CHROMATOGRAPHIC ANALYSIS OF PHOTOSENSITIZING DYES

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The studies reported here were undertaken with the initial objective of establishing the purity of several dyes which had been used in our investigations concerning their photochemical properties. Questions as to the purity of several dyes arose because in several instances our inability to demonstrate the photosensitizing properties of a dye was thought to be due to the presence of sensitizing impurities in dye samples used by other workers. In addition the determination of the spectroscopic properties of dyes, a field of investigation greatly stimulated by the development of laser systems, necessitates the use of pure dyestuffs.

There are several reviews[1–3] dealing with methods of dye purification in general, and with the chromatographic assay of dye purity in particular. For paper chromatography of thiazine dyes an excellent system was developed by TAYLOR[4,5]. It quickly became apparent, however, that the solvent systems generally used are extremely slow, and did not always give good resolution. In developing the solvent system reported here, our aim was to achieve a system which would give good separation of constituents in relatively short time periods, and one which could be applied to a wide variety of dye systems.

EXPERIMENTAL

The dyes used in this investigation are listed in Table I. Thionolin was prepared and purified by a method in the literature[6] and further purified by extraction of an acid solution with chloroform. Formamide (grade: "for vitamin assay"), *n*- and *tert.*-butanol, and acetone were purchased from Fischer as certified reagent grades. Most of the dyes were dissolved in 10% of the final volume absolute ethyl alcohol, and diluted to final volume with water; the final concentrations of the dyes were 1–4 mg per ml solution; 0.01–0.20 mg of dye (0.01–0.10 ml of solution) were applied to paper using a hypodermic syringe under a stream of air to minimize spreading of the spot. Thionolin and methylene violet were dissolved in methanol.

Solvents for development

Three solvent systems were used as described below:

A. *tert.*-Butanol–acetone–0.2 *M* HCl (aq.) (35:35:30; parts by volume). The apparent pH of this mixture as measured with a glass electrode is 1.9.

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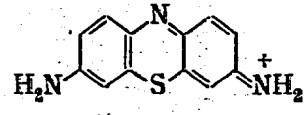
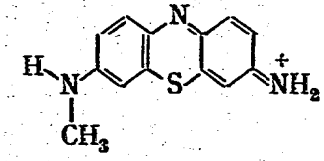
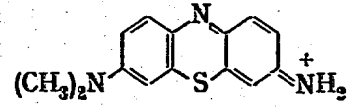
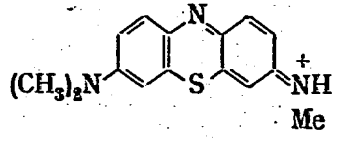
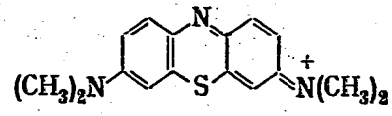
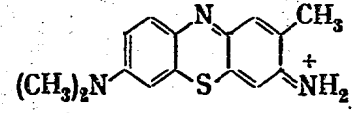
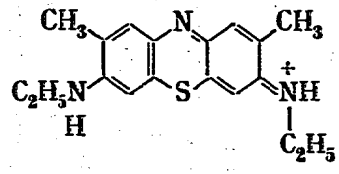
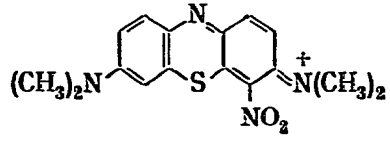
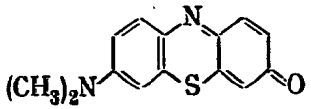
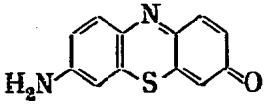
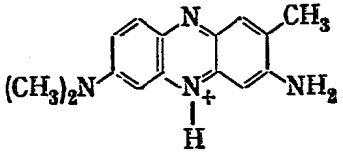
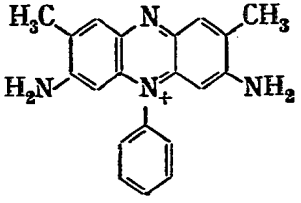
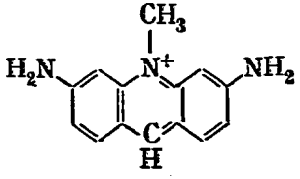
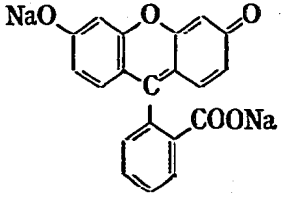
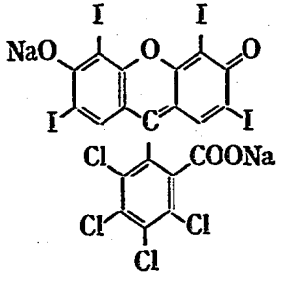
TABLE I

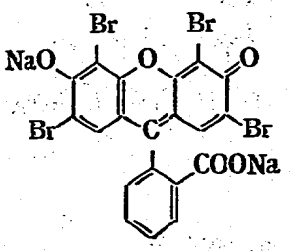
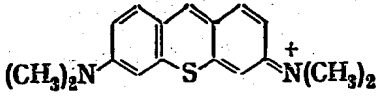
DYES USED FOR CHROMATOGRAPHY

Dye	*Structural formula*[1]	*Color Index No.*	*Certific'n No. (C) or lot No. (L)*	*Stated dye content (%)*	*Supplier*
Thiazines					
Thionine		5200	NT 20 (C)	85	National Aniline
Azure C		—	NAc 5 (C)	—	National Aniline
Azure A		—	NAz 16 (C)	93	National Aniline
Azure B		52010	NAb (C)	69	National Aniline
Methylene blue		52015	741220 (L)	88	Fisher Scientific
Toluidine blue O		52040	NU 12 (C)	76	National Aniline
New methylene blue N		52030	14642 (L)	—	National Aniline
Methylene green		52020	1623 p (L)	—	National Aniline

Methylene violet		—	NLv 8 (C)	70	National Aniline
Thionolin		—	—	—	Synthesized by authors according to ref. 6
Azines					
Neutral red		50040	N 129 (C)	83	Fisher Scientific
Safranine O		50240	NS 22 (C)	85	National Aniline
Acridines					
Acriflavine		46000	14435 (L)	—	National Aniline
Xanthenes					
Uranine		45350	731575 (L)	—	Fisher Scientific
Rose bengal		45440	724158 (L)	80	Fisher Scientific

(*continued on p. 134*)

TABLE I (*continued*)

Dye	*Structural formula*[7]	*Color Index No.*	*Certific'n No. (C) or lot No. (L)*	*Stated dye content (%)*	*Supplier*
Eosin Y		45380	732702 (L)	87	Fisher Scientific
Thiopyronines					
Thiopyronine		—	—	—	E. Merck AG.

B. *tert.*-Butanol–acetone–0.2 *M* NH_3 (aq.)–0.2 *M* HCl (aq.) (35:35:21:9). The apparent pH of this mixture is pH 9.0. This pH was chosen because it was found to prevent "ghosting" of components in the case of the alkaline thiazine dyes.

C. *tert.*-Butanol–*n*-butanol–0.2 *M* NH_3 (aq.) (40:30:30), with an apparent pH of 10.6.

Chromatographic development was carried out on wedge-shaped strips[7] of Whatman 3MM paper, 4 cm wide and 45 cm long, in a Chromatobox (Warner-Chilcott Laboratories). Chromatography was carried out in the dark, and the chambers were sealed with parafilm in order to minimize evaporation of the volatile solvent components.

In order to keep conditions constant, a standard developing time of eighteen hours was adopted for solvents A and B. With solvent C forty hours were necessary. At the end of these time periods the solvent front had reached to within two cm of the end of the paper.

RESULTS AND DISCUSSION

The results of the chromatographic analysis of thiazine dyes are listed in Table II. The value α is the absorbance at the absorption maximum in ml per mg of dye. If the different fractions in a dye sample do not differ greatly in molar extinction coefficient, the values of α can be taken as the relative molar proportions of the components of the mixture. Thus one can calculate that the methylene blue sample analyzed is contaminated with 2.3 % Azure A and 8.1 % Azure B. The reproducibility of the R_F values and of α are illustrated for the analysis obtained in the case of methylene blue, where the standard deviation of ten determinations is listed. For those fractions whose spectra showed a definite shoulder in the absorption maximum this wavelength is listed, together with the relative absorbance at this wavelength compared to that at the absorption maximum. These values frequently aid in the identification of the various fractions. It should be noted that the spectral data given were obtained by eluting the fractions into 50 % (v/v) aqueous formamide (in order to get quantitative elution) and that these spectra therefore differ from those obtained in acetate or in methanol: the shoulders in the absorption spectra are less pronounced. In the eighth column of the table are listed what we believe to be the identity of the various fractions obtained. These are based on absorption maxima, R_F values and the comparison of the spectra in acetate buffer or methanol with spectra reported in the literature.

Excellent separation of components was achieved both at alkaline and acid pH. None of the samples tested was pure, some containing as much as sixty per cent of contaminant. Our results are in overall agreement with those reported for thiazine dyes by TAYLOR[4,5] who used circular chromatography, and developed chromatograms using solvents based on cyclohexanone. Using this author's solvents in a closed system such as ours produced poor results: his method depends on the continued evaporation of the volatile solvent from the edge of the strip. It is therefore unsuited to the identification of dye components by their R_F values, since the values so obtained are all relative to the fastest-moving component.

Our identification of the various fractions occurring in those commercial samples of thiazine dyes which we analyzed, in general coincide with those assigned by TAYLOR[4,5]. Several other authors[3,8–10] obtain slightly different proportions of

TABLE II

CHROMATOGRAPHIC SEPARATION OF THIAZINE DYES

Dye	Amount (mg)	Developing solvent A (pH 1.9)					Identif.	Developing solvent B (pH 9.0)				
		R_F	λ_{max} (mμ)	λ_{sh} (mμ)	$\frac{OD\lambda_m}{OD\lambda_{sh}}$	α		R_F	λ_{max} (mμ)	λ_{sh} (mμ)	$\frac{OD\lambda_{max}}{OD\lambda_{sh}}$	α
Thionine	0.10	0.55	538	564	0.87	4.5	—	decomp.				
		0.50	602			190	Thionine	0.64	602	570	0.56	146.0
Azure C	0.01	0.47	610			36.0	Azure C	0.57	609			18.0
		0.45	627			50.0	Azure A	0.55	628			35.0
		0.33	647			36.0	Azure B	0.41	646			31.0
		0.21	661	620	0.60	15.0	Methylene blue	0.29	662	619	0.58	12.0
Azure A	0.02	0.45	618			48.0	—	0.58	616			23.5
		0.40	632			97.0	—	0.53	632			84.0
		0.31	643			27.0	—	0.43	643			23.5
		0.23	659	619	0.61	14.0	Methylene blue	0.30	660	617	0.65	11.0
Azure B	0.10	0.64	604	574	0.75	0.8	N-Me-thionolin					
		0.59	618	579	0.65	1.6	N,N-Di-Me-thionolin	0.78	—			—
		0.45	629			17.5	Azure A	0.58	629			15.5
		0.38	648			65.0	Azure B	0.52	647			55.0
		0.31	664	619	0.45	85.0	Methylene blue	0.44	664	620	0.49	72.0
Methylene blue	0.10	0.41 ± 0.01	629			5.4 ± 0.1	Azure A	0.55	629			4.3
		0.35 ± 0.01	648			19.1 ± 1.4	Azure B	0.49	647			15.8
		0.31 ± 0.01	665	620	0.50	213.0 ± 4.2	Methylene blue	0.45	663	619	0.45	203.0

Methylene violet	0.10	0.90	596	431	0.90	4.0	Thionol	0.84	614	583	0.67	127.0
		0.65	606	574	0.70	35.0	N-Me-thionolin	0.70	597			3.3
		0.58	619	581	0.61	101.0	N,N-Di-Me-thionolin	0.59	603			2.6
		0.44	603			4.6	Thionine	0.52	625			2.3
		0.38	629			3.0	Azure A	0.44	646			4.5
		0.32	647			5.5	Azure B	0.33	663	619	0.57	4.5
		0.26	662	620	0.55	6.9	Methylene blue					
Thionolin	0.10	0.69	592	560	0.84	128.0	Thionolin	0.79	593	560	0.86	122.0
Toluidine blue O	0.02	0.45	616			13.5	—	0.57	617			8.2
		0.43	632			47.0	—	0.53	632			41.5
		0.36	638			54.5	—	0.48	638			42.0
New methylene blue N	0.02	0.63	639			15.0	—	0.82	639			4.5
		0.60	633			92.0	New methylene blue N	0.80	635			12.5
								0.69	633			63.0
Methylene green	0.10	0.57	644			2.9	—	decomp.				—
		0.54	656	623	0.81	79.0	Methylene green	decomp.				—
		0.33	649			5.7	Azure B	0.44	648			4.9
		0.30	664	617	0.45	41.8	Methylene blue	0.38	663	619	0.48	33.3

TABLE III

CHROMATOGRAPHIC SEPARATION OF VARIOUS DYES

Dye	*Amount (mg)*	*Development*		R_F	λ_{max} *(mμ)*	α	*Fluorescence of band*	*Development*		R_F	λ_{max} *(mμ)*	α
		Solvent	*Time (h)*					*Solvent*	*Time (h)*			
Rose bengal	0.20	B	18	0.84	555	65.0	strong red	C	40	0.59	555	67.0
				(0.71	532	4.9	slight red			0.53	532	6.1
				0.64)	526	2.6	yellow			0.48	524	2.3
				0.45	518	0.4	very slight blue			0.41	517	0.4
										0.33	—	<0.1
Uranine	0.20						slight pink	C	40	0.52	505	0.3
							green			0.38	496	143.5
							pink			0.24	500	0.5
Eosin Y	0.20						yellow	C	40	0.51	524	106.0
							yellow-green			0.44	517	11.5
Acriflavine	0.10	A	18	0.66	—	<0.5	pink	B	18	0.75	—	<0.1
				0.59	460	6.7	orange-yellow			0.70	460	7.6
				0.56	450	60.0	yellow			0.66	450	61.0
				0.47	458	68.0	green-yellow			0.56	457	65.0
				0	—	—	reddish			—	—	—
Neutral red	0.10	A	18	0.50	526	1.8	red	B	18			
				0.47	540	85.0	dark red			0.702	540	
Safranine	0.10	A	18	0.85	525	17.3	red					
				0.78	528	81.0	red					
Thiopyronine	0.04	A	18	0.382	566	198.0	orange-red	B	18	sample decomposed		

contaminant, and, in some cases incorrectly designate the fast (pink) component in thionine as thionolin[8]. This unknown component is a weak base which can readily be extracted into chloroform from an aqueous solution at pH 9. The absorption spectra of the major component in the sample labelled "Azure A" in formamide, or in pH 5 acetate buffer do not resemble those of Azure A (absorption max. in formamide at 627–629 mμ).

By the use of the alkaline solvent ("solvent B") one obtains lower values for total absorbance (α) than in the acid system A. This is probably due to decomposition of several of the thiazine components at this pH. The more acid solvent is therefore to be preferred for the chromatographic separation of thiazines. Thionol, the fastest-moving component in methylene violet (solvent A) is resolved better in solvent system B.

The necessity for using pure samples in evaluating the photochemical reactivity of a dye is well illustrated by our observations on methylene green. On chromatography this dye sample was shown to contain at least forty per cent of contaminants, of which methylene blue is by far the major constituent. The ability of these fractions to sensitize the photopolymerization of calcium acrylate[11] is vastly different: when the components were used at equal absorptivity, whereas the methylene blue fraction caused polymer formation within seconds, the methylene green fraction caused only slight polymerization after six minutes. The decreased reactivity of the methylene green as compared with methylene blue can be ascribed to the presence of a nitro group[12]. Methylene green has been reported to be a good sensitizer for the photodynamic inactivation of viruses[13]. On the basis of our findings we would suspect that the dye sample used by this author was heavily contaminated with methylene blue, which was probably responsible for the photodynamic effect.

Table III gives results obtained for the chromatographic analysis of various photosensitizing dyes other than thiazines. Good separation was achieved with neutral red at acid pH, and for the acridine dye, acriflavine, at both acid and alkaline pH. Safranine O gave very poor separation in solvent system A. Increasing the polarity and the acidity of the resolving solvent may somewhat improve the resolution obtained. In the case of xanthene dyes, the alkaline butanol–acetone solvent (solvent B) gave poor resolution of the secondary bands, which were quite broad, although these were well-differentiated from the main component. Replacement of acetone with *n*-butanol and increasing the apparent pH of the mixture resulted in better separation of the contaminants, but the time needed to achieve resolution is twice as long. Our results agree with those obtained by others[14–16] using different solvent systems, in that eosin Y, for instance, produced two zones; the minor component is probably 2,4,5-tribromofluorescin[15]. Since the authors cited do not quote R_F values, it is difficult to decide whether the separations achieved are comparable.

None of the solvent systems used could usefully be applied to the analysis of triphenylmethane dyes: the polarity of the solvents is such that these dyes move with the solvent front. Reverse-phase chromatography, although it is a cumbersome technique seems best suited for the analysis of these dyes[17].

SUMMARY

Simple one-phase solvent systems were developed which are useful for the

chromatography of a variety of water-soluble acid and alkaline dyes. Using these solvents the purity of several commercial preparations of thiazine, azine, acridine, and xanthene dyes was assayed, and it was shown that such preparations can contain up to sixty percent of isomeric contaminants. These findings point to the need for purifying dye samples before their use in determining absolute quantum yields of photoreduction or photoxidation, spectral changes in absorption due to dye-binding, and spectroscopic properties.

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