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FLUORESCENT COLOURATION OF ORGANIC ANIONS WITH PINACRYPTOL YELLOW ON CELLULOSE LAYERS*

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SUMMARY

A fluorescent colouration of anionic substances with Pinacryptol Yellow (PY) on cellulose layers was investigated. It was found that strongly acidic organic anions such as R–O–PO_3^{2-}, R–O–SO_3^-, R–SO_3^-, R–PO_3^{2-}, RNH–SO_3^- and RNH–PO_3^{2-}, in which the substituent R is larger than n-C_3H_7, are positive to this reaction. It was also proved that both the increments of the anionic groups in a polyanionic compound and the degree of polymerization in a high-molecular anionic compound contribute positively to this phenomenon, and the presence of hydroxyl group(s) in an anionic compound produces a negative effect. The detection limits of various types of organic anions including natural or synthetic sulphated polysaccharides were examined on layers of different chromatographic media.

From the spectral examination, this fluorescent colouration was concluded to result from the formation of PY–organic anionic complexes on thin layers.

INTRODUCTION

In 1955, HOLNESS AND STONE[1] reported the use of the dye Pinacryptol Yellow (PY)** as a spray reagent for the detection of long-chained alkyl sulphates on paper chromatograms. Since then, PY has been widely used for detecting anionic detergents such as alkyl sulphates, alkyl sulphonates and alkylbenzene sulphonates[3,4]. This method has also been used for the detection of an N-substituted sulphamic acid, cyclamate[5].

This paper deals with an examination of the fluorescent colouration of organic anionic compounds with PY on cellulose layers in relation to their chemical structures.

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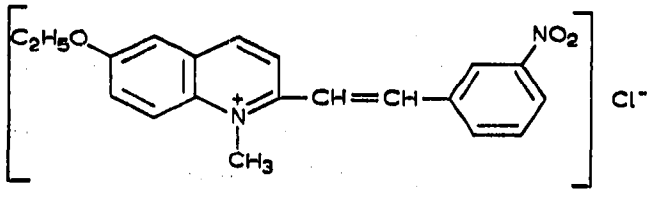
** Pinacryptol Yellow (abbreviated as PY)[2], which is commercially available in the chloride form, is a yellow powdery material and fluoresces orange under UV light. Its chemical structure is:



EXPERIMENTAL

Materials

All materials used were of analytical-reagent quality whenever possible, but otherwise of the best available grade. The following materials were prepared in this laboratory according to the references cited or by the procedures described: $ROSO_3Na$ (R = C_2H_5, *n*-C_3H_7, *n*-C_4H_9 or C_6H_{11}) and sodium pentaerythritol tetrasulphate were synthesized by sulphating ROH or pentaerythritol with chlorosulphonic acid in pyridine; RSO_3Na (R = *n*-C_3H_7, *n*-C_4H_9 or *n*-C_6H_{13}) by oxidizing RSH with performic acid; $RNHSO_3Na$ (R = C_2H_5, *n*-C_3H_7, *n*-C_4H_9 or *n*-C_8H_{17})[6]; $ROPO_3Na_2$ (R = C_6H_{11}, *n*-C_8H_{17} or $CH_3CH(OH)CHCH_3$) by phosphorylation of ROH with phosphoryl chloride in pyridine; potassium *trans*-2-hydroxycyclohexyl phosphate and sodium 2-hydroxypropyl phosphate by phosphorolysis of the corresponding oxide compound[7,8]; RPO_3Na_2 (R = C_2H_5, *n*-C_3H_7, *n*-C_4H_9 or *n*-C_6H_{13})[9]; $RNHPO_3Na_2$ (R = H or C_6H_{11})[10]; sodium *trans*-2-hydroxycyclohexyl sulphamate and sodium 2-sulphoamino-2-deoxy-D-glucose by sulphation of the corresponding hydroxylic amino compound with the pyridine–sulphur trioxide complex in aqueous alkali[11]; potassium D-glucose mono- and disulphate[12]; sodium D-glucose polysulphate and maltose polysulphate by sulphation of the corresponding saccharide with the triethylamine–sulphur trioxide complex in dimethylformamide[13]; and sodium dextran sulphate by sulphation of dextran with piperidine-N-sulphonic acid in dimethyl sulphoxide[14]. Keratosulphate and carrageenan were kindly supplied by Seikagaku Kogyo Co. Ltd., Tokyo, Japan.

PY salts of organic anionic compounds were prepared by mixing PY chloride with an equimolar amount of the sodium or potassium salt of an organic anion in water, followed by crystallization from ethanol or aqueous ethanol. Characteristics and analytical data of the PY salts obtained are as follows:

PY salt of	*Molecular formula*	*Characteristics*
Propyl sulphate	$C_{20}H_{19}N_2O_3 \cdot C_3H_7O_4S \cdot 2H_2O$	m.p. 202° (decomp.), yellow needles
Dodecyl sulphate	$C_{20}H_{19}N_2O_3 \cdot C_{12}H_{25}O_4S \cdot H_2O$	m.p. 180–182° (decomp.), yellow rod crystals
N-Cyclohexylsulphamic acid	$C_{20}H_{19}N_2O_3 \cdot C_6H_{12}NO_3S \cdot 2H_2O$	m.p. 266° (decomp.), yellow needles
Pentaerythritol tetrasulphate	$(C_{20}H_{19}N_2O_3)_3 \cdot C_5H_8O_{16}S_4 \cdot 2H_2O$	m.p. 252–254° (decomp.), fine yellow needles

In this investigation, Avicel SF (Funakoshi Pharmaceutical Co. Ltd., and Asahi Kasei Co. Ltd., Tokyo, Japan), a finely powdered product of Avicel for thin-layer chromatography (TLC), was used as the cellulose material for preparing thin layers. Polyamide (Polyamide Woelm TLC), silica gel (Silica Gel G, Merck), alumina (aluminum oxide, Woelm neutral), and DEAE cellulose (Serva DEAE-TLC) were also used for the TLC.

Examination of the fluorescent colouration of organic anionic compounds with PY on cellulose thin layers or papers

Samples were dissolved in distilled water and 1 μl of the test solution (5 μg sample) was spotted on the starting line 2.5 cm from the edge of the plate, which was

coated with Avicel SF (0.25 mm thick). The plate was developed ascendingly at 25° in a closed tank until the length of the run was 10 cm, by using one of the following solvents: (a) methanol–acetone–water (6:2:2); (b) ethyl acetate–conc. ammonia–acetone (1:1:8); and (c) isobutanol–conc. ammonia–water (7:1:2). The developed plate was dried in air and sprayed evenly with 0.1 % (w/v) PY chloride solution in 95 % ethanol, dried in the dark for 10 min, and observed for the appearance of a yellow to orange fluorescent spot against a pale greenish background under UV light (365 nm).

For the development of inorganic anions, acid polysaccharides and sulphated polysaccharides, paper electrophoresis was carried out on Toyo Roshi No. 51 filter-paper (12 × 25 cm) with pyridinium acetate buffer, pH 5.8 (pyridine–acetic acid–butanol–water, 5:1:5:250, v/v), and submitted to a potential gradient of 20 V/cm for 15–30 min. Five μl of the test solution (25 μg sample) were spotted on the starting line 4.5 cm to the cathodic side from the centre of the filter-paper. The developed paper was dried in air and placed in a closed tank filled with gaseous ammonia for 20 min at room temperature. The paper so treated was treated in a similar manner as for TLC.

The judgement as to whether a sample was positive or not to this fluorescent colouration was made according to the procedures described above.

Spectral measurement of samples in solution or on a glass plate

Fluorescence emission spectra of samples on glass plates were measured with an Aminco-Bowman spectrofluorimeter as follows. A small glass plate (1.2 × 4.8 cm) coated with Avicel SF was sprayed evenly with a solution of the sample to be tested. After being dried in the dark, the sprayed plate was inserted along the diagonal line of a quartz cell with a 10-mm light path to fix the surface of the plate at an angle of 45° to the light source of the fluorimeter, and the emission spectrum on the plate was measured under excitation at 405 nm. To obtain an adequate spectrum, the sensitivity of the fluorimeter (per cent of full-scale, pfs) was adjusted according to the concentration of the sample on the plate.

RESULTS AND DISCUSSION

On cellulose thin layers or papers, the anions positive to the fluorescent colouration with PY appear as an orange fluorescent spot against a pale greenish background under UV light. Various types of anions, including both organic and inorganic compounds, were developed on cellulose thin layers or paper, and examined for this fluorescent colouration. As shown in Table I, all the inorganic anions, carboxylic acids, hydroxylic acids and amino acids tested were negative, but the organic anions such as phosphates, phosphonates, sulphates, sulphonates and sulphamates, all of which are strongly acidic, were positive to this reaction. This result suggested that the fluorescent colouration with PY was specific for the strongly acidic organic anions.

The effect of substitution in these compounds on the fluorescent colouration was examined. The results in Table II show that a bulky substituent ($\geqslant n$-C_3H_7) in the organic anions was necessary for the positive reaction, and an increase in the chain-length of the substituent resulted in a more distinct reaction.

During the examination of this fluorescent colouration, we found that most

TABLE I

EXAMINATION OF FLUORESCENT COLOURATION OF INORGANIC AND ORGANIC ANIONS WITH PINACRYPTOL YELLOW ON CELLULOSE LAYERS

Sample	*Fluorescent colouration*[a]
Inorganic anions	
HPO_4^{2-}	—
SO_4^{2-}	—
NO_3^-	—
X^-[b]	—
SCN^-	—
XO_3^-[b]	—
$S_2O_3^{2-}$	—
$Fe(CN)_6^{3-}$	—
$Fe(CN)_6^{4-}$	—
Hydroxy acids	
Malic acid	—
Citric acid	—
D-Gluconic acid	—
Amino acids	
L-Aspartic acid	—
L-Glutamic acid	—
Carboxylic acids	
Caproic acid	—
Capric acid	—
Palmitic acid	—
Cyclohexyl phosphate	+
Cyclohexyl sulphate	+
Hexyl sulphonate	+
Hexyl phosphonate	+
N-Cyclohexyl sulphamate	+
N-Cyclohexyl phosphoroamidate	+

[a] In Tables I–III, the symbols + or — signify that a fluorescent orange spot is or is not detectable, respectively, at 5 μg on the cellulose layers after development, and inorganic anions at 25 μg on the cellulose layers after electrophoresis.

[b] X in both X^- and XO_3^- means halide.

TABLE II

INFLUENCE OF LENGTH OF THE SIDE-CHAIN IN ORGANIC ANIONS ON FLUORESCENT COLOURATION WITH PINACRYPTOL YELLOW ON CELLULOSE LAYERS

Organic anion	*Fluorescent colouration*[a]							
	$R = H$	CH_3	C_2H_5	$n\text{-}C_3H_7$	$n\text{-}C_4H_9$	$n\text{-}C_6H_{13}$ or C_6H_{11}	$n\text{-}C_8H_{17}$	$n\text{-}C_{12}H_{25}$
$RO\text{–}PO_3^{2-}$	—	—	—	+	+	+	+	
$RO\text{–}SO_3^-$	—		—	+	+	+		+
$R\text{–}PO_3^{2-}$			—	+	+	+		
$R\text{–}SO_3^-$		—	—	+	+	+		+
$RNH\text{–}SO_3^-$	—		—	+	+	+	+	
$RNH\text{–}PO_3^{2-}$	—					+		

[a] See footnote to Table I.

TABLE III

EFFECTS OF NEIGHBOURING HYDROXYL GROUP AND DEGREE OF ANIONIC GROUP SUBSTITUTION IN ORGANIC ANIONS ON FLUORESCENT COLOURATION WITH PINACRYPTOL YELLOW ON CELLULOSE LAYERS

Sample	*Fluorescent colouration*[a]
Influence of neighbouring hydroxyl group	
N-Cyclohexyl sulphamate	+
N-2-Hydroxycyclohexyl sulphamate	±
2-Sulphoamino-2-deoxy-D-glucose	−
Cyclohexyl phosphate	+
2-Hydroxycyclohexyl phosphate	±
Inositol 2-phosphate	−
α-D-glucose 1-phosphate	−
Propyl phosphate	+
2-Hydroxypropyl phosphate	−
α-Glycerophosphate	−
Influence of degree of anionic group substitution	
D-Glucose monosulphate	−
D-Glucose disulphate	−
D-Glucose trisulphate	+
Inositol 2-phosphate	−
Inositol hexaphosphate	+
Ethyl sulphonate	−
Ethylene disulphonate	+

[a] See footnote to Table I.

TABLE IV

FLUORESCENT COLOURATION OF NATURALLY OCCURRING SULPHATED AND NON-SULPHATED ACIDIC POLYSACCHARIDES WITH PINACRYPTOL YELLOW ON CELLULOSE LAYERS

Sample	*D.P.*	*D.S. (sulphate/unit monosaccharide)*	*Fluorescent*[a] *colouration*	*Detection limit of fluorescent colouration*[b] *(μg)*
Heparin		1.25	+	
Chondroitin 6-sulphate		0.50	+	
Keratosulphate		0.50	+	
Carrageenan		0.73	+	
Hyaluronic acid		0	−	
Pectic acid		0	−	
Dextran sulphate	244	0.12		5
Dextran sulphate	21	0.24		0.25
Dextran sulphate	244	1.46		0.025
Maltose polysulphate	2	2.4		0.025
D-Glucose polysulphate	1	3.4		0.025

[a] The symbol + or − signifies that a fluorescent orange spot is or is not detectable, respectively, at 25 μg on the cellulose papers after electrophoresis.

[b] The detection limit of samples was determined on the cellulose thin layers after being developed with methanol–acetone–water (6:2:2).

TABLE V

DETECTION LIMITS (μg) OF ORGANIC ANIONS SUBSTITUTED WITH *n*-HEXYL OR CYCLOHEXYL GROUPS WITH PINACRYPTOL YELLOW ON LAYERS OF DIFFERENT CHROMATOGRAPHIC MEDIA

The solvent systems used were: methanol–acetone–water (3:1:1) for Avicel SF, benzene–ethyl acetate–formic acid (5:10:2) for polyamide, acetone–10% ammonia (9:1) for silica gel, isopropanol–conc. ammonia (4:1) for alumina, and 0.5 *M* ammonium formate (pH 6.5) for DEAE-cellulose.

Organic anion	*Chromatographic medium*				
	Avicel SF	*Polyamide*	*Silica gel*	*Alumina*	*DEAE-cellulose*
$C_6H_{11}O-PO_3^{2-}$	2	1	40	5	30
$C_6H_{11}O-SO_3^-$	1	5	10	1	20
n-$C_6H_{13}-PO_3^{2-}$	3	5	20	1	20
n-$C_6H_{13}-SO_3^-$	0.5	5	20	5	30
$C_6H_{11}NH-SO_3^-$	0.5	1	40	0.5	30
$C_6H_{11}NH-PO_3^{2-}$	3	4	30	5	20

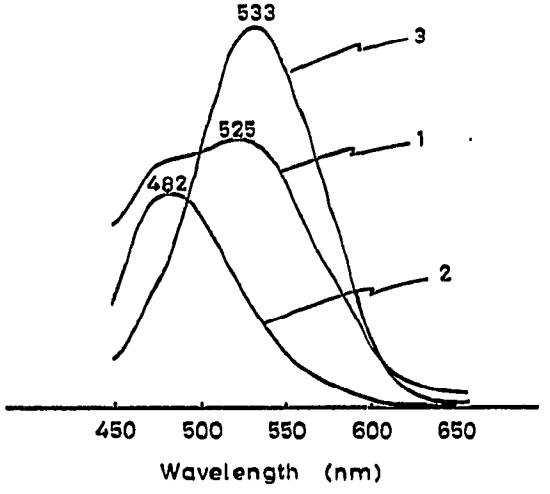
biologically important phosphate esters, such as D-glucose 1-phosphate and α-glycerophosphate, were negative to this reaction. As can be seen in Table III, the presence of neighbouring hydroxyl group(s) in the substituent of an organic anion markedly reduced its reactivity. On the other hand, an increase in the degree of anionic group substitution in an organic anion distinctly enhanced its reactivity.

In order to see how the degree of polymerization of a high-molecular anionic compound affects this reaction, an examination was made of compounds having no absorption band in both the UV and visible light ranges, such as acidic polysaccharides and synthetic polyanionic compounds. As expected, the hydroxylic compounds bearing carboxylic groups, such as pectic acid and hyaluronic acid, were negative, but all the sulphated compounds were markedly positive (Table IV). From a relationship between the reactivity and the degree of sulphate substitution (D.S.) of these sulphated polymerized compounds, it seems that the degree of polymerization (D.P.) contributes to their positive reaction. The results for synthetic dextran sulphates and related sugar sulphates also suggest this relationship (*cf.* the results for sulphated D-glucose in Table III). Moreover the results shown in Table IV indicate that this reaction can be applied to the detection of micro amounts of natural or synthetic sulphated polysaccharides.

The detection limits of various types of the organic anions on layers of different chromatographic media were examined, and the results obtained are shown in Table V. The orange fluorescence of the organic anions with PY could be best observed on cellulose and alumina layers, and to a lesser extent on a polyamide layer, but the layers with silica gel or DEAE-cellulose, both of which strongly absorb UV light, showed a marked decrease in the sensitivity.

As mentioned previously sodium cyclamate can be detected on cellulose layers as an orange fluorescent spot against a pale greenish background by spraying with PY[5]. Emission spectra of both the orange spot and the pale greenish background were measured on the layers directly under an excitation at 405 nm. The emission spectrum of the former (spectrum 1 in Fig. 1) had a maximum at 525 nm with a shoulder at 480 nm compared with that of the latter (spectrum 2 in Fig. 1) at 482 nm. On the other hand the cellulose layers sprayed with an ethanolic solution of crystalline

PY-cyclamate salt gave an orange fluorescence with a maximum at 533 nm (spectrum 3 in Fig. 1). Spectrum 1 in Fig. 1 appears to be identical with that formed by off-setting both spectra 2 and 3. If this is true, the spectral evidence in Fig. 1 suggests that the appearance of an orange fluorescent colouration on cellulose layers is due to the formation of PY-cyclamate salt from sodium cyclamate and PY chloride, and the cellulose layers, as well as polyamide or alumina layers, probably participate in this phenomenon as a medium. The PY salts prepared from various organic anions gave apparently different emission maxima on cellulose layers, as shown in Fig. 2, and their fluorescent colour tones are slightly different from each other.



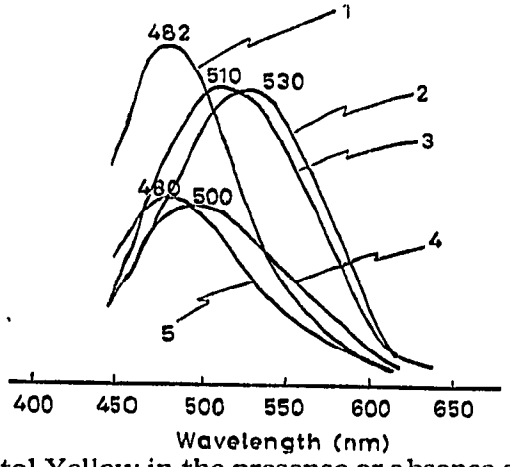


Fig. 1. Fluorescence emission spectra of Pinacryptol Yellow in the presence or absence of cyclamate on cellulose layers. Excitation at 405 nm; pfs = 30. 1 = Sodium cyclamate developed on a cellulose thin layer sprayed with 0.1% ethanolic PY; 2 = cellulose thin layer sprayed with 0.1% ethanolic PY; 3 = cellulose thin layer sprayed with 0.1% ethanolic PY-cyclamate.

Fig. 2. Fluorescence emission spectra of Pinacryptol Yellow salts of different organic anions on cellulose layers. Excitation at 405 nm; pfs = 10. 1 = PY chloride; 2 = PY salt of cyclamate; 3 = PY salt of dodecyl sulphate; 4 = PY salt of pentaerythritol tetrasulphate; 5 = PY salt of propyl sulphate.

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