DYE ADSORPTION BY MYCOBACTERIA[1,2]

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In the acid-fast reaction of mycobacteria, the first stage is adsorption of dye to the surface of the organism. Further penetration into the bacillary body frequently follows. Without adsorption there could be no staining, and fastness of dyes to acids or other agents is secondary.

Table 1, drawn from a large body of data, suggests that pH of solution and chemical type of dye may be of limited importance, and perhaps of no underlying significance. In addition to these factors, the adsorption of dye, with variations according to species, and influence of additives were studied.

MATERIALS AND METHODS

Dye adsorption.—This was measured by mixing a suspension of mycobacteria into a dye solution of known concentration for a standard period of time. Following centrifugation, the remaining dye content of the supernatant was measured photometrically.

All of the cultivated organisms used were harvested from the Proskauer and Beck medium after 4 to 5 weeks of growth at 37°C. Surface masses of wet organisms were homogenized by hand in saline and adjusted to a concentration of 100 mgm./ml. in Hopkins tubes. Homogenates were stored at 4°C until used, but the same homogenate was used for all determinations with a given dye. Except as specified, BCG was the test organism. It was used unheated. Cultures of *M. tuberculosis, M. bovis,* and *M avium* were heated at 100°C for 10 minutes before homogenization.

Dyes were prepared as a 0.1 per cent solution initially, without reference to molecular weight or dye content of sample. Few could be regarded as pure chemicals. From these solutions serial dilutions, usually 10 to 11, were made with equal amounts of distilled water until spectrophotometric sensitivity was lost, ordinarily at about 10^{-6}. With samples of low dye content sensitivity disappeared at 10^{-5}.

Aliquots of bacillary suspensions were mixed with 4 parts of suitable dilutions of dye, giving a final concentration of 20 mgm./ml. of microorganisms. These mixed suspensions were agitated in a Burrel shaker for 30 minutes at room temperature, and then centrifuged for 10 minutes at 10,000 g. Supernatants were analyzed spectrophotometrically for loss of dye adsorbed to the centrifuged bacilli. In the tables this is shown as percentages of the samples.

Estimations were made at the adsorption maxima of the dyes, which were obtained from Conn (2) or Lubs (7), or determined from the sample used, as with more than half those studied. Pyrex glass cuvettes were satisfactory for most of the work, but silica cells were needed for some of the highest dilutions, especially in the 410-450 lambda range. Analyses were made of 4 to 8 serial dilutions, but readings of adsorbence were disregarded when it was less than 10 per cent or more than 90 per cent. Analysis of several dilutions provided accuracy in controls.

Limitations.—Some problems of instability and low solubility of dyes yielded to use of small amounts of alcohols as solubilizers or stabilizers, or to performance of tests with minimal delay. Color changes of some dyes in the pH range of 6.7-7.0 were

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TABLE 1.—*Staining of mycobacterium spp. BCG.*

Dye	Solution	pH	Staining
Nile blue A	A[a]	3.9	4+
	B[b]	3.8	4+
	C[c]	7.3	4+
Orange 1	A	6.4	0
	B	6.4	0
	C	7.5	0
Safranin O	A	4.2	3+
	B	4.3	4+
	C	7.2	4+
Thiazine red	A	8.6	0
	B	7.9	0
	C	7.8	0

[a]A = aqueous solution, 1% dye.
[b]B = Ziehl-Neelsen formula, 5% phenol, 10% alcohol, 1% dye.
[c]C = Hanks' solution, 1% dye.

too provoking for perseverance. This eliminated dyes of the gallamin type from study. Fluorescence was an obvious problem with rhodamine B, thioflavine T, and probably auramine O. Gross coloration of bacillary sediment was not accompanied by increased transmittance of the supernatant. Measurements at low dilutions required elaborations beyond the scope of this work. Measurement of adsorption according to pH of solution was sometimes indicated, but done only in a few cases, as with brilliant cresyl blue. Adsorption of dyes to glass or plastic tubes was avoided when necessary by use of silicone treated glassware.

Dye nomenclature.—Dyes are identified by their color index (C.I.) numbers and the names, are those in common biologic use ([2]), Fieser and Fieser ([3]), and Lubs ([7]).

Acid and basic dyes.—Definition of a dye as acid or basic depends upon its ionization, and in no way upon pH of an aqueous solution of the dye. A basic dye supplied as its hydrochloride salt will be acid in solution; yet the dye will continue to ionize basically because its auxochrome groups are basic. Some acid dyes, manufactured by sulfonation of basic dyes, will contain mixtures of acid and basic dyes. The use of sulfanilic acid or chicago acid as an intermediate may yield a dye with both acid and basic auxochromes. Because the sulfonic ion is more strongly acid than the amino auxochrome is basic, these dyes are dominantly acid. They are listed here as acid dyes with basic radicals. Acid++ in Table 2 indicates an acid dye containing two basic auxochrome groups per molecule. Substitutions in basic groups do not alter basic characteristics unless ionization is greatly reduced.

Staining procedures.—Acid-fast stains were carried out chiefly in traditional manner, without use of heat for fixation, and staining in the cold in Coplin jars for 30 minutes. The standard dye content was 1 per cent. Water-fastness was studied, as well as acid-fastness (1 per cent HCl in 70 per cent alcohol), and 4 staining solutions were used in every case, (1) aqueous, (2) 5 per cent phenol-10 per cent alcohol, (3) Hanks' solution, and (4) Hanks' solution containing 1 per cent phenol and 2 per cent alcohol. The pH of the staining solutions was measured routinely.

Estimation of depth of staining was referred to that of sections simultaneously stained with solutions of new fuchsin, which was by definition the 4+ control. It has been suggested previously and is here confirmed, that intensity of staining is related to dye intensity, and color sensitivities of the human eye ([4]). Dyes in the central part of the visual spectrum received higher staining ratings from observers than those with absorption maxima below 480 or above 620 lambda. Accordingly, the degree of acid-fastness, from trace to 4+, is an estimation rather than a true measurement.

RESULTS

The results, shown in Table 2, indicate that dyes are adsorbed by mycobacteria in accord with their anionic dissociation. Basic dyes are adsorbed; acid dyes are not. Acid dyes with basic auxochromes are frequently adsorbed, and may stain bacilli weakly acid-fast, but usually do not. Some variations from these findings are discussed under the separate types of dyes. Chemical type of dye is unimportant, except in the case of the hydroxy fuchsins, which merit special attention.

Nitro dyes.—No adsorption of the 3 dyes used occurred. They are not greatly different from one another chemically. Bacilli are not colored at all.

Benzidine dyes.—These are so-called because prepared from benzidine; all are acid dyes, but 6 of the 7 used also contain free amine groups. Significant adsorption occurred, which tended to disappear as the concentration increased. Staining, however, was poor. The dyes washed out easily in water as well as acid-alcohol. The pH of all of these dyes in solution is 8.0 or above for 1 per cent solutions, regardless of buffering. Congo red gave a little staining at pH 8.2, but not at pH 7.5. Erie garnet B, which gave moderate staining, contains one amino group.

Stilbene dyes. — No adsorption was observed, and no staining proved possible.

Thiazole dyes. — Only the basic thioflavine T was adsorbed or stained bacilli. Aqueous solutions gave a pH of 3.0 and in Hanks' solution the pH was 6.6. The pH of the solution was a factor, adsorption being greater at the higher pH, and a pH close to 7.0 was needed to produce moderate staining of bacilli. Measurements of adsorption were affected by fluorescence and difficult to reproduce.

Acridine dyes.—All are basic, all were adsorbed, and all stained bacilli well. The staining was found to be much better when solutions were buffered close to pH 7.0, with phosphine G and acriflavine.

Eurhodine dye.—Neutral red has long been recognized as a basic dye which stains acid-fast bacilli well. It was adsorbed well.

Safranins.—With the exception of indulin, a sulfonated safranin and an acid dye, all are basic and stained acid-fast bacilli well. Safranins are not as brilliant to the eye as fuchsins.

Azosafranins.—These two dyes have azo chromophores added to the safranin complex, and Janus black B has a hydroxy group in this chromophore. These two dyes went well over the 90 per cent adsorption level at 10^{-5} dilutions, proving as heavily adsorbed as any in the series.

Aposafranin.—Azocarmine G of this group is an acid dye. It was not adsorbed and staining was impossible.

Nigrosin.—This is evidently a mixture of azine dyes of uncertain composition. The formulas call for a basic dye. The sample was somewhat adsorbed in higher dilution.

Ketone imine dye.—Auramine O, strongly basic, has been much used for mycobacteria because of its fluorescence. The adsorption observed may actually have been greater, with fluorescence lowering the measured spectrophotometric reading.

Mono-azo dyes.—These dyes constitute a somewhat heterogeneous group, not easily characterized. All are acid dyes. Only 2 were adsorbed and stained, both of them having basic groups.

Dis- and poly-azo dyes.—The 4 similar dyes, bismarck browns and chrysoidin Y, are basic, were adsorbed, and stained bacilli well. Chrysoidin Y is the mono-azo congener of the diazo bismarck browns, and is included here because of its chemical similarity. Its adsorption was measurably less, and it stained BCG poorly, but *M. lepraemurium* well.

The adsorption of chicago blue gave a reading observed with a number of the acid dyes with basic components. At higher dilutions adsorption occurred, but in the more concentrated solutions, there was none. No staining should occur from a 1 per cent solution, nor was any observed.

Direct green G stained some mycobacterial species well. It has 2 free amino groups per molecule, and is the only poly-azo dye on the list.

Oxazine dyes.—These dyes followed the general pattern. The basic dyes were adsorbed, the acid dye not. Staining of bacilli corresponded to the adsorption. Brilliant cresyl blue at pH 3.4 appeared to coat bacilli with a monolayer, outlining organisms clearly, but without penetration of the bacillary body. At pH 6.5 much heavier staining was observed.

Indamin dye.—Toluylene blue was the only satisfactory one studied; it did not have much color intensity, but was well adsorbed.

Thiazine dyes.—All the basic dyes of this group followed the pattern. The azures were adsorbed according to their methylene blue component. Problems of solubility and color change gave only fair results with brilliant alizarin blue.

Aryl methane dyes.—With the exception of malachite green all the basic dyes of this group were adsorbed and stained bacilli. The acid dyes did not. Malachite green oxalate was adsorbed as though it were an acid dye with a basic component. Conn lists it as a weakly basic dye. It is felt that both of the samples investigated had the color-base somewhat altered in rendering the salts water-soluble.

Two samples of acid fuchsin differed markedly from a sample studied some 25 years ago ([4]) which gave no staining of mycobacteria. This dye is prepared by direct sulfonation of basic fuchsins, and the adsorption and staining are attributable to residual fuchsins contaminating the sample, or to incomplete sulfonation. A similar situation existed with methyl green (C.I. 11, 106) which is manufactured from crystal violet.

Wool green stained BCG slightly at pH 4.6, but not at pH 7.0, where the adsorption study was carried out. It failed to stain *M. lepraemurium* at either pH.

TABLE 2.—*Dye adsorption by mycobacteria (++ = Basic groups in acid dyes)*

Dye	C. I. No.	Acid-base	Lambda	Per cent adsorption							
				10^{-6}	–	10^{-5}	–	10^{-4}	–	10^{-3}	Acid-fast
1. Nitro Dyes											
1. Picric acid	10,305	Acid	405		0	0	0	0	0		0
2. Martius yellow	10,315	Acid	445		0	0	0	0	0		0
3. Naphthol yellow	10,316	Acid	430		0	0	0	0	0		0
2. Benzidine Dyes											
1. Congo red	22,120	Acid^{++}	497		6	30	45	38			1^{+}
2. Benzoazurin	24,140	Acid	569		22	22	13	6			0
3. Brilliant congo red	23,570	Acid^{++}	505		16	26	32	28			Trace
4. Evans blue	23,860	Acid^{++}	595			10	3	3			0
5. Erie garnet B	22,145	Acid^{++}	510		41	45	39	16	4		1^{+}
6. Benzopurpurin 4B	23,500	Acid^{++}	525		28	36	15	4			0
7. Benzopurpurin 10B	24,100	Acid^{++}	525		5	16	13	2	0		0
3. Stilbene Dyes											
1. Brilliant yellow	24,890	Acid	493		0	0	0	0	0	0	0
2. Mikado yellow	40,006	Acid	415			0	1	3	0		0
4. Thiazole Dyes											
1. Primulin	49,000	Acid	420		0	0	0	0	3		0
2. Titan yellow	19,540	Acid	430		0	3	4	3	0		0
3. Thiazine red	14,780	Acid	505		0	0	0	0	0		0
4. Thioflavine T	49,005	Basic	410				(a)				2^{+}
5. Thioflavine S	49,010	Acid	400		0	0	14	14			0

5. Acridine Dyes

1. Acridine orange	46,005	Basic	497			24	58	65	50		3^+
2. Acridine yellow	46,025	Basic	455	68	75	87	84	60			2^+
3. Phosphine GN	46,045	Basic	485		35	41	66	65	44		3^+
4. Acriflavine	46,000	Basic	450	59	19	34	25				4^+
5. Acriflavine HCL (mixture)	46,000	Basic	445		15	24	20				4^+

6. Eurhodine Dye

1. Neutral red	50,040	Basic	650		26	30	50	72	78		3^+

7. Safranins

1. Neutral violet	50,030	Basic	560			9	35	42	47		3^+
2. Indulin scarlet	50,080	Basic	500		22	38	64	70	70		3^+
3. Safranin O	50,240	Basic	530			30	26	26	10		3^+
4. Safranin bluish	50,210	Basic	530		14	17	26				3^+
5. Amethyst violet	50,225	Basic	598		38	32	29	27			3^+
6. Indulin	50,400	Acid	550			0	2	11	2		0

8. Azosafranins

1. Janus black B	11,825	Basic	600				85	92	98	85	4^+
2. Janus green B	11,045	Basic	615			47	70	90	75	38	4^+

9. Aposafranin

1. Azocarmine G	50,085	Acid	512		0	0	0	0	0		0

Dye	C. I. No.	Acid-base	Lambda	Per cent Adsorption 10^{-6}	–	10^{-5}	–	10^{-4}	–	10^{-3}	Acid-fast
10. Complex Azine Dye											
1. Nigroin, w. sol.	50,420	Basic	650			19	21	10	1		Trace
11. Ketone Imine Dye											
1. Auramine O	43,815	Basic	430	10	10	15	28	26	20		3^{+}
12. Mono-azo Dyes											
1. Methyl orange	13,025	$Acid^{+}$	460		0	0	0	0			0
2. Fast yellow	13,015	$Acid^{+}$	490			0	0	0	0		0
3. Diamond flavine G	14,135	Acid	372		0	12	13	2			0
4. Orange 1	14,600	Acid	476		0	0	0	0	0		0
5. Eriochrome viol.	15,670	Acid	510		0	0	6	3			0
6. Ponceau 2R	16,150	Acid	499			0	0	0	0		0
7. Orange G	16,230	Acid	476			0	0	0	0		0
8. Chromatrope 2 R	16,570	Acid	510			0	0	0	0		0
9. Azo-acid blue	16,180	Acid	570			0	0	0	0		0
10. Roccellin	15,620	Acid	510		0	8	15				0
11. Diamond blue 3B	14,835	Acid	530	0	0	0	0	0			0
12. Amaranth	16,185	Acid	525		0	0	0	0			0
13. Brill. ponceau	16,255	Acid	505		0	0	0	0			0
14. Eriochrome blk-T	14,645	$Acid^{++}$	565		31	30	34	23			3^{+}
15. Eriochrome bl-Blk	15,705	$Acid^{++}$	540			20	21	18	8		1^{+}

13. Dis- and Poly-azo Dyes

1. Bismarck brown Y	21,000	Basic	463		17	18	36	52			3^+
2. Bismarck br. 53B	21,010	Basic	463		28	36	50	67			3^-
3. Bismarck brown R	21,030	Basic	463		49	46	40	58			3^+
4. Chrysoidin Y	11,270	Basic	415		8	13	26				3^+
5. Brill. purp. R	23,510	Acid^{++}	460			27	14	16	10		Trace
6. Biebrich scarlet	26,905	Acid	503		0	0	0	0	0		0
7. Napth. bl-blk	20,470	Acid$^+$	620			0	0	3	0		0
8. Chlorzaol black E	30,235	Acid^{++}	600			19	26	23			Trace
9. Niagara sky blue	24,400	Acid^{++}	605		0	0	6	6			0
10. Trypan blue	23,850	Acid^{++}	580		0	4	6	0			0
11. Dianil blue 2R	23,690	Acid	568		13	15	11	7			0
12. Diamond black F	——	Acid	562		25	25	23	8			0
13. Crocein scarlet	27,290	Acid	510		0	0	0	0			0
14. Cyanin blue G	26,380	Acid$^+$	555		62	75	71	60	24		1^-
15. Toluylene orange	23,380	Acid^{++}	405		15	21	31	23			0
16. Chicago blue	24,380	Acid^{++}	627			37	36	28	9	0	0
17. Direct green G	30,315	Acid^{++}	630		30	34	33	26			1^-

14. Oxazine Dyes

1. Celestin blue	51,050	Basic	654		Color changes						1^+
2. Brill. cresyl bl.	51,010	Basic	625			0	5	15	14		2^+
3. Meldola's blue	51,175	Basic	573			14	18	17	20		2^-
4. Delphine blue	51,120	Acid$^+$	585				0	0	4	2	0
5. Nile blue A	51,180	Basic	640			77	74	74	48		4^+

15. Indamin Dye

1. Toluylene blue	49,410	Basic	620			18	56	73	65		2^+

Dye	C. I. No.	Acid-base	Lambda	Per cent Adsorption							
				10^{-6}	—	10^{-5}	—	10^{-4}	—	10^{-3}	Acid-fast
16. Thiazine Dyes											
1. Thionin	52,000	Basic	598		2	13	39	81			3+
2. Methylene green	52,020	Basic	660			61	54	35			2+
3. Methylene blue HCL	52,015	Basic	665		14	18	38	42			2+
4. New methyl bl-N	52,030	Basic	588			35	62	70	85		2+
5. Brill. aliz. bl	52,055	Acid+	630			2	9	20	20		0
6. Toluidine blue	52,040	Basic	620		9	18	46	54	50		3+
7. Azure A	——	Basic	630				27	44	56		2+
8. Azure B	——	Basic	595			26	32	61	75		2+
9. Azure C	——	Basic	610			17	45	60			2+
17. Aryl Methane Dyes											
1. Malachite green oxal.	42,000	Basic (weak)	618			66	55	55	26	5	0
2. Rhoduline blue 6G	42,025	Basic	590	70	62	58	37	20			3+
3. Crystal violet	42,555	Basic	590	62	58	55	37	20			4+
4. Acid fuchsin	42,685	Acid	545		25	35	35	37			1+
5. Ethyl violet	42,600	Basic	596	88	88	85	76	63	28		4+
6. Alkali blue	42,750	Acid++	594		87	83	76	57	32		0
7. New fuchsin	45,625	Basic	546		25	46	54	78	55		3+
8. Anil. bl, w. sol.	42,755	Acid	595			79	83	80	45		0
9. Fast green ex. bl.	42,038	Acid+	630		0	0	0	0			0
10. Alphazurin 2G	42,051	Acid+	595		0	0	0	0	0		0
11. Fast green FCF	42,053	Acid	630	0	0	0	0	0			0
12. Guinea green B	42,085	Acid+	620		0	0	7	6			0
13. Eriogla ıcin	42,090	Acid	630			0	0	0	0		0
14. Light green SFY	42,095	Acid	635			6	4	0			0
15. Brill. mill. gr.	42,100	Acid	630			0	0	0	0	0	0

16. Victoria blue B	44,045	Basic	619		70	86	91	86	84		4^+
17. Night blue	44,085	Basic	625			58	60	60	67		3^+
18. Alk. fast green	44,025	$Acid^+$	640		0	0	0	0	0		0
19. Wool green	44,090	$Acid^{++}$	634		0	0	0	0	0		1^+
18. Xanthene Dyes											
1. Fluorescein	45,190	Acid	530		0	0	0	0			0
2. Rhodamin B	45,170	Basic	557				(a)				4^+
3. Rhodamin 6G	45,160	Basic	525	35	42	45	32				3^+
4. Pyronin Y	45,005	Basic	525	35	42	45	32				4^+
5. Pyronin B	45,010	Basic	555	48	28	16	19				1^+
6. Eosin Y	45,380	Acid	515	0	0	1	0				0
7. Erythrosin bl.	45,430	Acid	525		0	10	9	2			Trace
8. Phloxin B	45,410	Acid	547		12	13	6	1			2^+
9. Fast acid blue R	45,205	Acid	534		0	0	2	4	2		0
10. Violamin R	54,190	Acid	530	0	0	0	3				0
19. Anthraquinone Dyes											
1. Alizarin green	65,170	Acid	630			13	13	6	2		0
2. Alzarin blue-blk	63,615	$Acid^{++}$	590		38	25	24	15	4		Trace
3. Alizarin saph. A	62,055	Acid	649			6	4	4	0		0
4. Anthraqu. viol. R	61,710	Acid	555		0	0	0	3	6		0
5. Anthracene blue	58,805	Acid	595		0	0	2	1	0		0
6. Anthrac. blue SWR	58,605	Acid	510			12	27	33	16	0	0
Miscellaneous Dyes											
1. Pinacyanole	——	Basic	608	63	68	75	92	70	24		3^+
2. Quinoline yellow	47,005	Acid	440	0	0	0	0	0	0		0
3. Alcian blue	74,240	Basic??	600			13	29	65	97	71	3^+

(a) measurement of adsorption unsatisfactory because of autofluorescence.

Xanthene dyes.—Conn ([2]) tells the story of Mallory's discovery that phloxin B was "the best *eosin* I have found. . . ." Clearly an acid dye, the sample at hand was significantly adsorbed and stained bacilli variably a light pink shade. Erythrosin gave similar inconsistencies. The rhodamines and pyronines, all basic dyes, were well adsorbed. Rhodamin B adsorption could not be measured spectrophotometrically because of fluorescence. It was felt that the activities of phloxin B and erythrosin bluish resulted from contamination of the samples with basic pyronins.

Anthraquinone dyes.—Nothing of interest was exhibited in this group. The poor water solubility of many other members of this class eliminated them from the study.

Miscellaneous dyes.—Pinacyanole at pH 6.7 stains BCG purple and *M. lepraemurium* blue. It undergoes a color change at a slightly higher pH. Alcian blue has a low dye content and high salt content. Its structure is uncertain; it is manufactured from phthalocyanin pigments by ammonification. It is probably a basic dye.

Hydroxy aryl methane dyes.—The metachromatic effects of staining mycobacteria with eriochrome cyanin R have been reported ([5]). It was not recognized at the time that some of the color differences were attributable to the organism studied. The importance of phenol is even more striking with rosolic acid, and eriochrome azure blue was erroneously reported not to stain mycobacteria, BCG not having been tested.

With 2 of these dyes the presence of phenol is significant for stain-

TABLE 3.—*Hydroxy aryl methane dyes.*

Dye	Per cent adsorption by BCG	Staining			
	pH 3.6	BCG		*M. lepraemurium*	
		pH 3.6	pH 6.6	pH 3.6	pH 6.6
1. Rosolic acid Lambda 460	A[a] 27	3+ Blue	2+ Blue	0	0
	ZN[b c]	3+ Mixed Blue-Yellow	3+	4+ Yellow	2+ Yellow
2. Eriochrome cyanin R C.I. 43,820 Lambda 525	A 37	3+ Mixed Blue-Orange	1+	1+ Yellow-Orange	Trace
	ZN 22	1+ Blue	1+ Mixed	4+ Yellow-Orange	3+
3. Eriochrome azure Blue C.I. 43,830 Lambda 430	A 69	1+ Mixed	2+ Blue	0	0
	ZN 46	2+ Mixed Blue-Orange	2+	0	1+ Orange

[a]A = Aqueous solution.
[b]ZN = Ziehl-Neelsen formula.
[c]Absence of measured adsorption because of fluorescence.

TABLE 4.—*Dyes staining mycobacteria 2+ or better.*

Dye type	Number	Basic	Acid+	Acid	Uncertain
1. Thiazole	1	1	..	..	..
2. Acridine	5	5	..	..	..
3. Eurhodine	1	1	..	..	..
4. Safranins	5	5	..	..	..
5. Azosafranins	2	2	..	..	..
6. Ketone-imine	1	1	..	..	..
7. Mono-azo	1	..	1	..	..
8. Poly-azo	4	4	..	..	..
9. Oxazine	3	3	..	..	..
10. Indamin	1	1	..	..	..
11. Thiazine[a]	5	5	..	..	..
12. Aryl methane	6	6	..	..	..
13. Xanthene	4	3	..	1	..
14. Hydroxy aryl methane	3	..	..	..	3
15. Miscellaneous	2	2	..	..	..
Total	44	39	1	1	3

[a]Azures A, B, C, excluded as methylene blue additives.

ing, although less dye was adsorbed from the Ziehl-Neelsen solutions than from the aqueous, the dye strengths being equal.

All 3 hydroxyfuchsins can be adjusted to produce metachromatic effects (Table 3), and red shades have been observed. Sharp differences occurred between staining of *M. lepraemurium*, and of BCG. It is not known whether these dyes ionize as acids or as bases, and they present an unresolved puzzle. Molecular rearrangement on contact is probable.

Staining of mycobacteria demonstrating acid-fastness (Table 4) agreed well with the finding that adsorption was dependent upon anionic dissociation.

The exceptions do not disturb the thesis of the importance of basicity, in consideration of the impurities of many dyes, and of possible anionic alteration resulting from solubilizing as salts.

Rate of adsorption and staining.—The shortest time interval measurable was 2 minutes, which meant brief shaking and rapid centrifuga-

TABLE 5.—*Rate of adsorption by BCG at* 0.8×10^{-5} *dye.*

Time	Per cent adsorption	
Minutes	Safranin 0	New methylene blue
2	21	45
5	26	43
10	30	40
15	32	54
30	38	58
60	41	61
90	40	47
120	37	45

tion at 12,000 g. Both dyes studied (Table 5) were so rapidly adsorbed that more than 50 per cent of the maximal adsorption had already taken place in the minimum interval.

BCG grown on the Proskauer-Beck medium stains acid-fast with equal rapidity, regardless of time, being stained almost as heavily in 2 as in 20 minutes. Contrariwise, *M. leprae* and *M. lepraemurium* derived from tissues stain much more slowly. These large variations could stem from varying rates of penetration of dye into bacillary bodies, under different biologic conditions, or the staining rate of bacilli may be conditioned by anionic adsorptions already present.

Variations in pH.—Variation of pH is significant to staining of some dyes. Three examples are noted (Table 6). A great many more examples were observed in which variation of the pH made no differ-

TABLE 6.—*Staining of mycobacteria species by different types of dyes.*

Dye	pH	Staining	pH	Staining
Amethyst violet	5.7	2+	6.9	4+
Methylene blue	3.6	Trace	7.4	3+
Acridine orange	3.6	0	6.15	3+

TABLE 7.—*Effects of phenol on adsorption and staining.*

Dye	Type of dye	Per cent adsorption		Staining	
		Water	5% phenol	Water	5% phenol
1. Martius yellow	Nitro	0	0	0	0
2. Benzopurpurin 10B	Benzidine	15	15	0	Trace
3. Thioflavine T	Thiazole	10	10	2+	2+
4. Acridine orange	Acridine	41	38	3+	3+
5. Neutral red	Eurhodine	55	62	3+	3+
6. Safranin 0	Safranin	32	11	3+	3+
7. Janus black B	Azosafranin	82	83	3+	3+
8. Azocarmine G	Aposafranin	0	2	0	0
9. Nigrosin	Complex azine	12	11	Trace	Trace
10. Auramine 0	Ketone-imine	(a)	(a)	3+	3+
11. Eriochrome blue black R	Mono-azo	30	20	2+	1+
12. Bismarck brown 53-B	Diazo	33	19	3+	3+
13. Toluylene orange	Diazo	19	18	Trace	Trace
14. Meldola's blue	Oxazine	8	8	2+	2+
15. Toluylene blue	Indamin	19	27	2+	2+
16. Methylene blue Cl	Thiazine	10	10	3+	3+
17. New fuchsin	Aryl methane	33	46	4+	4+
18. Crystal violet	Aryl methane	21	61	4+	4+
19. Brilliant milling green	Aryl methane	3	4	0	0
20. Pyronin Y	Xanthene	8	22	3+	3+
21. Azure blue B	Hydroxymethane (pH 3.6)	69	46	1+	2+
22. Eriochrome cyanin R	Hydroxymethane (pH 3.6)	37	22	1+	3+
23. Pinacyanole	Cyanin	72	97	4+	4+
24. Alcian blue	Solubilized pigment	92	96	3+	3+

ence; equally good stains have been observed over a wide range. The few variations evidently relate to dyes, not to bacillary factors.

Effect of phenol.—Lartigue and Fite (6) showed that phenol did not alter new fuchsin chemically, and that improved staining could be obtained with several organic agents. Solubilizing effects were suggested, but no direct action on dye or bacilli.

This work was extended to include dyes of nineteen types (Table 7). Bacilli were stained from 1 per cent solutions in both water and the Ziehl-Neelsen formula. Adsorption was measured at 0.8×10^{-5} dye concentration in similar solutions.

Phenol had no effect on adsorption or staining with 14 of 24 dyes. It increased the adsorption of 5 dyes and decreased it in the case of 5 others. Blind reading of the degrees of acid-fastness showed no differences in 30-minute staining intensities, with the significant exception of the hydroxy fuchsins. In these the measured adsorption was *decreased* by phenol, while the staining increased. Molecular rearrangement of these dyes in the presence of phenol is suggested, rather than effect upon bacilli.

COMPARISON OF ADSORPTION AND STAINING OF DIFFERENT SPECIES OF MYCOBACTERIA

The experiments showed that differences in adsorption and staining might be expected according to mycobacterial species. A larger test run was carried out accordingly.

For staining purposes, 1 per cent solutions were used as previously, but only the staining in the Ziehl-Neelsen formula, followed by washing in 1 per cent HCl in 70 per cent alcohol, is reported here. Excerpts from more than 9,600 readings, recorded in Table 8, are reasonably representative of the group. In addition to the 8 mycobacteria reported, 5 others were studied, viz., another avian strain and 4 nonpathogens. No significant information would be added by inclusion of the results with these microorganisms.

Many variations, of minor or moderate degree, were observed among the different species studied. *M. fortuitum* stands forth, from its staining by some acid dyes, as evidently significantly different in its reactions. Acid-fastness of mycobacteria is not uniform for species grown under the same conditions, and no *one* organism behaves consistently throughout the dye spectrum.

Adsorption by a group of 30 basic and acid dyes with basic groups was determined, comparing that by $H37R_v$ with those by *M. fortuitum*, *M. smegmatis*, *M. bovis*, and *M. avium*. These revealed relatively little that was new. The basic dyes were usually adsorbed equally by all 5 organisms. Poor staining and poor adsorption, as by *M. fortuitum* with acridine orange, harmonized, and *M. smegmatis* adsorbed an occasional dye poorly, as in the case of Meldola's blue, staining poorly also.

TABLE 8.—*Staining of mycobacteria by various dyes.*

Part I—Acid dyes

Dye	pH of sol.	*M. leprae*	*M. lepraemurium*	BCG	*M. tuberculosis*	*M. bovis*	*M. avium*	*M. fortuitum*	*M. smegmatis*
1. Amaranth	6.5	0	0	0	0	0	0	0	0
2. Azocarmine G	5.2	0	0	0	0	0	0	0	0
3. Benzoazurin	7.5	Trace	Trace	0	1+	0	0	3+	3+
4. Biebrich scarlet	7.1	0	0	0	Trace	0	0	Trace	Trace
5. Brilliant ponceau 5R	7.8	0	0	0	0	0	0	1+	2+
6. Brilliant yellow	7.4	0	0	0	Trace	0	0	Trace	1+
7. Chromatrope 2R	6.7	0	0	0	0	0	0	3+	Trace
8. Crocein scarlet	6.5	0	0	0	0	0	0	Trace	Trace
9. Diamond bl. F	7.4	0	0	0	0	0	0	Trace	0
10. Fast acid blue R	7.6	0	0	0	0	0	0	0	0

Part II—Basic dyes

Dye	pH of sol.	*M. leprae*	*M. lepraemurium*	BCG	*M. tuberculosis*	*M. bovis*	*M. avium*	*M. fortuitum*	*M. smegmatis*
1. Acridine orange	3.7	4+	4+	2+	4+	1+	1+	1+	Trace
2. Acridine yellow	3.3	1+	0	2+	3+	0	0	Trace	1+
3. Amethyst violet	5.75	4+	4+	4+	4+	1+	2+	1+	1+
4. Auramine 0	4.7	4+	4+	2+	4+	4+	4+	1+	0
5. Crystal violet	4.7	4+	3+	4+	4+	2+	4+	2+	Trace
6. Ethyl violet	4.9	4+	3+	3+	4+	2+	2+	3+	1+
7. New fuchsin	6.5	4+	4+	4+	4+	3+	3+	3+	2+
8. Meldola's blue	4.55	4+	3+	2+	3+	4+	4+	3+	1+
9. Methylene blue	3.6	3+	2+	2+	2+	2+	2+	4+	2+
10. Night blue	3.0	4+	2+	1+	3+	2+	1+	3+	1+
11. Neutral red	4.6	4+	2+	3+	2+	2+	2+	3+	2+
12. Nile blue A	3.8	4+	2+	4+	2+	Trace	Trace	Trace	1+
13. Rhodamine B	3.25	3+	4+	3+	4+	4+	4+	3+	0

Part III—Acid dyes with two anxochrome groups

Dye	pH of sol.	*M. leprae*	*M. lepraemurium*	BCG	*M. tuberculosis*	*M. bovis*	*M. avium*	*M. fortuitum*	*M. smegmatis*
1. Benzopurpurin 4B	8.050	0	0	0	0	0	0	1+	2+
2. Benzopürpurin 10B	8.1	0	Trace	0	0	0	0	Trace	Trace
3. Brilliant milling green	4.6	1+	Trace	1+	0	0	0	Trace	1+
4. Brilliant purpurin R	7.5	0	0	0	0	0	0	1+	2+
5. Chicago blue	5.85	0	0	0	0	0	0	2+	Trace
6. Chlorazol black	8.75	0	0	0	0	0	0	3+	Trace
7. Direct green	7.8	4+	0	0	Trace	0	0	3+.	Trace
8. Guinea green B	4.7	0	0	0	Trace	0	0	Trace	Trace
9. Wool green	4.6	0	0	2+	1+	0	0	1+	Trace

DISCUSSION

"Acid-fastness is a property of the intact cell" [Topley and Wilson (8)], and is dependent upon the ionic status of the intact cell. Because mechanical rupture of the mycobacterial cell is followed immediately by loss of acid-fastness, this property cannot derive from any chemical substance in any part of the cell. Evidently an ionic equilibrium within the bacterial cell is essential for adsorption and retention of dye. The intact cell wall is necessary for preservation of this equilibrium.

Penetration of dye into the bacterial cell also may depend upon this equilibrium, which is sensitive to the biologic integrity of the cell. Inadequately stained mycobacteria show uneven staining, rather than diffuse even coloration, a fact suggesting that the ionic status is not equal in all parts of the mycobacterial cell, and probably not a surface factor.

The adsorption of basically ionized substances by mycobacteria explains the progressive acidity of cultures of mycobacteria. In addition, the necessity of anionic dissociation by effective chemotherapeutic agents is emphasized.

SUMMARY

Dyes are adsorbed to the surfaces of mycobacteria according to the anionic components of the auxochrome groups of the dyes. Basic dyes are uniformly adsorbed. Acid dyes are not adsorbed. Acid dyes with basic auxochromes are adsorbed, especially in higher dilutions, but are not fast to acid. Basic dyes are fast to water, alcohol, and acids. Adsorption takes place rapidly, reaching a maximum in one hour, waning thereafter. Some differences are observed among mycobacterial species both in adsorption and staining. The chemical nature of the dye is not significant, except in the case of the hydroxy aryl methanes, which exhibit unusual reactions. The pH is not critical between 3.4 and 8.2, although a few dyes are adsorbed better at one or another extreme.

RESUMEN

Los colorantes son adsorbidos a las superficies de las micobacterias de acuerdo a los componentes aniónicos de los grupos auxocrómicos de los colorantes. Colorantes básicos son uniformemente adsorbidos, especialmente en altas diluciones, pero no son estables para los ácidos. Los colorantes básicos son estables para el agua, alcohol y ácidos. La adsorción toma lugar rapidamente, llegando al máximo en una hora, desapareciendo luego. Se observaron algunas diferencias entre las especies micobacterianas tanto en la adsorción como en la coloración. La naturaleza química del colorante no es significativa, excepto en el caso de los hidroxil-aril-metanos, los cuales exhiben reacciones inusuales. El pH no es crítico entre 3.4 y 8.2, aunque unos pocos colorantes se adsorben mejor en uno u otro de los extremos.

RÉSUMÉ

Les colorants sont adsorbés par la surface des mycobactéries en fonction des constituants anioniques de leurs groupements auxochromes. Les colorants basiques sont uniformément adsorbés. Les colorants acidees ne le sont pas. Les colorants acides dotés

d'auxochromes basiques sont adsorbés, particulièrement aux dilutions les plus élevées, mais ne sont pas acido-résistants. L'adsorption a lieu rapidement, atteignant son maximum en une heure, et disparaît progressivement ensuite. En ce qui concerne l'adsorption et la capacité à être colorées, certaines différences s'observent entre diverses éspèces de mycobactéries. La nature chimique du colorant n'importe guère, sauf dans le cas des méthanes hydroxy-aryles, qui témoignent de réactions inhabituelles. Entre les valeurs de 3.4 à 8.2, le pH ne constitue pas un facteur critique, quoique certains colorants soient adsorbés davantage à l'un ou l'autre extrême de cette marge de variation.

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