[CONTRIBUTION FROM THE CANCER RESEARCH LABORATORY, UNIVERSITY OF FLORIDA]

The Spectrophotometric Determination of Ionization Constants of Basic Dyes[1]

BY SIEGFRIED WOISLAWSKI[1a]

RECEIVED JUNE 5, 1953

The ionization constants of 14 basic dyes belonging to 5 chemical classes: azo, azines, xanthenes, oxazines and thiazines were determined spectrophotometrically. Fairly good agreement for the ionization constants by the three different methods (spectrophotometric in buffered 50% alcohol, titration in water and in 50% unbuffered alcohol) is obtained for Nile Blue A, Bismarck Brown Y, Rhodamine B, Chrysoidin Y, Neutral Red and Pyronine B. With the exception of Acridine Red these are all the weaker bases. Higher values were found for the other dyes by the spectrophotometric method and these paralleled the distribution ratios in buffer–benzene. The data indicate that the ionization constants not only vary with the method and medium, but also that the order of basicity is different in different media.

Ray and Jung[2] have determined the ionization constants of basic dyes from titration data in 50% alcoholic solutions. Woislawski[3] redetermined these ionization constants by titration in aqueous medium. Discrepancies were noted between his values and those found by Ray and Jung.

The present study was undertaken because it was hoped that the determination of the ionization constants of the dyes by a different method, namely, from spectrophotometric data in buffered 50% alcoholic solutions, might help to resolve these discrepancies. The spectrophotometric method was chosen because Noyes[4] has demonstrated that the colors of indicators are a valid measure of their degree of ionization, unless changes in the structure of the molecule take place. The loss or gain of a hydrogen ion seems to be accompanied by a change in light absorption. The extent of ionization of weak bases (and acids) may therefore be calculated from the changes in the absorption spectra.

Experimental

A stock solution of concentration 1×10^{-4} M of the dyes in 95% alcohol was prepared. To 10 ml. of the stock solution was at first added either 50 ml. of N HCl, 0.1 N HCl, phthalate buffer pH 3.0, acetate buffer pH 5.0, phosphate buffer pH 6.7, borax–HCl buffer pH 8.2, 0.1 N NaOH or N NaOH, which was then diluted to the mark with 95% alcohol. The final concentration of the dyes was 1×10^{-5} M. In both the buffered and unbuffered solutions the pH became more alkaline upon dilution with alcohol. The absorption spectra were measured using a Beckman model DU spectrophotometer. Spectra were measured from 400–700 mμ at 24 ± 1°. For the determination of pH a Beckman pH meter (model G) with glass electrode 1190T and calomel electrode 1170 was used. When the pH rose above 10, a Beckman electrode 1190E was used. Because the pK_a cannot be converted to pK_b with any degree of accuracy in 50% alcoholic solutions, only pK_a values are reported in this paper. The pK_a of the dyes was determined by the formula of Flexser, *et al.*[5]

$$pK_a = pH_m + \log \frac{E_B - E_m}{E_m - E_{BH^+}}$$

where E_B is the optical density of the uncharged molecule of the dye, E_{BH^+} that of the charged ion, and E_m represents the density of the mixture of the two species; pH_m is the pH of the solution containing a mixture of the two species. For very strong bases it was not possible to determine the pK_a because the dyes changed color about pH 13 which is beyond the limit of the pH meter. In those regions where the concentrations of the uncharged molecule and charged ion were approximately equal, additional spectra for solutions which differed only by a few tenths of a pH unit in either direction were determined. Sometimes the calculations would yield several pK_a values. In such cases the optical density was plotted *versus* the pH and the pK_a determined from the resulting sigmoid curve. Methylene blue and methylene green undergo hydrolytic cleavage in very strong alkaline solutions with the formation of oxazones. The slowness of the change of color, and the inability to restore the original color upon addition of acid were interpreted as chemical changes and not as ionization phenomena. For this reason it was not possible to determine the pK_a for methylene blue, and the value given for methylene green is reported with some reservation.

Results and Discussion

In most dyes (except no. 3, 4, 7, 14, Table I) the absorption band decreases in intensity (hypsochromic effect) when changing the pH from the acid to the alkaline region. In many dyes (no. 1, 3, 4, 6, 8, 9, 12, 14) the absorption band shifts toward lower wave lengths (hypsochromic effect) under these conditions. In some dyes (no. 1, 6, 8, 9, 10, 11, 12, 14) we noticed both a change in intensity and a shift of the absorption band toward lower wave lengths under the same conditions. Sometimes the intensity of the absorption band remains constant over a large pH range, and changes are only observed at high alkalinity (no. 9, 11, 12, 13, 14). There are two exceptions to these rules among the 14 dyes that have been used in this study. Pyronine B (no. 7) increases its intensity from pH 1.7 to 7.8, and then decreases with higher alkalinity. In Brilliant Cresyl Blue (no. 10) the absorption band shifts at pH 11.3 toward the lower wave lengths and at pH > 13 the shift of the absorption band is reversed toward longer wave lengths (bathochromic effect). If one or more of the curves for a given compound does not intersect at a common point, it indicates the presence of an additional form. This may be caused by an impurity originally present or formed under the experimental conditions (hydrolysis or oxidation).

Let us consider the 3 azines Neutral Red, Neutral Violet and Safranin O. The spectral curves of the indicator Neutral Red (no. 6) show that from pH 1.6–3.9 (which correspond to the acid form, BH+) there is a single maximum at λ 540 mμ. At pH 6.49, corresponding to E_m, we notice two maxima of equal optical density, namely, at λ 540 and λ 460 mμ. It is obvious that we are dealing here with a mixture of the acid and basic forms of the indicator. At pH 8.03, we notice again a single

(1) This investigation was supported by research grant C-1308 from the National Cancer Institute, National Institutes of Health, Public Health Service.

(1a) Deceased April 16, 1953. Requests for reprints should be addressed to the Laboratory. Photographs of the spectra are also available.

(2) F. E. Ray and M. L. Jung, *Brit. J. Cancer*, **5**, 358 (1951).

(3) S. Woislawski, *Proc. Soc. Exp. Biol. Med.*, **79**, 390 (1952).

(4) A. A. Noyes, THIS JOURNAL, **32**, 815 (1910).

(5) L. A. Flexser, L. P. Hammett and A. Dingwall, *ibid.*, **57**, 2106 (1935).

maximum at λ 460 mμ, which represents the optical density of the free base E_B. From *p*H 10.6–11.28 the optical density does not change, and the spectral curve does not intersect the isosbestic point at λ 483 mμ; therefore we do not consider the latter for the calculation of pK_a. If we use the curves at *p*H 1.6, 6.49 and 8.03 for the calculation of pK_a we find that pK_a equals 6.77, Table I. The spectral curves also show a lateral shift of the absorption band toward the shorter wave lengths with increasing alkalinity. Neutral Violet (no. 8) behaves much the same as Neutral Red although it differs from it chemically in that a methyl group is replaced by a dimethyl-*p*-phenylenediamino group. From *p*H 1.1–4.07 there is a single maximum at λ 540 mμ and the optical density does not change. At *p*H 6.07 the density decreases, and at *p*H 7.08 we noticed the appearance of a second maximum at λ 460 mμ whose intensity equals that at λ 560 mμ. At *p*H 8.08 there is again a single maximum at λ 460 mμ. At *p*H 10.6–11.28 there is another spectral curve that does not intersect the isosbestic point at λ 480 mμ, and is therefore not used for the calculation of pK_a. Using the curves at *p*H 1.6, 7.08 and 8.08 we find that pK_a = 7.31. Safranin O (no. 13) differs from Neutral Red and Neutral Violet since it does not show any indicator properties which may be due to the *meso*-phenyl ring. There is no significant change in optical density between *p*H 0.7 and 11.4. As we see from the curves the optical density at λ 530 mμ decreases in 0.1 *N* NaOH and *N* NaOH with simultaneous increases in absorption in the regions of λ 460 and λ 555 mμ. Because these alkalinities are beyond the range of the *p*H meter it is not possible to calculate a pK_a for such a strong base. This is in agreement with the observation by Woislawski,[3] who was unable to calculate a pK_a for Safranin O from titration data in aqueous medium.

Let us consider the two azo dyes Chrysoidin Y and Bismarck Brown Y. Chrysoidin Y (no. 5) does not change in optical density between *p*H 1.6–3.83; thereafter it decreases to *p*H 8.04. Again there is no significant change in optical density between *p*H 8.04–11.1. The color changes its quality to the naked eye, accompanied by a slight shift in the absorption band toward smaller wave lengths (10 mμ). There is an isosbestic range at λ 415 mμ. On plotting *p*H *versus* optical density one obtains a pK_a value of 5.5. Bismarck Brown Y (no. 2) shows that the optical density changes between *p*H 1.6–8.1. There is no significant change in density between *p*H 8.1–10.9 We notice an isosbestic point at λ 4.15 mμ (similar to Chrysoidin Y) and an isosbestic range between λ 473–480 mμ. On plotting *p*H *versus* optical density one obtains a pK_a value of 5.5. Both azo dyes, Chrysoidin Y and Bismarck Brown Y, are subject to chelation, and this may explain their relatively low basicity.

Let us consider the three xanthenes Acridine Red, Rhodamine B and Pyronine B. Acridine Red (no. 4) shows that the density does not change between *p*H 1.6–3.8. At *p*H 5.0 we notice a lateral shift toward the shorter wave lengths. At *p*H 6.0 there is a further shift toward the shorter wave lengths. The optical density then does not change between *p*H 6.0–11.3. There is an isosbestic point at λ 548 mμ. The pK_a was 5.1. Rhodamine B (no. 3) also shows lateral shifts toward the shorter wave lengths at *p*H 4.45 and 6.01. From *p*H 6.01–12.9 the intensity does not change significantly. There is an isosbestic point at λ 552 mμ, close to that of Acridine Red. Because Rhodamine B contains a carboxyl group it should be much less basic than Acridine Red. This difference is pronounced in aqueous medium but is less in alcoholic medium where the ionization of the carboxyl group is repressed. In Pyronine B (no. 7) we noticed an exception to the rule that the optical density decreases with increasing alkalinity of the medium. The optical density first increases from *p*H 1.7–7.8, where it reaches a maximum and then decreases with increasing alkalinity. The calculation of pK_a leads to the logarithm of a negative number. If we neglect the negative sign, then pK_a 7.1 which is in close agreement with the value obtained from titration data in aqueous medium. On plotting *p*H *versus* optical density one obtains a bell-shaped curve; the pK_a was 6.9, and there is possibly a second pK_a of about 10.1.

Let us consider the two oxazines Brilliant Cresyl Blue and Nile Blue A. Brilliant Cresyl Blue (no. 10) shows two isosbestic points at λ 535 and λ 665 mμ. Because of the low tinctorial power of the dye, the concentration was doubled (2×10^{-5} *M*). The optical density did not change from *p*H 1.6–7.86; from there it decreased to *p*H 10.4. At *p*H 11.3 we notice an hypsochromic effect and at *p*H > 13 a reversed shift of the absorption band toward longer wave lengths (bathochromic effect); this is in contrast to previous observations. Because the colors are not stable in these strongly alkaline solutions a pK_a cannot be calculated with any degree of accuracy in this region. The attempted calculation of pK_a in this region leads to the logarithm of a negative number (as previously mentioned for Pyronine B). Using the spectral curves at *p*H 7.86, 9.71 and 10.4 for the calculation of pK_a, we find that pK_a 9.9. In Nile Blue A (no. 1) we noticed at first a decrease in optical density from *p*H 0.6–7.85. At *p*H 9.7 the absorption band shifts toward shorter wave lengths, and at *p*H 11.3 a further shift in the same direction is observed. If we consider the spectral curves at *p*H 0.6, 1.54 and 7.85, which intersect the isosbestic point at λ 600 mμ we find that pK_a equals 1.6. Nile Blue A has a second isosbestic point at λ 560 mμ, and if we consider the spectral curves that intersect the latter isosbestic point, namely, at *p*H 7.85, 9.70 and 11.3 we obtain a pK_a' 9.7.

The following four thiazines were investigated: Methylene Blue, Methylene Green, Thionine and Toluidine Blue O. Methylene Blue has an isosbestic point at λ 595 mμ, and the optical density does not change from *p*H 0.6–9.65. In 0.1 *N* and *N* NaOH the color changes slowly and irreversibly toward violet. Since the constitution of Methylene Violet is known, this change is due to the hydrolytic replacement of a dimethylimino group by oxygen with the formation of an oxazone, which is a weaker base. We also notice from the spectral curves that the 0.1 *N* NaOH solution is violet;

it absorbs more in the blue than in the red region. The reverse is true for the N NaOH solution which is red violet. It was not possible to calculate a pK_a for Methylene Blue in such strong alkaline solutions; this is in agreement with previous observations by Woislawski[3] from titration data in aqueous medium. Methylene Green (no. 9) differs chemically from methylene blue by an ortho nitro group which has a pronounced effect on the spectral transmittance. The optical density does not change from pH 1.6–8.1. From pH 8.1–10.5 the optical density decreases with increasing alkalinity. At pH 12.4 the color changes to violet and shows an absorption maximum at λ 560 mμ (similar to methylene blue). It is therefore possible that methylene green undergoes a hydrolytic cleavage similar to methylene blue. The presence of an ortho nitro group would tend to facilitate such a cleavage. There is an isosbestic point at λ 572 mμ. The pK_a was 9.7. Thionin (no. 12) has an isosbestic range between λ 533 and 543 mμ. The optical density does not change from pH 1.6–8.2. From there the intensity decreases with higher alkalinity. At pH 11.3 the color changes from violet to red violet, and we observe two absorption maxima, namely, at λ 510 and at λ 600 mμ. At pH 12.47 the color becomes red and we noticed only one maximum at λ 510 mμ. The pK_a was 11.2. Toluidine Blue O (no. 11) does not change the optical density from pH 1.6 to 8.14 (similar to Thionin). From there the intensity decreases with increasing alkalinity. At pH 10.9 the solution is violet and we notice two maxima, namely, at λ 540 mμ and at λ 630 mμ. At pH 12.06 the solution is pink and there is only one maximum at λ 530 mμ. There is an isosbestic point at λ 563 mμ. The pK_a was 10.8. In Table I, column 3 are listed the spectrophotometric pK_a values arranged in the order of increasing basicity in buffered 50% alcoholic solutions. In column 4 are listed the pK_a values from titration data in aqueous medium, as determined by Woislawski.[3] In column 5 are listed the pK_a values in unbuffered 50% alcohol, as determined by Ray and Jung[1] from titration data.

Fairly good agreement for different methods is obtained for Nile Blue A, Bismarck Brown Y, Rhodamine B, Chrysoidin Y, Neutral Red and Pyronine B. With the exception of Acridine Red these are all the weaker bases. Higher values are found for the other dyes by the spectrophotometric method. We notice that the spectrophotometric pK_a values largely parallel the buffer/benzene distribution ratios, column 6, as determined by Woislawski.[3] The slight discrepancy between Neutral Violet and Pyronine B may well be within the margin of the error. Neutral Violet has a slightly larger pK_a value than Pyronine B, but a somewhat smaller buffer/benzene ratio. A more serious discrepancy is that Safranin O appears to be more basic than Thionin from spectrophotometric data, whereas the distribution ratios point to the reverse order. This discrepancy may be due to the limitations of the spectrophotometric method for very strong bases. The data show that the ionization constants not only vary with the method and medium, but also that the order of basicity is different in different media. This is contrary to the assumption that a series preserves the same order of basicity in different media. The dissociation constant of a compound, therefore, depends not only on its basicity, but also on other factors of the medium.

TABLE I

COMPARISON OF THE BASICITY OF DYES DETERMINED BY DIFFERENT METHODS

No.	Compound	pK_a (buffered 50% alcohol)	pK_a[a] (water)	pK_a[b] (unbuffered 50% alcohol)	Buffer[a] benzene
1	Nile Blue A	1.6(9.7)	3.1	2.4	[c]
2	Bismarck Brown Y	4.5	4.8	5.0	[c]
3	Rhodamine B	4.6	3.7	3.2	[e]
4	Acridine Red	5.1	8.1	3.1	[e]
5	Chrysoidin Y	5.5	5.0	5.3	[c]
6	Neutral Red	6.8	6.7	6.5	[e]
7	Pyronine B	7.1	6.9	7.7	1.96
8	Neutral Violet	7.3	6.6	3.2	0.68
9	Methylene Green	9.7	4.1	3.2	2.50
10	Brilliant Cresyl Blue	9.9	4.6	3.2	2.70
11	Toluidine Blue O	10.8	7.9	7.5	2.90
12	Thionine	11.2	6.8	6.9	11.60
13	Safranin O	..	...	6.4	5.60
14	Methylene Blue	..	...	3.8	[d]

[a] Ref. 3. [b] Ref. 1. [c] 100% in benzene. [d] 100% in buffer. [e] Colorless.

GAINESVILLE, FLORIDA