensity ratio (BPCA/PCA)
Whatman #1 paper	1.4	240	170
NaOAc/paper	100	420	4.2
KI/paper	1.2	550	460
NaOAc + KI/paper	50	270	5.3
Whatman GF-C (glass fiber)	0	0	- - -
NaOAc/Whatman GF-C	4.5	0.8	0.2

[a] All data after 24 h drying. Whatman #1 filter paper was employed.

of the S_1–T_1 energy gap on phosphorescence and delayed fluorescence.

Moisture Sensitivity and the Effects of Support Treatment. Initial investigations into the RTP of the pteridines showed that only very weak phosphorescence could be observed from a few of the compounds when spotted on regular Whatman #1 filler paper (Table I). It was absolutely necessary to dry these samples overnight in a desiccator in order to observe appreciable RTP emission, and exposure of the dry sample to the humidity of air quenched the greater part of the RTP emission within seconds. Although RTP samples can be fairly sensitive to quenching by moisture (*9*, *10*, *13*), the pteridines are much more sensitive to this effect than other compounds investigated by us. The unusual moisture sensitivity found in this study is probably due to the powerful hygroscopic characteristics of many of the pteridines (*20*). Had an extensive drying period not been initially attempted, the RTP of the pteridines might have been entirely overlooked.

von Wandruszka and Hurtubise have reported that RTP from compounds adsorbed on sodium acetate (NaOAc) is relatively insensitive to quenching by moisture (*11*, *15*), and Jakovljevic has enhanced the RTP of cinoxacin (a potent antimicrobial drug) by using paper supports impregnated with a number of agents (*18*). In an attempt to reduce moisture problems and increase the RTP signal of the pteridines, NaOAc treated paper supports were employed. The results obtained with this sample preparation technique are quite dramatic. Practically all of those pteridines, which show no RTP on paper, are phosphorescent on the NaOAc impregnated support (Table I). Compounds, which exhibit only weak phosphorescence when adsorbed on paper, yield brilliant phosphorescence on NaOAc treated paper. A 70-fold enhancement is noted for PCA (Table II). Furthermore, a 5-min drying period in an oven at 100 °C is sufficient to observe visible phosphorescence from the pteridines on NaOAc treated paper, and the dry samples show RTP in humid air for several minutes before being quenched by moisture. The RTP enhancement provided by NaOAc treatment might be due to the fact that NaOAc can hydrogen bond to the phosphor (*11*); therefore, the acetate impregnated paper sample matrix should provide increased immunity against collisional deactivation processes.

The effects of several different support pretreatment techniques on the RTP emission intensity of PCA are reported in Table II. As a reference of comparison, 4-biphenylcarboxylic acid (BPCA) is employed because it is one of the strongest room temperature phosphors on paper (*9*, *10*). BPCA RTP samples are also much less sensitive to quenching by moisture than PCA samples.

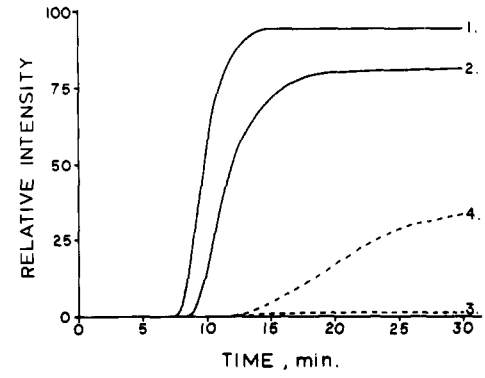


Figure 6. Room temperature phosphorescence intensity plotted as a function of drying time for 4-biphenylcarboxylic acid (BPCA) (——) and pterin-6-carboxylic acid (PCA)(- - -). The wet sample was placed in the instrument cell compartment at time equals 0 min and drying was enacted by flushing in dry Ar. The signal was monitored continuously as the sample dried. (1) BPCA adsorbed on paper. (2) BPCA adsorbed on sodium acetate treated paper. (3) PCA adsorbed on paper. (4) PCA adsorbed on sodium acetate treated paper. The relative intensities of curves 3 and 4 (- - -) are multiplied by a factor of 10

As indicated in Table II, NaOAc treatment of the paper support provides a much larger signal enhancement for PCA than BPCA. This difference can be partially understood in light of the RTP behavior of the compounds on NaOAc. Since PCA and BPCA do not show RTP when adsorbed on untreated Whatman GF-C glass fiber (Table II), comparison of PCA and BPCA on NaOAc treated Whatman GF-C indicates the RTP emission that would be expected from the samples studied on NaOAc (Table II). NaOAC provides a better support than paper for PCA; however, NaOAc functions as a very poor support for BCPA. These data are consistent with the dramatic difference in the NaOAc enhancement effect observed between PCA and BPCA. The data in Table II also indicate that NaOAc treated paper is vastly superior to either paper or NaOAc as a RTP support for PCA.

As might be expected, the hygroscopic nature of NaOAc slows the drying of NaOAc-treated RTP samples. Observation of the RTP emission from BPCA and PCA samples as they dry in the instrument cell compartment under dry Ar illustrates this phenomenon (Figure 6). BPCA on untreated paper (curve 1) shows a rapid RTP signal growth which levels off in about 15 min. The stabilization of the RTP emission gives an indication that the sample desiccation is nearly complete. The NaOAc treated BPCA sample (curve 2) shows a much slower growth in the RTP signal under the same conditions which implies that sample drying is occurring at a slower rate. After 1 h in the cell, the RTP intensity of the NaOAc treated BPCA sample is still below the intensity of the untreated sample. Given a 24-h drying period in desiccators, the NaOAc treated sample exhibits almost twice the RTP emission of the untreated sample (Table II).

PCA on untreated paper (curve 3) yields negligible RTP emission after 1 h of drying in the instrument cell. This is due to the extremely hygroscopic nature of the sample and the relatively weak emission obtained from the sample on untreated paper. PCA can be dried in the cell on NaOAc treated paper (curve 4) (Figure 6); however, the drying is very slow as compared to RTP samples on untreated paper. Although NaOAc treatment does slow the drying of RTP samples, once dry, the NaOAc matrix affords some protection of PCA samples from quenching by moisture (*31*).

One of the more unusual results in the support impregnation study is the effect of heavy atom treatment (KI) on various samples. The external heavy atom effect commonly enhances the quantum efficiency of phosphorescence at the expense of fluorescence, although exceptions have been noted (*29*). BPCA on KI treated paper exhibits the expected en-

hancement in RTP emission (Table II). On the other hand, all other samples reported in Table II show declines in RTP emission when compared to the equivalent non-KI treated samples. In the case of PCA samples, the heavy atom effect did produce the desired enhancement in the intersystem crossing rate as evidenced by a higher ratio of phosphorescense to delayed fluorescence in the KI treated sample (Figure 4). However, the phosphorescence intensity gain concomitant with the increase of the intersystem crossing rate is not realized (see intensities in Table II). This seems to indicate that the nonradiative decay rate from the lowest excited triplet to the ground singlet state is enhanced with respect to the radiative triplet decay process.

Several factors might be partially responsible for the declines in the RTP emission intensity observed in several of the KI treated samples. KI might disrupt the integrity of the sample matrix, possibly by interfering with the hydrogen bonding network between the phosphor and support. This would increase the likelihood of collisional deactivation processes (nonradiative decay of the triplet state). The results of KI treatment on BPCA samples adsorbed on NaOAc/ treated paper seem to indicate something unusual has occurred within this sample matrix. The addition of KI to BPCA samples adsorbed on paper illustrates that the heavy atom effect provides a 2.3-fold enhancement of the RTP emission. NaOAc treatment of BPCA samples on paper also shows a 1.8-fold RTP emission enhancement; therefore, one might expect even greater signal enhancements by impregnating the sample with both KI and NaOAc. This is not the case. BPCA samples treated with both agents actually yield a much lower RTP emission than samples treated with only one agent (Table II). Obviously some interaction has occurred in the samples impregnated with both agents which is unfavorable to the radiative pathway of triplet state deactivation. As mentioned earlier, the RTP emission from PCA was adversely affected by KI treatment of samples adsorbed on paper or NaOAc impregnated paper, even though the KI produced the desired enhancement of the intersystem crossing rate.

The RTP emission from KI treated samples was also noted to be more sensitive to the quenching effects of moisture (*31*). This factor might also play a part in the reduction of the RTP emission from KI treated samples. The increase in moisture sensitivity of these samples also lends evidence that the presence of KI has somehow altered the sample matrix. A weakening of the hydrogen bonding between the phosphor and the support matrix would allow moisture to more readily displace the phospher molecules from the support and might account for the intensity reductions and increased moisture sensitivity observed. Finally, since the mechanism of the heavy-atom effect is not fully understood, various other unknown factors could come into play (*29*). RTP might well be put to use in shedding more light on the nature of the heavy atom effect.

Analytical Potential. The analytical applicability of RTP to the pteridines was demonstrated by constructing a calibration plot for PCA using the NaOAc treated support. For maximum reproducibility and lowest detection limit, the samples were dried overnight and equilibrated 1 h in the instrument cell before making measurements. A detection limit of 1 ng and a range of linearity extending from 1 ng to slightly above 100 ng is obtained by this method. The phosphorescence background generally limited the detection limit to an analyte signal approximately two times that of the background emission. The relative standard deviations of the measurements are excellent (typically around 3.5% for triplicate measurements). Although the procedure used requires considerable drying time, this can be shortened significantly (with some loss in reproducibility and detection limit) by applying in-cell drying techniques reported by us earlier (*10*).

Our data clearly indicate the quantitative ability of the RTP technique for the pteridines. Application of an automated RTP system such as that described by Winefordner et al. (*19*) should also allow rapid RTP analysis of the pteridines with virtually no sample handling involved. An automated system would cope beautifully with the moisture problems encountered with the pteridines since the sample could be transported directly from the drying compartment to the instrument cell compartment without being exposed to moist air. Finally, the extraordinary selectivity of the RTP technique for the pteridines should offer an excellent potential to avoid complex separations in many biological analyses.

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