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SOLUBILIZATION AND FLUORESCENCE EMISSION SPECTRA OF SUBSTANCES GENERATED BY THERMAL TREATMENT OF CARBOHYDRATES ON SILICA GEL

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SUMMARY

Reproducible solubilization of fluorescent derivatives of glucosamine and glucose from heated silica gel adsorbent is reported. The fluorescence emission spectra of these compounds were also measured. It was found that the fluorescent compounds remain stable from −20 to 30°C within a wide pH range (4–10). The presence of an adsorbent such as silica gel, paper or glass microfibre paper is essential for the generation of fluorescent derivatives. The fluorescence excitability was found to be reversible under certain conditions, at least for glucosamine.

INTRODUCTION

Since the initial observation that glucosamine could be easily rendered visible on paper chromatograms on heating without reagents, several advances have been made, as follows. A thermal–UV method was developed for the detection and recovery of organic compounds on paper chromatograms[1]. This procedure is based on the appearance of visible spots, detected under UV or visible light, on heating the paper after chromatography, and gives a high recovery. Moreover, with this procedure it is possible to detect N-acetyl amino acids, which is difficult with other methods[2].

The procedure was then extended to thin-layer chromatography (TLC) on silica gel plates[2]. A characteristic temperature-dependent pattern was observed under UV and visible light for a variety of organic compounds[3]. In addition, chemically related substances generated specific fluorescent colours. However, the fluorescent derivatives could not be properly analysed owing to the difficulties encountered in solubilizing them from the silica gel matrix. Thus, to have the fluorophores in a soluble state in a reproducible and stable form is important to study the properties of these compounds.

In this paper, we report the reproducible solubilization of glucosamine- and glucose-derived fluorophores from a silica gel matrix. These sugars, which are widely distributed in nature, were selected because information about their properties using

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the thermal–UV method is well established[1–3]. In addition, we describe other important properties, such as the fluorescence emission spectra of the derivatives and the reversibility of the fluorescence excitability of glucosamine-derived fluorophores under certain conditions.

EXPERIMENTAL

Materials

Glass-based silica gel 60 thin-layer plates (20 × 20 or 5 × 20 cm, 0.25 mm thick) were purchased from Merck (Darmstadt, G.F.R.). The plates were washed overnight using methanol–water (1:1). Whatman No. 1 paper was used.

[U-^{14}C]Glucose (285 Ci/mol) and [6-^{3}H]glucosamine (10 Ci/mol) were obtained from New England Nuclear (Boston, MA, U.S.A.). Glucosamine and glucose were reagent-grade materials from Sigma (St. Louis, MO, U.S.A.).

Analytical procedures

For precise heating of the plates, a Packard Model 804 gas chromatograph oven equipped with a Model 873 temperature controller was used, with which the desired temperature was achieved in about 30 sec in all instances. Usually the starting temperature for heating was 10°C below the setting temperature.

The plates, papers and tubes (cooled or hot) were observed under visible and longwave UV light (366 nm). The temperature of the plate used to study the reversibility of fluorescence was measured using a Fluka Model 8020 A meter, interfaced with a Fluka Model 80 T 152 temperature probe.

Radioactivity was measured in a Beckman Model 8100 scintillation counter after drying the adsorbent and solvent with air at room temperature, resuspending with 2 ml of water and 10 ml of Bray solution[4].

Fluorescence measurements were made with an SLM Model 4800 spectrofluorimeter interfaced with a conveniently programmed Hewlett-Packard Model 9825 computer and recorded with a Hewlett-Packard Model 7225 A plotter. Emission spectra were obtained at room temperature. Excitation of fluorescence was carried out at 320 nm for glucosamine and 336 nm for glucose. These wavelengths were selected to obtain the maximum fluorescent radiation for each compound. The resolution in both monochromators was set at 4 nm in all the measurements.

A blank was measured at the end of each experiment and subtracted from the emission spectra. Spectra were corrected for instrument response using a series of correction factors supplied by the manufacturer. The intensity of the excitation light was monitored with a reference photomultiplier tube.

RESULTS

Role of the adsorbent in the generation of fluorescent compounds

To determine if the adsorbent is required during the thermal treatment for the induction of fluorescence of glucosamine and glucose, the following experiment was performed. About 10 mg of glucosamine and glucose were placed separately on a glass plate without adsorbent and the plate was heated from 50 to 200°C in steps of 10°C with 5 min at each temperature. The substances were examined under visible

and UV light after each heating step. Non-heated substances were simultaneously observed as a control. Fluorescence was not detected for either glucosamine or glucose over the entire temperature range tested. Under visible light a yellow colour appeared at 90°C for glucosamine and at 140°C for glucose, as would be expected for a charring reaction.

As previously described, fluorescent compounds from the above-mentioned carbohydrates were generated during thermal treatment when they were spotted on paper[1], silica gel and glass microfibre paper[2]. The present results demonstrate the absolute requirement for an adsorbent for fluorescence excitability.

These observations are compatible with previously described transformations of organic compounds due to secondary interactions between the substances and the adsorbent[5–7].

Solubilization of the fluorescent material

Zones of 1 cm^2 on a silica gel plate were spotted separately with the following solutions: 0.5 ml of water, 1.5 mg of glucosamine in 0.5 ml of water and 1.5 mg of glucose in 0.5 ml of water. In some instances about 20,000 cpm of [^{3}H]glucosamine or [^{14}C]glucose were added to the corresponding solutions before spotting. The plate was dried with air at room temperature and then heated at 60°C for 10 min as described under Experimental. After heating, the silica gel zones corresponding to water and glucosamine were scraped off and quantitatively transferred into glass tubes. The plate was subsequently heated at 120°C for 10 min and the procedure was repeated for glucose- and water-spotted zones.

In all instances, the extraction was carried out by vortexing the silica gel mixed with 1 ml of methanol–water (1:1) and centrifuged at 1040 *g* for 15 min. The extraction was repeated four times. The solvent fractions were pooled for fluorescence spectra measurements.

Table I summarizes the results obtained in the different steps of the extraction procedure in which radioactivity and fluorescence were monitored in the solutions

TABLE I

SOLUBILIZATION OF THE FLUORESCENT COMPOUNDS GENERATED BY HEATING GLUCOSAMINE AND GLUCOSE ON SILICA GEL

GlcN = glucosamine; Glc = glucose. + = Easily detected under visual inspection; ± = weak detected; − = not detected.

Extraction step	*Radioactivity remaining in the adsorbent, %*		*Fluorescence of the dried adsorbent**		*Solunilized radioactivity, %*		*Fluorescence of the washing solutions**	
	GlcN	*Glc*	*GlcN*	*Glc*	*GlcN*	*Glc*	*GlcN*	*Glc*
Initial	100	100	+	+	0	0	−	−
1st	27	21	+	+	72.5	79	+	+
2nd	3.3	2.2	±	±	23.5	18.5	+	+
3rd	0.8	0.5	−	−	2.2	1.3	±	±
4th	0.27	0.23	−	−	0.4	0.2	−	−
5th	0.26	0.23	−	−	0	0	−	−

* Fluorescence was monitored by visual inspection, except in the 5th step. See text for details.

and in the dried adsorbents. The fluorescent solutions from glucosamine and glucose revealed strong blue and yellow colours, respectively, under UV light.

As shown in Table I, the radioactivity remaining in the previously heated silica gel after washing was about 0.25% of the amount initially spotted, for both glucosamine and glucose. The solution from the last washing step cannot be distinguished from the blank [methanol–water (1:1)] when examined for spectral analysis under the conditions described below. These results indicate that the substances responsible for both the radioactivity and fluorescence were almost quantitatively dissolved from the silica gel.

The fluorescent solutions apparently remain unchanged for at least 2 weeks at either −20°C or 30°C. Moreover, the solutions could be dried with air at 25°C and redissolved without noticeable changes in colour or intensity. The residues of the dried solutions were also stable for at least 1 week and their colours did not differ from those of the corresponding solutions.

To determine the effect of pH on the fluorescent stability of the solutions, the latter were made acid (pH 4) or alkaline (pH 10) by addition of hydrochloric acid and sodium hydroxide, respectively. After 1 h at room temperature, no changes in colour or apparent intensity were observed under UV light in either instance.

Fluorescence emission spectra

The spectral curves for the glucosamine- and glucose-derived fluorescent compounds were obtained from four separately processed spots for each sugar as described above. The emission spectra for glucosamine and glucose are shown in Fig. 1, each curve being the result of the subtraction of the values for the heated adsorbent containing the sample from those of the heated silica gel without the sample (blank). The heating temperatures of the blank were 60 and 120°C for glucosamine and glucose, respectively. In each separate experiment a single peak was obtained. With

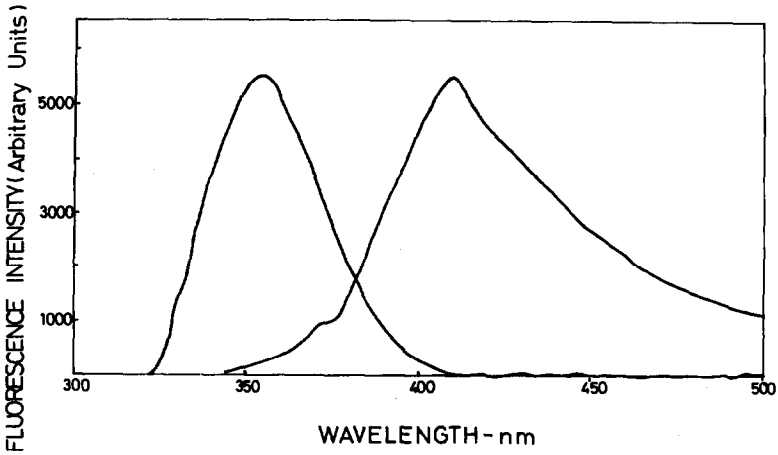

Fig. 1. Fluorescence emission spectra of solubilized fluorescent compounds derived from glucosamine and glucose. Excitation wavelengths: 320 nm for glucosamine and 336 nm for glucose. The resolution in both monochromators was set at 4 nm. Emission peaks are localized at 357 nm for glucosamine and 410 nm for glucose. The glucose curve was normalized to that of glucosamine (original glucose peak intensity = 3600 arbitrary units).

glucosamine derivatives the peak appeared at 357 nm whereas for glucose derivatives it was at 410 nm.

The spectral analysis of methanol–water (1:1) does not give a significative fluorescent response at the excitation wavelengths selected for maximum intensity emission for glucosamine and glucose.

Spectra from quadruplicate samples corresponding to one sugar were virtually identical with respect to emission peak wavelength and the peak intensities, differing only about ± 5% from the mean, thus indicating that the entire procedure was highly reproducible.

Reversibility of fluorescence

A paper disc was immersed for a few seconds in a 10 g/l solution of glucosamine in water and dried with air at room temperature. The paper was heated by pressing for about 15 sec on a metallic surface at 130°C and immediately examined under UV light. A strong characteristic blue fluorescence was generated in the zone of the paper that was in contact with the hot metal. After 5–10 min at 25°C, all the fluorescence disappeared. This experiment was repeated many times using the same paper. If the heating time was increased to about 1 min, the fluorescence became stable. It appeared that the fluorescent compounds required a minimum heating time at a given temperature to become stable. On the other hand, a paper heated as above but without glucosamine did not show any fluorescence after heating.

This is the first evidence for reversibility of the fluorescence induced by heating organic compounds spotted on a solid adsorbent.

DISCUSSION

The results obtained demonstrate the reproducible and almost quantitative solubilization of fluorophores derived from glucosamine and glucose on heating the carbohydrates in silica gel. Previous efforts by this[1,2] and other groups[7,8] to obtain reproducible solubilization were unsuccessful. In the previous work, the specific temperature-dependence pattern was not determined. The establishment of this parameter by the thermal–UV method was very useful in achieving a high recovery of the originally spotted substances after detection[3]. This suggested the possibility that also the fluorescent derivatives could be solubilized and obtained with high recovery, as shown in this work. This solubilization and the preservation of the fluorescent properties clearly indicate that the silica gel matrix was not required to stabilize the fluorophores.

Using chemical reagents together with the thermal–UV procedure, other groups were able to quantitate the fluorescence derived from organic compounds in the dry solid state[5,9]. Accurate measurement of the fluorescence emission spectra was difficult on the silica gel plates, owing to the irregular distribution of the substance in the spot[5] and light scattering[10]. The exhaustive separation of the fluorophores from the matrix, achieved in this work, opens up the possibility of the quantification of the fluorescent compounds in solution. Our results reveal for the first time the reproducible fluorescence emission spectra of glucosamine and glucose derivatives in solution.

The usual chromatographic properties of a particular compound together with

the characteristic temperature-dependent detection pattern[3] and the precise determination of the fluorescence emission spectra allow a more complete discrimination of chemically related compounds as reported for glucosamine and glucose. This will be particularly suitable for other chemically related substances that also generate different fluorescent colours at specific temperatures, *e.g.*, methyl glucosides. In this instance, the change in the position of a methyl group determines the colour shift observed after thermal–UV detection[3]. These properties may be used to confirm the identification of organic compounds. It should be pointed out that after all the procedures described here, the originally spotted sample is recovered, retaining most if not all of its characteristic properties. Further, the fact that fluorophores remain stable in solution may allow the study of the structure of these compounds, thus permitting the possibility of studying the mechanism of the chemical reactions of fluorescence induction.

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