237. *Studies in the Light Absorption of Dyes. Part III.*[1] *Absorption Spectra of Dyes in Solution and Adsorbed in Solid Films.*

By C. H. Giles, S. M. K. Rahman, and (in part) D. Smith.

The absorption spectra have been examined of a large number of dyes of various chemical classes, including " reactive " dyes, in aqueous and non-aqueous solution, and adsorbed in solid transparent films. Almost all dyes have two absorption (*x* and *y*) wavebands. The shorter (*y*) waveband increases in height relative to the longer (*x*) with increase in concentration, in nearly all cases. This is attributed to aggregation. There is also a shift of both peaks with change in solvent (" solvatochromic " effect). The statistical significance of the method is examined.

The relation between colour and constitution of dyes has been discussed by many authors,[2] but the identification of absorption bands with aggregated states has received little attention except in the case of the cyanine photographic sensitising dyes (see, *e.g.*, ref. 2*c*) and certain basic dyes,[3-5] especially Methylene Blue.[3]

Lewis and Calvin [4] suggested that the absorption spectrum of a substance with a planar or almost planar molecule should consist of bands corresponding severally to electronic oscillations along the three perpendicular axes of the molecule. The shorter the axis, the shorter the wavelength of its band. This suggestion was confirmed experimentally by Lewis and Bigeleisen [5] for several basic dyes. When the molecules aggregate they may pack face-to-face, and the axis normal to the faces, originally one of the shorter ones, is lengthened. Thus the aggregate should have an intensified *short* waveband. This is in fact found with a number of dyes which display two well-defined absorption bands,[3,4] *e.g.*, Methylene Blue (C.I. 52,015).

The cyanine dyes, which have been studied exhaustively, show, *e.g.*, the main absorption band of the monomeric form of the dye (often termed the " *M*-band ") in dilute aqueous solution or in non-aggregating solvents, *e.g.*, pyridine. A shoulder on the short-wave side of this band becomes more pronounced (" *D*-band ") as the concentration in water increases, and is usually attributed to a dimeric form. This band shifts gradually to shorter wavelengths with increase in concentration, owing to the formation of larger aggregates (and is then called the " *H*-band "). In presence of certain electrolytes an entirely new, sharp, band appears on the long-wave side of the *M*-band. This is known as the " *J*-band," and is attributed to chain-like aggregates in which the planar dye molecules are packed face-to-face with a layer of water molecules between each pair of dye molecules.

Lewis and Bigeleisen also detected second-order *x*-bands in absorption spectra of some dyes.[6] The wavelengths of these bands are about half those of the first-order bands, whereas the *y*-bands are usually at only slightly shorter wavelengths than the *x*-bands.

Little is known about the occurrence of these bands in the spectra of other dyes, except vat dyes in non-aqueous solvents; [7] and apart from the investigations upon cyanine dyes

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[1] The paper by Campbell, Cathcart, Giles, and Rahman, *Trans. Faraday Soc.*, 1959, **55**, 1631, is considered to be Part II of this series.

[2] See, *e.g.*, (*a*) Maccoll, *Quart. Rev.*, 1947, **1**, 16; (*b*) Lewis, *J. Amer. Chem. Soc.*, 1945, **67**, 770; (*c*) Dickinson, *Phot. J.*, 1950, **90***B*, 142; *J. Phot. Soc.*, 1954, **2**, 50 (cyanine sensitising dyes); (*d*) Knott, *J.*, 1951, 1024; (*e*) Brooker, *J. Amer. Chem. Soc.*, 1951, **73**, 5332, 5350, 5356 (merocyanine dyes); (*f*) Brooker, White, Heseltine, Keyes, Dent, and Van Lare, *J. Phot. Sci.*, 1953, **1**, 173 (cyanine sensitising dyes); (*g*) Brode, Gould, and Wyman, *J. Amer. Chem. Soc.*, 1953, **75**, 1856 (azo-dyes, effect of solvents); (*h*) Peters and Sumner, *J. Soc. Dyers and Colourists*, 1956, **72**, 77 (anthraquinone dyes); (*i*) Carroll and West, " Fundamental Mechanisms of Photographic Sensitivity," Butterworths, London, 1951.

[3] Rabinowitch and Epstein, *J. Amer. Chem. Soc.*, 1941, **63**, 69; Lemin and Vickerstaff, *Trans. Faraday Soc.*, 1947, **43**, 491; Förster and König, *Z. Electrochem.*, 1957, **61**, 344; Campbell and Giles, *J. Soc. Dyers and Colourists*, 1958, **74**, 164.

[4] Lewis and Calvin, *Chem. Rev.*, 1939, **25**, 273.

[5] Lewis and Bigeleisen, *J. Amer. Chem. Soc.*, 1943, **65**, 2102.

[6] Lewis and Bigeleisen, *J. Amer. Chem. Soc.*, 1943, **65**, 2107.

[7] Moran and Stonehill, *J.*, 1957, 765, 779.

TABLE 1. *Absorption data* (x- *and* y-*bands*) *for dyes in various media.*

Dye	Medium *	$\lambda_{max.}$ † y	$\lambda_{max.}$ † x	O.D. at $\lambda_{max.}$ (x) in 1 cm. cell	O.D. ratio $y : x$
	Anthraquinone dyes				
C.I. 62,085	1 : 1 H_2O–pyridine	5800	6250	0·602[1]	0·92
	,, ,,	5800	6250	1·300	0·93
	,, ,,	5800	6250	1·460	0·94
	0·2M-Phenol	5800	6200	0·264	0·95
	,,	5800	6200	1·276	0·97
	H_2O	5800	6100	0·349[2]	0·96
	,,	5800	6100	0·918	0·99
	,,	5800	6100	1·259	0·98
	Gelatin	5850	6250	0·125	0·92
	,,	5850	6250	0·270	0·96
	,,	5850	6250	0·804	1·02
	,,	5850	6250	1·080	1·05
C.I. 63,010	1 : 1 H_2O–pyridine	6000	6450	0·167	0·91
	,,	6000	6450	0·749[3]	0·91
	Gelatin	(6100)	6500	0·108	0·93
	,,	(6100)	6500	0·250	0·94
	,,	(6100)	6450	0·355	0·95
	Monoazo dye, unsulphonated				
p-Dodecylaniline $\longrightarrow$ 2-naphthol	Ethylmethyl-cellulose film	4200	4800	0·315	0·80
	Collodion film	4200	5000	0·920	0·65
(This dye is more highly aggregated in the hydrophilic than in the hydrophobic film.)					
	Monazo-dyes, sulphonated				
Aniline $\longrightarrow$ naphthionic acid	Pyridine	4400	4750	0·344[4]	0·89
	MeOH	(4200)	4600	0·447[5]	0·76
	H_2O	(4200)	4600	0·459[6]	0·80
	,,	(4200)	4600	1·760	0·85
	,,	(4200)	4600	2·46	0·91
	M-NaCl	(4200)	4600	0·557	0·83
C.I. 16,255	MeOH	5100	(5300?)	1·150[7]	1·11
	Gelatin (dyed in 0·5M-phenol)	5150	(5450)	1·000	1·28
	Gelatin (dyed in 0·25M-phenol)	5150	(5450)	2·910	1·29
	Gelatin	5150	(5450)	1·580	1·30
C.I. 18,050	H_2O	5050	5300	0·447[8]	1·01
	,,	5050	5300	0·720	1·01
	,,	5050	5300	0·955	1·01
	,,	5050	5300	3·048	1·03
p-Dodecylaniline $\longrightarrow$ *N*-acetyl-H-acid	H_2O	5150	5450	0·110[28]	1·00
	,,	5150	5450	0·902	1·02
	,,	5150	5450	3·480[30]	1·09¶
	Gelatin (dyed in 2M-phenol)	5200	5550	0·825	1·13
	Gelatin	5200	5550	0·081	1·10
	,,	5200	5550	0·435	1·16
	,,	5200	5550	0·880	1·23
	Monazo-dyes, sulphonated				
Aniline $\longrightarrow$ R-acid	MeOH	4850	5050	0·305[9]	1·03
	H_2O	4950	5050	0·580[10]	1·04
	Gelatin	4950	(5150)	0·280	1·09
p-Dodecylaniline $\longrightarrow$ R-acid	MeOH	4950	(5200)	0·200[11]	1·12
	H_2O	5000	(5270)	0·280[12]	1·10
	0·2M-phenol	4950	(5350)	0·190	1·14
	Gelatin	5050	(5350)	0·190	1·08
Dye IV §	H_2O	4100	(4400)	0·349[13]	1·27
	,,	4000	(4400)	0·569	1·50
	Gelatin	4000	(4400)	0·086	1·38
Dye V §	H_2O	4050	(4400)	0·182	1·59
	,,	4050	(4400)	0·445	1·66
	Gelatin	4000	(4400)	0·185	1·75
Dye VI §	1 : 1 H_2O-pyridine	4800	(5300)	0·137	2·31
	H_2O	4800	(5300)	0·165	2·54
	Gelatin	4800	(5300)	0·147	2·56
Dye VII §	1 : 1 H_2O–pyridine	4900	(5300)	0·078[14]	1·46
	Gelatin (+CTAB)	4800	(5300)	0·135	1·74
	H_2O	5000	(5300)	0·211[15]	1·13
	Gelatin	4950	(5300)	0·168	1·42
(CTAB = cetyltrimethylammonium bromide; this cationic agent increases the association of the dye.)					
	Bis- and tris-azo-dyes, sulphonated				
C.I. 23,910	0·2M-Phenol	5050	(5450)	0·162	1·23
	,,	5050	(5450)	0·730	1·49
	H_2O	5100	(5450)	0·165	1·34
	,,	5100	(5450)	0·780	1·60
C.I. 24,140	Pyridine	(5500)	5930	0·760[16]	0·77
	MeOH	(5500)	5750	0·680[17]	0·93
	Cellulose	5850	(6350)	0·690	1·27
C.I. 24,410 (Sky Blue FF)	1 : 1 H_2O–pyridine	(5900)	6300	0·160[18]	0·83
	,,	(5900)	6300	0·771	0·83
	,,	(5900)	6300	1·520	0·84
	H_2O	(5900)	6200	0·061[29]	0·87
	,,	(5900)	6200	0·084[31]	0·89
	,,	(5900)	6200	0·346[32]	0·89
	,,	(5900)	6200	1·250[33]	0·89
	,,	(5900)	6200	1·490[34]	0·94
	Cellulose	6100	6600	0·294[35]	0·80
	,,	6100	6600	0·820	0·80
	,,	6100	6550	1·900	0·89
	Gelatin	(6100)	6250	0·165	0·96
	,,	(6100)	6250	0·552	0·98
	,,	(6100)	6250	0·956	0·98
	,,	(6100)	6250	1·800	1·04
Copper complex of C.I. 24,410	1 : 1 H_2O–pyridine	(6200)	(6600)	0·139	1·01
	,,	(6200)	(6600)	0·561	1·03
	,,	(6200)	(6600)	1·480	1·07

	Bis- and tris-azo-dyes, sulphonated				
	H_2O	(6150)	(6750)	0·133[19]	1·40
	,,	(6250)	(6750)	0·424	1·35
	,,	(6250)	(6750)	1·260	1·36
	Cellulose	(6300)	(6650)	0·137[36]	0·99
	,,	(6300)	(6650)	0·555	1·00
	,,	(6300)	(6650)	0·678	1·06
C.I. Direct Blue 66 (trisazo)	1 : 1 H_2O-pyridine	(5900?)	6400	0·118[20]	0·67
	,,	(5900?)	6500	0·540	0·70
	,,	(5900?)	6500	1·49	0·70
	H_2O	(6000?)	(6300?)	0·102[21]	0·90
	,,	(6000?)	(6300?)	0·554	0·91
	,,	(6000?)	(6300?)	1·680	0·96
	Cellulose	(5900?)	6400	0·218[37]	0·69
	,,	(5900?)	6400	1·100	0·71
	,,	(5900?)	6400	2·230	0·79
	Gelatin	(5900?)	6350	0·222	0·80
	,,	(5900?)	6350	0·335	0·82
	,,	(5900?)	6350	1·000	0·86
Polar Yellow R	0·2M-Phenol	(4100)	(4400)	0·150	0·97
	,,	(4100)	(4400)	1·370	1·06
	Cellulose	(4100)	(4400)	0·420	0·98
	H_2O	(4100)	(4400)	0·245	1·02
				0·1400 l.$^{-1}$	
	,,	(4100)	(4400)	1·150	1·02
				0·7200 l.$^{-1}$	
	Gelatin	(4100)	(4400)	0·323	1·03
	,,	(4100)	(4400)	1·370	1·06
	Metal-unsulphonated ligand (1 : 2) *dyes*				
Irganol Blue BS	H_2O	(5750)	6200	0·091[22]	0·85
	,,	(5750)	6200	0·364	0·86
	,,	(5750)	6200	1·400	0·88
	Pyridine	(5850)	6250	0·116[23]	0·85
	,,	(5850)	6250	0·414	0·90
	,,	(5850)	6250	1·060	0·91
	Gelatin	(5850)	6250	0·104	0·93
	,,	(5850)	6250	0·310	0·94
	,,	(5850)	6250	1·430	0·96
Irgalan Orange RL (C.I. Acid Orange 86)	H_2O	4800	(5300?)	0·062[38]	1·65
	,,	4800	(5300?)	0·283[39]	1·73
	,,	4800	(5300?)	1·105[40]	1·70
	Gelatin	4900	(5400?)	0·475	1·54
	,,	4900	(5400?)	0·980	1·54
	,,	4900	(5400?)	1·720	1·56
	Pyridine	5000	(5400?)	0·590[24]	1·40
	,,	5000	(5400?)	1·370	1·43

	Reactive dyes for cellulose				
Cibacron Rubine R	H_2O	(4950)	5250	0·125[25]	0·76
	,,	(4950)	5250	0·498	0·80
	,,	(4950)	5250	0·995	0·83
	,,	(4950)	5250	1·990	0·86
	Cellulose (after alkali-treatment)	(5050?)	(5500?)	0·111	0·91
	,, ,,	(5050?)	(5500?)	0·545	1·01
	,, ,,	(5050?)	(5500?)	1·300	1·08
Cibacron Scarlet 2G	H_2O	5000	(5290?)	0·158	1·10
	,,	5000	(5200?)	0·586	1·14
	,,	5000	(5200?)	1·820	1·14
	2% Na_2CO_3 (cooled after boiling 30 min.)	4950	(5200?)	0·204	1·23
	,, ,,	4950	(5250?)	0·541	1·28
	Cellulose (after alkali-treatment)	5100	(5350)	0·068	1·02
	,, ,,	5100	(5350)	0·73	1·12
	,, ,,	5100	(5350?)	1·46	1·12
Procion Brilliant Blue H7G (phthalocyanine derivative)	H_2O	6200	6600	0·074[41]	0·43
	,,	6200	6600	0·382[42]	0·68
	,,	6200	6600	1·340[43]	0·81
	Cellulose	6250	6630	0·162	0·57
	,,	6250	6630	0·494	0·65
	,,	6250	6630	1·460	0·66
	2% Na_2CO_3 (after boiling 30 min.)	6300	6630	0·106	0·68
	,, ,,	6300	6650	0·410	0·71
	Pyridine	6070	6700	0·162[26]	0·25
	,,	6070	6700	0·632	0·28
	,,	6050	6650	1·300	0·34
	Triphenylmethane dye				
C.I. 42,000 (Malachite Green)	H_2O	(5750)	6200	0·098[27]	0·47
	,,	(5750)	6200	0·368	0·46
	,,	(5750)	6200	2·460	0·53
	Cellulose	(5750)	6300	0·790	0·45
	Cellulose acetate	(5750)	6300	0·363	0·33
	Gelatin	(5750)	6300	0·361	0·39
	,,	(5750)	6200	1·080	0·39

* Solvent ratios are v/v.

† Wavelengths are given in parentheses where the peak is flat or in the form of a shoulder; a query indicates a very indefinite peak.

Concns. can be determined from the following data for optical densities. For concns. of 0·01 g./l., O.D. (x) = (1) 0·312, (2) 0·277, (3) 0·330, (4) 0·450, (5) 0·442, (6) 0·237, (7) 0·521, (8) 0·385, (9) 0·660, (10) 0·338, (11) 0·346, (12) 0·316, (13) 0·349, (14) 0·622, (15) 0·608, (16) 0·332, (17) 0·325, (18) 0·110, (19) 0·223, (20) 0·268, (21) 0·253, (22) 0·186, (23) 0·194, (24) 0·285, (25) 0·361, (26) 0·169, (27) 0·320. For 0·004 g./l., O.D. (x) = (28) 0·110, (29) 0·061. Also, O.D. (x) = (30) 3·48 at 0·20 g./l., (31) 0·084 at 0·0056 g./l., (32) 0·346 at 0·0348 g./l., (33) 1·250 at 0·1419 g./l., (34) 1·490 at 0·1682 g./l., (35) 0·922 at 11·92 g./kg.,‡ (36) 0·860 at 5·03 g./kg.,‡ (37) 0·832 at 4·35 g./kg.,‡ (38) 0·062 at 0·0033 g./l., (39) 0·283 at 0·0185 g./l., (40) 1·105 at 0·432 g./l., (41) 0·074 at 0·0034 g./l., (42) 0·382 at 0·0191 g./l., (43) 1·340 at 0·1063 g./l.

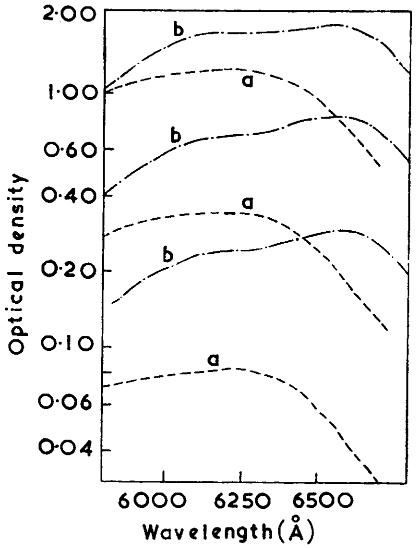
‡ 0·0015 in. gauge Cellophane film. The molar extinction coefficients of these dyes in cellulose appear to be 2—3 times their values in water.

§ See Cameron and Giles, *J.*, 1957, 3140.

¶ x-Band flatter than at lower optical densities.

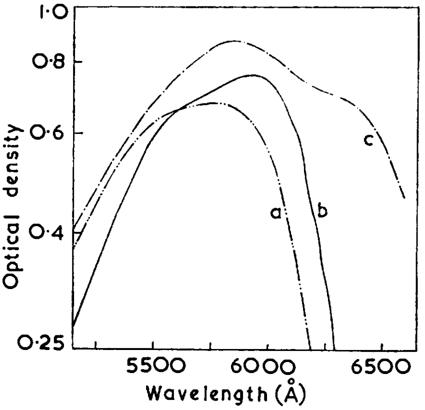
in silver halide-gelatin emulsions (see, *e.g.*, refs. 2*c*, *f*), little previous work on the occurrence of *x*- and *y*-bands in spectra of *adsorbed* dyes has been reported. In connection with a study of the photo-tendering properties of anthraquinone dyes Moran and Stonehill [7] described the *x*- and *y*-bands in spectra of vat dyes dyed on cellulose acetate films, but they did not investigate the effect of changing the concentration.

FIG. 1. *Absorption spectra of a (direct cotton) bisazo-dye* (C.I. 24,410, *Sky Blue FF*) *in various media.*



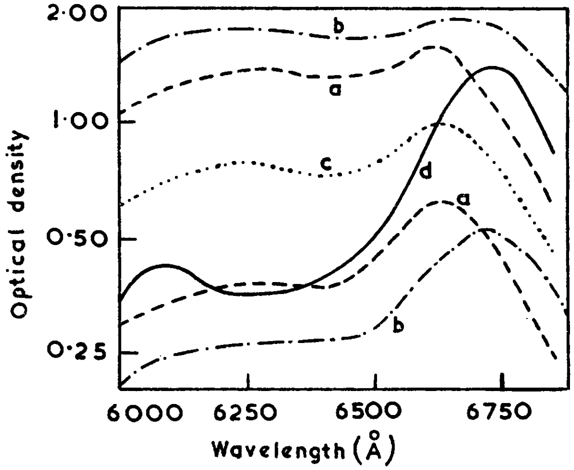
a, *Water*; b, *dyed regenerated cellulose film.*

FIG. 2. *Absorption spectra of a (direct cotton) bisazo-dye* (C.I. 24,140) *in various media.*



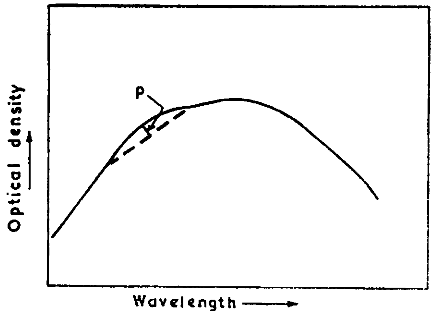
a, *Methanol*; b, *pyridine*; c, *dyed regenerated cellulose film.*

FIG. 3. *Absorption spectra of a reactive dye (Procion Brilliant Blue* H7G) *in various media.*



a, *Water*; b, *dyed regenerated cellulose film*; c, *alkaline solution, after boiling*; d, *pyridine.*

FIG. 4. *Illustrating the method of estimating the position of a subsidiary absorption peak. The peak is at the intersection of the curve with the perpendicular* p. *This method was checked by graphically adding peaks of different heights.*



We have examined the visible absorption spectra of dyes of various chemical classes, in solution in water and other solvents and adsorbed in transparent films (see Table 1). In particular the changes in the shapes of the absorption spectra with concentration have been recorded, in terms of the ratio of heights of the two bands that appear in almost all cases (cf. Figs. 1—3). The significance of the results was analysed statistically (see Appendix). The conclusions may be summarised thus:

(i) Most dyes of all classes have two absorption bands in the visible region, which we tentatively identify with Lewis and Calvin's x and y bands,* because they are close together and because increase in dye concentration, either in solution or adsorbed in solid films, or change from a non-associating solvent (*e.g.*, methanol or pyridine) to an associating one (*e.g.*, water) almost always increases the height of the band of shorter wave-length relative to that of longer wavelength (in only a very few cases the height remains constant, or decreases very slightly). In many cases the bands are so close as to make one of them difficult to locate with precision; it appears as a very slight bulge on the short-wave side (occasionally on the long-wave side) of the main peak: the approximate position of this type of band was estimated as shown in Fig. 4.

(ii) Both bands persist in all media, but there is in most cases a " solvatochromic " shift of both bands with change of solvent.†

(iii) The similarity in behaviour with increase in concentration of each dye in solution or adsorbed in films is evidence that the adsorbed dye is at least partly aggregated. This confirms the result of the examination of " Beer's law curves " of dyed films reported earlier.[1]

(iv) The change in height of the y-band relative to that of the x-band with increase in concentration is small (*ca.* 5% for a 100-fold increase in concentration).

(v) The main (x) band of many dyes shifts to longer wavelengths when the dye is adsorbed on cellulose. In the adsorbed state the dye molecules are probably aligned parallel to the cellulose chains, and separated therefrom by a layer of water molecules.[9] Krasovitskii and Pereyaslova [10] found that $\lambda_{\text{max.}}$ for benzidine direct cottton dyes is frequently much higher on regenerated cellulose sheet than in solution, and attributed this to increased conjugation in the dye molecule, caused by improved planarity.

Reactive Dyes.—These recently introduced dyes [11] differ from most others in forming a covalent bond with the fibre molecule itself. The present results show that they are aggregated in the solid substrate, a conclusion previously reached from a study of light-fading rates.[12] Their intermolecular forces are therefore sufficiently powerful to cause aggregation even when one point on the dye molecule is covalently attached to the fibre molecular chain. During the reaction with the fibre the system is, of course, in an aqueous medium, and the fibre molecules accessible to dye will be solvated by water and flexible enough to accommodate themselves to the positions taken up by the aggregated dye molecules.

EXPERIMENTAL

Dyes.—These were laboratory or commercial products recrystallised three times from 3 : 2 v/v ethanol–water; after two recrystallisations the apparent molar extinction coefficients did not increase, and this was taken to indicate complete removal of inorganic impurities. The two azo-pyrazolone dyes were obtained in an already purified form. Malachite Green was

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* The azo-dyes used are *o*-aminoazo- or *o*-hydroxyazo-compounds and cannot readily show azo–quinone imine or *cis–trans* changes. This was confirmed by examination of Orange II (sulphanilic acid ⟶ 2-naphthol) solutions: the $x : y$ band ratio does not change with variation in pH or on prolonged intense illumination.

† Kundt's rule [8] is that an increase in refractive index of solvent causes the absorption waveband to move to longer wavelengths. It is not always obeyed, and opposite effects are sometimes found amongst dyes of close similarity of structure. *E.g.*, Sky Blue FF (C.I. 24,410) obeys the rule in water and pyridine, but Benzopurpurine (C.I. 23,500) behaves in the opposite sense.

[8] Kundt, *Ann. Phys. Chem.*, 1878, **4**, 34.

[9] Giles and Hassan, *J. Soc. Dyers and Colourists*, 1958, **74**, 846.

[10] Krasovitskii and Pereyaslova, *Doklady Akad. Nauk S.S.S.R.*, 1954, **98**, 71; through *J. Soc. Dyers and Colourists*, 1955, **71**, 193.

[11] Vickerstaff, *J. Soc. Dyers and Colourists*, 1957, **73**, 237.

[12] Baxter. Giles and Lewington, *J. Soc. Dyers and Colourists*, 1957, **73**, 386.

recrystallised twice from dilute hydrochloric acid. Distilled water was used for solutions for spectrophotometric measurements.

As controls for the cellulose films dyed with reactive dyes, dye solutions which had been boiled with alkali were employed. Alkali-boiling in absence of cellulose replaces the reactive chlorine atoms in the dye molecule by hydroxy-groups. In presence of cellulose the chlorine atoms are replaced by the ether link to the cellulose.

Ageing Effects, etc.—To prepare the aqueous solutions, the dye was first dissolved in a minimum of distilled water (cold for most dyes, boiling for a few of difficult solubility, *e.g.*, metal-complex dyes) and then diluted, as required, with cold water. Readings were then taken as soon as possible, usually within 30 min. of dilution. Tests were, however, made to determine whether there are any ageing effects, by examining the solutions (kept cold) at different periods up to 24 hr. With sulphonated dyes there was no change, except in one or two cases, where, over a period of several hours, the $y : x$ peak ratio increased by $<2\%$. The metal-complex dye solutions, however, which had been prepared by diluting the hot solution, showed a slight decrease in aggregation with time; the weakest solutions changed at the lowest rate. Thus a weak solution of Irgalan Orange RL with optical density 0·119 at 4800 Å decreased in $y : x$ ratio by about 15% over 24 hr.

To discover whether any of the spectral changes recorded could be caused by traces of metal ions, two dyes (C.I. 18,050, 24,410) were examined in 0·1% aqueous ethylenediaminetetra-acetic acid solution. The spectra were the same as in water.

Preparation of Films.—The methods have been described.[1]

Instruments.—Unicam S.P. 500 and S.P. 600 spectrophotometers were used, by one operator.

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Appendix

By D. Smith

To determine the significance of the optical-density ratios which form the basis of the present work, one dye (C.I. 18,050) was examined at three concentrations, the ratios of optical density at the two $\lambda_{\text{max.}}$ for each solution being determined three times. The whole operation was repeated by four different operators (S.P. 500 instrument). The results are shown in Table 2.

TABLE 2. *Replication results.*

Operator	A		B	
Concn.	Ratio *	Sum	Ratio	Sum
a	1·067, 1·067, 1·064	3·198	1·058, 1·059, 1·062	3·179
b	0·987, 0·985, 0·987	2·959	0·987, 0·987, 0·987	2·961
c	0·970, 0·955, 0·955	2·880	0·969, 0·970, 0·985	2·924
Total		9·037		9·064

Operator	C		D		
Concn.	Ratio	Sum	Ratio	Sum	Total
a	1·079, 1·079, 1·079	3·237	1·055, 1·056, 1·056	3·167	12·781
b	0·983, 0·983, 0·985	2·951	0·978, 0·963, 0·970	2·911	11·782
c	0·952, 0·968, 0·953	2·873	0·955, 0·955, 0·955	2·865	11·542
Total		9·061		8·943	36·105

* Optical-density ratio, y-band/x-band maximum.

The analysis of variance gives $\sigma_0^2 = 0{\cdot}000025$ and hence the standard deviation for each ratio is $\pm 0{\cdot}005$. Therefore, the average percent error of each ratio is $\pm 0{\cdot}5$.

To find if the difference $(x_1 - x_2)$ in the ratio for two concentrations of a dye is significant at the 5% level, when the x's are the average of two readings, the t-test can be used Thus,

$$t = \frac{x_1 - x_2}{\left(\frac{\sigma_0^2}{2} + \frac{\sigma_0^2}{2}\right)} = \frac{x_1 - x_2}{\sigma_0} \text{ with 24 degrees of freedom}$$

At the 5% level of significance the difference in ratios is significant if $t \geqslant 2{\cdot}06$.

Therefore, x_1 is significantly different from x_2 if $(x_1 - x_2) \geqslant \sigma_0 t \geqslant 0{\cdot}005 \times 2{\cdot}06 \geqslant 0{\cdot}01$, which is approximately the smallest difference recorded in Table 1.

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