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Note

Use of phosphorescence in the detection of sub-nanomolar quantities of 4-methylumbelliferone and its derivatives on chromatograms

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Derivatives of 4-methylumbelliferone (4-methyl-7-hydroxycoumarin, MU) are now very important as fluorogenic substrates for the estimation of enzyme activities. Although more than thirty such derivatives have already been described for this purpose[1], others are required to extend the range of the assays and thereby help to solve urgent problems in clinical diagnosis. Chromatographic methods can be valuable in following the preparation of such compounds and in the examination of their purity[2,3]. It has been the practice in this laboratory over many years to detect compounds of MU after chromatography by means of their native fluorescence or of the fluorescence of MU liberated by various treatments of the chromatograms[2]. Experience shows, however, that the detection of such compounds using their fluorescence (excitation and fluorescence maxima near 317 nm and 375 nm respectively)[1,2] is insensitive, is hazardous to the eyes, and the treatment of the chromatograms by heat, acids, bases or enzyme preparations is erratic in performance and destructive to the compounds of interest.

We now describe a novel approach to the detection of MU and some of its important derivatives on chromatograms using low-temperature phosphorescence. The experimental method involved is very sensitive, much less hazardous to the eyes, and is non-destructive of the compounds of interest. Some original observations are also included on the spectroscopic basis of the method and these suggest that this experimental approach should find wider application.

EXPERIMENTAL

Materials

Methylumbelliferone (Koch-Light Labs., Colnbrook, Great Britain) was recrystallised three times from hot ethanol and was then homogenous when chromatographed on Whatman No. 1 paper in of *n*-butanol–ethanol–water (4:1:5, v/v) and viewed under ultraviolet light[2]. 4-Methylumbelliferyl 2′-acetamido-2′deoxy-β-D-glucoside (I) and 4-methylumbelliferyl β-D-galactoside (II) were prepared as described previously[2,4]. Chromatography was carried out ascendingly using Whatman No. 1, No. 20 or No. 3MM papers, or aluminium foil-backed cellulose thin layers [Merck

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(Darmstadt, G.F.R.) 5552 plates without fluorescent indicator]. The plates were washed (pre-run) extensively with absolute ethanol before use.

Detection of luminescent zones on chromatograms

In Fig. 1 are illustrations of the experimental arrangements developed for the detection of luminescent (fluorescent and phosphorescent) zones on electropherograms, or on thin-layer and paper chromatograms.

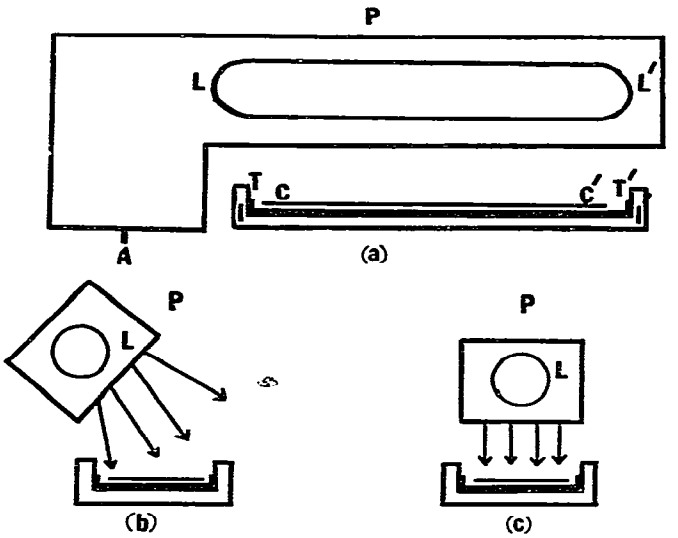

Fig. 1. Schematic diagrams (a: side view; b and c: end views) of experimental arrangements for fluorescence (b) and phosphorescence (c) observation or photography of zones on chromatogram (CC′) resting in a shallow, insulated (I) metal tray (TT′). Observation or photography were carried out from a point P during (for fluorescence) or after (for phosphorescence) irradiation at 313 nm by the strip mercury lamp (LL′) pivoted about A.

A chromatogram (CC′) was placed in a metal tray (TT′) and the observation and photography of fluorescent zones were carried out from a point P vertically above the tray (Fig. 1b), using a Hanovia mercury strip lamp (LL′; fitted with a 313 nm optical interference filter) inclined at an angle of incidence of about 45°. For the corresponding observation and photography of phosphorescent zones, chromatograms were immersed in a thin layer of liquid nitrogen and the mercury lamp was placed vertically above the tray (Fig. 1c). The lamp was then swung away rapidly by hand (about the pivot at A) before observation or photography.

Measurement of phosphorescence

Phosphorescence excitation and emission spectra were determined at 77 °K on the substances of interest dissolved in water–1,2-ethanediol (1:1, v/v) using a Baird Atomic Fluorospec SF100E spectrophotofluorimeter with the phosphorimetric acessories (quartz Dewar flask, optical chopper etc.) supplied by the manufacturer. Phosphorescent spectra or scans of such zones on the chromatograms were performed on the same Baird-Atomic instrument but using accessories developed at the University of Loughborough for this purpose[5].

Other spectral measurements on chromatograms

Fluorescent emission and radiation scattered from the surface of chromatograms were measured (at room temperature at an angle of incidence of 23°) using the versatile, highly sensitive, single-beamed spectrofluorimeter constructed in this laboratory[6].

RESULTS AND DISCUSSION

The phosphorescence excitation and emission spectra of MU and the glycoside (I) were determined at 77 °K in an aqueous ethanediol glass. It can be seen (Figs. 2a, b) that both MU and derivative I were excited maximally at about 325 nm and phosphoresced maximally near 485 nm. The corresponding galactoside (II) gave essentially similar results and zones of all three of these substances also showed similar spectral characteristics on chromatograms at 77 °K.

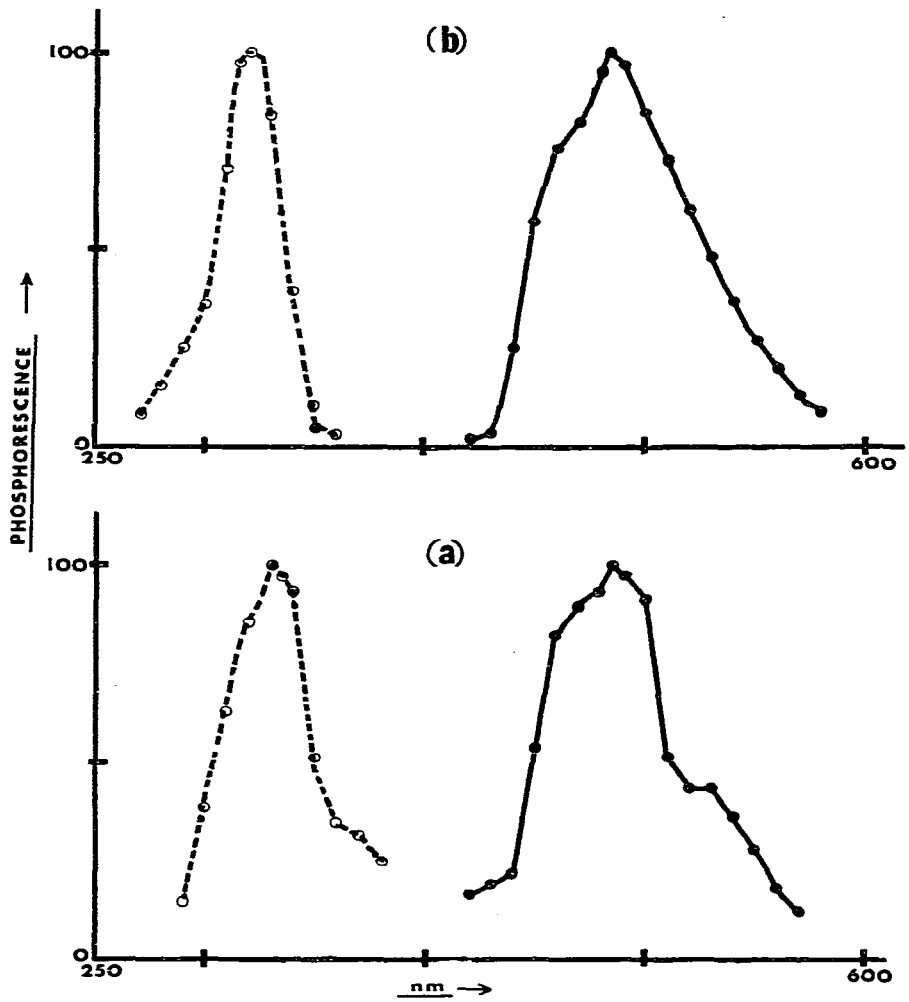

Fig. 2. Phosphorescence excitation (○- - -○) and emission (●—●) spectra of (a) MU and (b) glycoside I determined at 77 °K in aqueous ethanediol glasses.

Preliminary experiments entailing the rapid closing of the primary shutter of the measuring instrument showed a consequent decay of the phosphorescence of MU and the glycoside (I) with half-lives for both substances about 1 sec; the phosphorescence of contaminants residual in the pre-washed chromatograms was of much shorter half-life under these conditions.

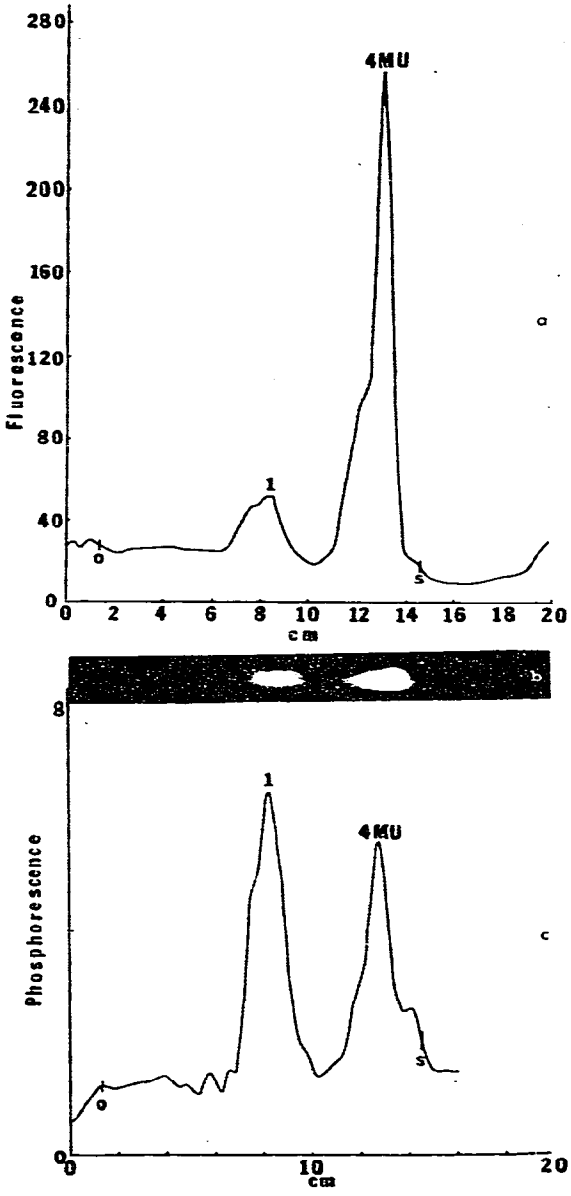

Fig. 3. (a) Fluorescence scan at 380 nm and room temperature of the chromatogram illustrated at (b) which had been developed on Whatman No. 20 paper bearing zones of MU (5 nmoles) and the glycoside I (4 nmoles). (c) Phosphorescence scan at 485 nm of the same chromatogram illustrated in (b) and maintained at 77 °K. The samples of MU and the glycoside were applied at a point corresponding to 0 on the traces and the solvent front reached a point indicated at S. In each case the zones were irradiated at 325 nm.

A photograph of the fluorescent zone of MU and the glycoside after chromatography is to be seen in Fig. 3b and a fluorescence scan of these zones is shown in Fig. 3a. The wavelengths employed in the scan (excitation, 325 nm; emission, 380 nm) were peculiarly favourable to the glycoside yet the fluorescence readings for this compound were surprisingly small. After carrying out fluorescence measurements on MU at comparable concentrations to those of the glycoside, and on the glycoside at smaller concentrations than that used in Fig. 3a, it was concluded that neither an "inner filter" nor a concentration-dependent quenching effect was involved. It seems therefore that the low levels of fluorescence readings for the glycoside must be due to a relatively small quantum efficiency in zones on dried chromatograms, but that the poor sensitivity of the visual detection of the fluorescent glycoside (see Table I) must also be attributed to the diminuitive sensitivity of human scotopic vision near 380 nm (*cf.* ref. 7). The results of phosphorescence scans (excitation, 325 nm; emission, 485 nm) are shown in Fig. 3c and, in contrast to the corresponding fluorescence measurements (Fig. 3a) the intensities of phosphorescence of MU and the glycoside are comparable. Integration under these emission curves (corrected for the slight difference in the concentrations employed) revealed that the glycoside was about twice as phosphorescent as MU under these conditions.

TABLE I

LOWER LIMITS OF MU AND THE GLYCOSIDE (I) DETECTABLE USING INSTRUMENTAL AND VISUAL MEANS

Detection method	*Compound*	*Lower limit of detection of compound (moles)*	
		*In aqueous solution**	*On Whatman No. 20 chromatogram***
Fluorescence	MU	$2 \cdot 10^{-14}$	$1 \cdot 10^{-10}$
	Glycoside I	$1 \cdot 10^{-13}$	$15 \cdot 10^{-9}$
Phosphorescence	MU	—	$2 \cdot 10^{-10}$
	Glycoside I	—	$5 \cdot 10^{-11}$

* Amount of compound in 1 μl giving reading, under conditions of Leaback and Walker[2], equal to control reading.

** Amount of compound (normally applied to chromatogram from 1 μl solution) detectable by eye using the apparatus illustrated in Fig. 1. Similar results were obtained on Whatman No. 1 or No. 3MM chromatographic papers or cellulose thin-layer chromatograms (Merck).

The lower limits of detectability of MU are about the same by either the phosphorescence or fluorescence procedures, whereas the limits for the detection of the glycoside are more than two orders of magnitude lower using phosphorescence (see Table I). Such results may in part be attributable to differences in various quantum efficiencies, but the difficulties of detecting small amounts of fluorescent emission (at about 380 nm) in the presence of intense excitation radiation scattered from the chromatographic material, must also be important. This can be illustrated by reference to Fig. 4b, where the relatively large quantities of glycoside present (2

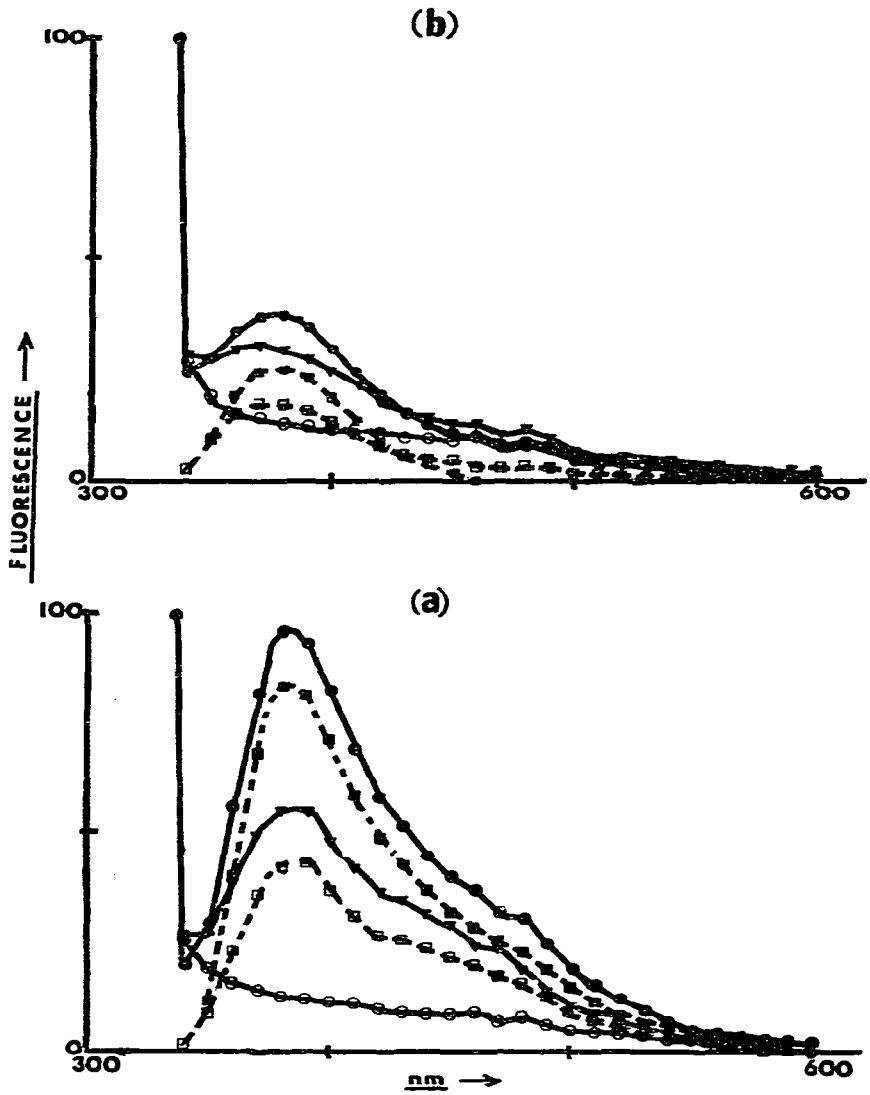

Fig. 4. "Fluorescence" readings against wavelength (actually fluorescence plus scattered incident radiation) for chromatograms developed on Whatman No. 20 paper during irradiation with 325 nm light for (a) zones containing zero (○- - -○); 2.5 nmoles (▲—▲) and 5 nmoles (●—●) of MU, (b) zones containing zero (○—○); 2 nmoles (●—●) and 4 nmoles (○- - -○) of the glycoside I. Corresponding curves representing the difference between the zones containing MU or the glycoside are represented by broken lines: □- - -□ and ■- - -■.

or 4 nmoles per zone) gave readings of intensity comparable to the background (mainly scattered) radiation.

The results in Fig. 4a show that MU fluoresces maximally near 380 nm on dried chromatograms. This emission can be identified as that of unionised MU by its similarity to that (380 nm) of the MU conjugates, and by its dissimiliarity to that (450 nm) of the MU anion[1,2]. This is the first report of the fluorescent emission of unionised MU and contrasts with the strong photo-ionisation[1] of MU that takes

place in solution even at pH values well below its ground-state pK' value of 7.8. Calculations for MU based on the Weller[8] equation (as modified by Bridges *et al.*[9] and using available optical absorption data[1]) gave an estimated excited state pK' of 1.5 for MU. This rationalises the absence of fluorescence from the unionised form of MU in solution (even under moderately acidic conditions)[10] and the unexpected emission of unionised MU on the air-dried chromatograms must be due to the paucity of water and/or to the adsorption of the phenol on the cellulose fibres. This phenomenon could complicate any quantitation of MU on chromatograms by fluorescence measurements, and indicates that assays of MU itself should be carried out in solutions by existing fluorimetric methods[1,2]. On the contrary, mixtures of MU derivatives are best examined after chromatography by the phosphorescence procedures described here and work is in progress to make the approach quantitative.

The advantages of phosphorescence methods for the detection of luminescent substances on chromatograms, and the fundamental and instrumental bases for these advantages, do not seem to have been recognised in recent extensive accounts of fluorescence and phosphorescence methods of analysis[11–13].

ACKNOWLEDGEMENTS

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