THE STAINING ACT: AN INVESTIGATION INTO THE NATURE OF METHYLENBLUE-EOSIN STAINING

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The various theories of the nature of the act of staining of animal cells, tissues and products, which have been put forward by different writers fall into three groups: (1) that the process of staining is a chemical reaction between the dye on the one hand, and the cell or tissue elements on the other; (2) that in staining the dye is adsorbed by the tissue elements stained; and (3) that the dye is present in the form of a solid solution in the stained material. The problem remains, however, still unsettled, further data respecting individual stains being much to be desired.¹

For studying the nature of an individual staining act methylen-blue-eosin was selected as being a dye which is peculiarly suitable for the purpose. This substance is a salt which is formed by the union of the basic radical of methylenblue and the acid radical of eosin. When it is allowed to act upon cells or tissues some of the elements stain blue, others red. For example, with an alcoholic or aqueous solution of methylenblue-eosin a polynuclear leucocyte exhibits blue staining of the chromoplasm of the nucleus, while at the same time the oxyphile granules of the cytoplasm stain deep red, precisely similar blue staining being obtained with methylenblue and the red staining with eosin. In the case of methylenblue-eosin, provided that hydrolysis of the dye can be excluded, the act of staining can only be explained as due to a chemical reaction between dye and cell elements. If hydrolysis is not avoided then both adsorption and solid solution of the free acid and the free base may possibly occur and produce the staining effect.

1. Cf. W. M. Bayliss, 'On some aspects of adsorption phenomena,' *The Bio-Chemical Journ.*, 1906, Vol. I, p. 175.

As it seemed not improbable that an investigation of the colour and spectroscopic characters of methylenblue-eosin might throw light upon the presence or absence of hydrolysis of the dye, this was first undertaken. In this investigation the condition of methylenblue-eosin in solution in alcohol forms the primary object of study, but attention is also given to various points respecting alcoholic and watery solutions of methylenblue, eosin and allied substances, since these data have a direct or indirect bearing on the subject of enquiry. It will be convenient to commence with the latter two dyes.

COLOUR AND SPECTROSCOPIC CHARACTERS

Methylenblue is tetramethyldiamidothiodiphenylamin chloride, having the formula

and the molecular weight 371, or after freeing from water at 100° C., 317·36. This salt crystallises in dark blue microscopic plates; from solution in water it is precipitated by sodium chloride and by zinc chloride. The form employed in the present work was the double salt with zinc chloride, having the formula $2\text{C}_{16}\text{H}_{18}\text{N}_3\text{SCl} + \text{ZnCl}_2 + \text{H}_2\text{O}$, and the molecular weight 787·96, or when anhydrous 770·08. The sample employed (rectif., for injection *intra vitam*, Grüber) contained 9·12% S, (theory requiring 8·17%). Methylenblue, if allowed to separate out slowly from alcoholic or aqueous solution crystallises in needles and prisms having a bronze lustre on the surface, but when rapidly evaporated it forms a film, which under the microscope does not exhibit crystals, but is amorphous in character. In alcoholic and in aqueous solution of ·0001 N to ·00001 N concentration (·0079% to ·00079%) it forms a characteristic blue liquid, no difference of colour between alcoholic and aqueous solutions being recognisable to the naked eye, though on spectroscopic examination a marked difference is recognisable. Thus in alcoholic solution of $2·80 \times 10^{-5}$ N

concentration, in a layer 1 cm. thick, a very dark band appears in the red, while between this band and the solar line D a lesser degree of absorption occurs (Fig. 1, A) ; in aqueous solution a narrower very dark band is seen and a second less dark band running up to D (Fig. 1, H).

Water soluble eosin, the sodium salt of tetrabromfluorescein, $C_{20}H_6Br_4O_5Na_2 + 5H_2O$, to which the constitutional formula

has been assigned,¹ has a molecular weight of 776.2 or, without water of crystallisation 686.2. The sample employed contained after drying 41.7% Br, theory requiring 47.2% Br. From watery solution it crystallises out in triclinic red crystals, exhibiting a greenish surface lustre. Its colour in .001 N concentration (.078%) in alcohol is yellowish red with a strong light-yellowish green fluorescence ; in water it is of a darker brownish red colour and shows a slighter olive green fluorescence. In .00001 N concentration the same difference in respect of fluorescence is seen, but the colour in alcohol is a vivid pink, in water a slightly brownish pink.² The spectrum in alcohol in 2.45×10^{-5} N concentration, in a layer 1 cm. thick shows a very dark band in the green about the solar line E, together with a second lighter band towards F, as shown in Fig. 1, B ; in aqueous solution of the same concentration a broader very dark band nearer the blue end of the spectrum is seen, but no second absorption band is observed (Fig. 1, F).

Alcohol soluble eosin ($C_{20}H_6Br_4O_5$), the free acid corresponding to the foregoing salt, receiving the formula

1. The exact constitution of the eosin molecule is not yet established. In accordance with current views of the nature of the chromophore group, the quinone ring is exhibited in the formulae given.

2. The above statements refer to the colour of solutions in test-tubes. A layer of eosin solution of .01 N concentration, .5 mm. thick, has the same colour as a layer of eosin solution of .0002 N, 25 mm. thick.

has a molecular weight of 643.0. The sample used contained after drying 47.6% Br, theory requiring 48.3% Br. From alcoholic solution it crystallises on slow evaporation in yellowish red crystals,

Fig. 1. ABSORPTION SPECTRA. In absolute alcohol : A, methylenblue, in 2.78×10^{-5} N concentration ; B, water soluble eosin, in 2.45×10^{-5} N concentration ; C, alcohol soluble eosin, in 2.50×10^{-5} N concentration ; D, methylenblue-eosin, in 2.80×10^{-5} N concentration. In water : E, methylenblue-eosin, in 2.80×10^{-5} N concentration ; F, water soluble eosin, in 2.45×10^{-5} N concentration ; G, alcohol soluble eosin, in 2.50×10^{-5} N concentration ; H, methylenblue, in 2.78×10^{-5} N concentration. The liquids were viewed in a layer 1 cm. thick.

having the formula $C_{20}H_8Br_4O_5C_2H_6O$; on rapid evaporation a film forms in which no crystals are recognisable; from alcoholic solution to which hydrochloric acid has been added, in red alcohol-free

crystals. The colour of a '001 N solution in alcohol is a deeper pink than that of the sodium salt in the same solvent, and is accompanied by a slight yellowish green fluorescence; in '0003 N concentration in water its colour is a deeper brownish pink with a slight olive-green fluorescence. In alcohol in '00001 N concentration it shows a brilliant slightly violet pink colour, and in water in the same concentration a slight brownish tint is added, the same difference as before in respect of fluorescence being seen. The spectrum of alcohol soluble eosin of 2.50×10^{-5} N concentration in a layer 1 cm. thick in alcohol (Fig. 1, C) shows a very dark broad band in the green and to the side of this, nearest the blue end of the spectrum, a second lighter narrower band, both bands being slightly nearer the red end of the spectrum than the corresponding bands of water soluble eosin, whose dark band is not so wide. In aqueous solution in the same concentration (Fig. 1, C), the absorption spectrum differs from that of the sodium salt in water in the same concentration (Fig. 1, F), in the diminished width of the dark band which is displaced towards the red, an exceedingly faint second absorption band being seen towards F.

Methylenblue-eosin was first prepared by Romanowsky¹ (1891), who observed that when concentrated solutions of methylenblue and eosin were mixed a precipitate formed, which, when dissolved, furnished a selective stain for the nuclear chromoplasm of the Plasmodium malariae. Ziemann² (1898), who made use of the same stain, observed that the precipitate was soluble in excess of the methylenblue, as also of the eosin solution. Rosin³ (1899), found that similar precipitates were obtainable from mixtures of other so-called basic and acid dyes. None of these observers, however, investigated the chemical composition of Methylenblue-eosin. Jenner⁴ (1899), who made use of this dye for staining blood-films, found that it dissolved to the extent of about '5% in the methyl alcohol, was less soluble in ethyl alcohol, and

1. *Zur Frage der Parasitologie und der Therapie der Malaria*, 1891.

2. 'Über Malaria und andere Blutparasiten,' etc., Jena, 1898, Fischer. Also *Centralbl. f. Bakteriöl.*, 1898, No. 25.

3. 'Über eine Gruppe von Anilinfarbstoffen, ihre Bedeutung für die Biochemie der Zelle und ihre Verwendbarkeit für die Gewebsfärbung,' *Berl. kl. Wochenschr.*, 1899, 36 Jahrg., p. 251.

4. 'A new method for rapidly fixing and staining blood,' *Lancet*, 1899, Vol. I, p. 370.

much less soluble in water¹. He regarded methylenblue-eosin as a salt, since he observed that, no matter what the proportions in which methylenblue and eosin were mixed might be, the precipitate formed always possessed the same melting point, namely, 227° C. The precipitate, he further observed, consisted of brilliant grass-green crystals if alcoholic solutions were used (with rapid evaporation a film forms in which no crystals are recognisable), but was amorphous and showed a metallic green colour if aqueous solutions were employed. Jenner did not determine the composition of methylenblue-eosin, and though he mentions that the most abundant precipitate is formed when methylenblue and eosin are mixed in the proportion of 8.0 to 8.3 parts of the former and 10 parts of the latter, yet this statement, in the absence of further details as to the composition and purity of the methylenblue and eosin employed, does not enable a conclusion to be reached as to the composition of the precipitate. The absence of a sharp indication of accurate admixture of methylenblue and eosin renders this method of attempting to determine the relative proportions required unsatisfactory. It may be pointed out that if the proportion of two molecules of methylenblue ($2C_{16}H_{18}N_3SCl + ZnCl_2 + H_2O$, mol. wt. 788) and one of eosin ($C_{20}H_6Na_2Br_4O_5 + 5H_2O$, mol. wt. 776) are employed, the ratio of the former to the latter, by weight, would be 10.2 : 10; if the proportion is one and a half to one, the ratio becomes 7.55 : 10.

The composition of methylenblue-eosin² was therefore further investigated, two methods being employed. In the first place by means of the colorimetric observations described below it was ascertained that the proportions in which methylenblue and eosin react to produce methylenblue-eosin were two molecules of the former ($C_{16}H_{18}N_3SCl$) and one molecule of the latter ($C_{20}H_6Na_2Br_4O_5$). In the second place, by ultimate analysis, the percentages of sulphur and bromine respectively in methylenblue-eosin dried to constant weight at 100° C. were found to be 5.12 and 23.50, a second analysis giving

1. It dissolves in water to the extent of about .02 per cent. at 16° C.

2. Methylenblue-eosin is quite free from chlorine and zinc on the one hand, and from sodium, potassium and ammonium on the other, so that the possibility of its being an admixture of methylenblue and eosin is altogether excluded.

5.35 and 23.84 The formula $(C_{18}H_{18}N_3S)_2C_{20}H_6Br_4O_5$ (mol. wt. 1204.4), which requires 5.28% of sulphur and 26.34% of bromine, therefore represents the required relation.

The colour of methylenblue-eosin in absolute alcohol in .001 N concentration (.12%) is dark purple with a strong dark yellowish green fluorescence; in .00001 N concentration, bluish purple with the same strong fluorescence. In aqueous solution in the latter concentration its colour is reddish purple with a very slight dark olive green fluorescence. In alcoholic solution of 2.8×10^{-5} N concentration in a layer 1 cm. thick, methylenblue-eosin exhibits a spectrum (Fig. 1, D) showing absorption bands both in the red and the green, the former being identical with that of methylenblue in corresponding concentration (Fig. 1, A), the latter with that of water soluble eosin in corresponding concentration. In aqueous solution in the above concentration and thickness, a different absorption spectrum is seen, in the red a very dark band touching Fraunhofer's line B being seen with a lighter absorption area on each side; while in the green a very dark band centred about *b* is seen, with a lesser degree of absorption towards F but without a second lighter band as in alcohol. Methylenblue-eosin, therefore, in water shows bands which are different from those exhibited by methylenblue and eosin respectively in aqueous solution.

If methylenblue-eosin is dissolved in a test-tube of alcohol in amount sufficiently small to produce a convenient depth of colour¹, no difficulty is experienced in ascertaining the respective amounts of methylenblue and of water soluble eosin, which require to be added to a second tube of alcohol in order to produce the same quality and intensity of colour, which the first exhibits. If the eosin is employed in the alcohol soluble form, the colour of the first tube can be very nearly matched but complete equality of colour is not attainable. In water, also, a methylenblue-eosin solution may similarly be matched with methylenblue and water soluble eosin, but when alcohol soluble eosin is employed matching is attended with difficulty, for although

1. Only dilute solutions can be employed owing to the intense colour of the dyes. The concentrations given in Table II will be found suitable in most cases.

immediately after mixing the latter two dyes in suitable proportions a fair equality of colour is obtainable yet the colour rapidly changes, the mixed liquid becoming bluer until at the end of twenty-four hours it has assumed the appearance of a methylenblue solution, the eosin having separated out in the form of a red precipitate, which has settled at the bottom of the test-tube. It appears, therefore, that in this case methylenblue-eosin is not formed, a simple mixture of the two dyes resulting and the alcohol soluble eosin being after a time 'salted out' by the methylenblue. In two cases, however, namely when methylenblue and water soluble eosin are employed in alcohol or in water, equality of colour is attainable both as regards tint and depth. The smallest variation of colour which, under the most favourable conditions in respect of light, of background and of freedom of the eyes from fatigue, is recognisable with certainty is represented by a variation of about $2\frac{1}{2}\%$ in the amount of the solutions of methylenblue or water soluble eosin required to produce equality. When equality of colour has been attained the spectra are found to be identical also. The concentration of methylenblue and of water soluble eosin, which was found after repeated trials to give equality, is shown in Table 2, Exps. 1 and 2. The composition of the methylenblue-eosin employed has been already referred to (p. 411); the sample of methylenblue, $2(\text{C}_{16}\text{H}_{14}\text{N}_3\text{SCl}) \cdot \text{ZnCl}_2$, used was found on analysis (p. 407) to contain 9.12% of sulphur (theoretically 8.17% is required), and the eosin salt, $\text{C}_{20}\text{H}_6\text{Br}_4\text{O}_6\text{Na}_2$, contained (p. 408) 41.7% of bromine (theoretically 47.20%). It is clear, from the results in Table 2, that in the formation of methylenblue-eosin two molecules of zinc-free methylenblue, $\text{C}_{16}\text{H}_{14}\text{N}_3\text{SCl}$, and one molecule of eosin take part. It is to be noted that the slight defect of the eosin radical already noted in the methylenblue-eosin employed has its counterpart in the concentrations required to match (48×10^{-5} N instead of 54×10^{-5} N, Table 2, Exps. 1, 3, 5, 7). The cause of these differences of concentration obtaining when equality of colour was reached is presumably to be attributed to the formation of small amounts of leuco or other derivatives of feeble colour intensity; no indication of the presence of such compounds is, however, afforded by the analyses made (pp. 407-

411). It would appear that methylenblue and methylenblue-eosin are more liable to deteriorate on keeping than is eosin.

If, instead of adding methylenblue and eosin to the same liquid these dyes are dissolved separately in the concentrations given above and are placed in glass cells 3 cm. thick, one in front of the other, the transmitted light can then be compared with that of a solution of methylenblue-eosin of similar concentration contained in a third cell of the same dimensions as the former. Examined in this way in alcoholic solution the colour of methylenblue-eosin is found to be accurately matched, when the same concentrations are employed as in the test-tube experiment, if eosin in the form of the sodium salt is employed ; if, however, eosin in the form of the free acid is used, the colour of the apposed solutions, though approximating very nearly to that of the solution of methylenblue-eosin is not quite identical with it (Table 2, Exps. 2 and 4). When watery solutions are employed, the colour of the apposed liquids is too blue compared with that of the single liquid, whichever form of eosin is used, the same concentrations being employed as for the test-tube experiments above described ; if, however, the concentration of the eosin is sufficiently increased (Table 2, Exps. 7 and 8), then although a very slight increase or diminution of the concentration of the eosin causes the colour of the apposed cells to become too red or too blue, nevertheless an absolute identity of colour is not attainable. In this connection reference should be made to Table 1, in which an attempt is made to exhibit the relative colour of the dyes employed in a form suitable for ready comparison.

If the spectra of the apposed liquids are studied (Fig. 1), it is found that in all cases the spectra are the sum of those of the individual liquids, as is to be expected, when it is borne in mind that eosin has no absorptive effect whatever at the red end of the solar spectrum, nor does methylenblue absorb any light in the central part of the solar spectrum. It will now be readily understood that identity of colour is obtainable only when methylenblue-eosin is matched with methylenblue and water soluble eosin, alcohol being the solvent employed. The spectroscope thus affords a valuable means of checking and con-

firming the conclusions arrived at by the eye. The approximation to equality attained by increasing the strength of apposed watery solutions of eosin (Table 2, Exps. 7 and 8; cp. Fig. 1, D to F) is reached by increasing the absorption of light towards the solar line F, but no true equality of colour is possible.

Before proceeding to consider the bearing of the above data upon the nature of the staining act of methylenblue-eosin, it will be of advantage to make a brief reference to the hydrolysis of organic salts. Water, which possesses a very high dielectric constant and at the same time exhibits an extremely low degree of ionisation, has the property of hydrolysing salts formed by the combination of (1) a weak base with a strong acid, or (2) a weak acid with a strong base, or (3) a weak base with a weak acid. Thus anilin hydrochloride in '03 N concentration is hydrolysed to the extent of 2·6%, while urea hydrochloride in the same concentration is hydrolysed to as much as '95%¹; potassium cyanide in '02 N concentration is hydrolysed to the extent of 2·3%²; anilin acetate in '01 N concentration is hydrolysed to the extent of 55%.³ From these illustrations which can be multiplied it is obvious that, before attempting to explain the nature of methylenblue-eosin staining it is essential to ascertain how much this dye is hydrolysed by the solvent employed.

In solution in alcohol the spectroscopic characters of methylenblue-eosin show that this dye is not hydrolysed into methylenblue (free base) and eosin (free acid) to any recognisable extent, for the absorption spectrum of methylenblue-eosin in the green coincides with that of water soluble eosin, and no modification due to the presence of alcohol soluble eosin can be detected (Fig. 1, A to D). The question now arises, what is the smallest amount of alcohol soluble eosin, the addition of which (in an apposed cell) can be recognised spectroscopically. This addition may be put at about 25% of the concentration of the methylenblue-eosin examined ($2\cdot8 \times 10^{-5}$ N), when the spectroscopic appearance is observed, but when the colour

1. Shields (1893), *Zeitschr. f. physik. Chem.*, Bd. XII, s. 167; Walker (1899), *ibid.*, Bd. XXXII, s. 137.

2. Walker (1889), *Zeitschr. f. physik. Chem.*, Bd. IV, s. 319.

3. Arrhenius and Walker (1890), *Zeitschr. f. physik. Chem.*, Bd. V, s. 18.

is judged with the naked eye an addition of 10%, or under extremely favourable circumstances of 2½%, can be recognised. If methylenblue (with or without the addition of an equivalent amount of potassium hydroxide), or alcohol soluble eosin is employed in alcoholic solution in one-tenth of the concentration in which methylenblue-eosin is ordinarily used (*i.e.* '005 N or '6%)¹ for staining purposes in alcohol, the respective blue and red differential staining cannot be obtained or is exceedingly faint; to obtain staining equal to that of methylenblue-eosin in the same period of time, the concentration must equal or, better still, exceed that of the methylenblue-eosin stain. Since, therefore the latter dye in alcoholic solution is not hydrolysed to an extent sufficient to furnish a possible explanation of its staining power, the conclusion follows that its staining act is a chemical reaction in which the dye molecules are broken up, and not a purely physical process. It is interesting to observe that no recognisable hydrolysis of water soluble eosin in 2.5×10^{-5} N concentration in alcohol is obtainable, for if sodium hydrate dissolved in absolute alcohol is added no change of colour or spectrum can be detected. Owing to the relatively low value of the dielectric constant of alcohol (26 for ethyl alcohol) compared with that of water (80) ionisation of the dyes need not be taken into account.

In water, methylenblue-eosin presents an absorption spectrum in the green (Fig. 1, H) differing slightly but distinctly from that of water soluble eosin (Fig 1, G), while its absorption spectrum in the red differs from that of methylenblue in the form of chloride (Fig. 1, E) or of free base (Fig. 2, α). Since the absorption spectrum of methylenblue-eosin is not compounded of that of methylenblue and water soluble eosin, colour matching by apposition is not possible as in the case of alcoholic solutions, and the only means of judging of the existence of hydrolysis is by means of spectroscopic examination. Unfortunately this, while failing to afford evidence of eosin in the form of free acid, is not sufficiently delicate (*cp.* p. 413) to afford proof that hydrolysis, if present, would be in so small a degree that the possibility

1. In methyl alcohol (Jenner's stain). In water the concentration is usually about one-fifteenth of this amount.

of the red and blue staining being a physical process would be altogether excluded. The colour and spectrum of methylenblue chloride in water, it may be observed, do not differ from those of the free base in water (Fig. 2, α). The spectrum of water soluble eosin in 2.5×10^{-5} N concentration in water affords no evidence of hydrolysis, for on adding sodium hydrate no change is produced; in the stronger solution used for staining (0.05 N) the degree of hydrolysis must be insignificant, since the degree of hydrolysis is inversely proportional to the square root of the concentration.¹ It does not, however, follow that the production of alcohol soluble eosin in watery solution of methylenblue-eosin would be relatively less in the 0.05 N concentration used for staining than in the concentration (2.8×10^{-5} N) submitted to spectroscopic examination, for in the case of a salt formed, as is methylenblue-eosin by the combination of a weak base with a weak acid, the amount of hydrolysis occurring may be independent of the concentration.² In such a case also a marked degree of ionisation may be present. Thus anilin acetate in watery solution is not only hydrolysed to about 55% in all concentrations, but the undissociated fraction is nearly completely ionised in centinormal concentration. Ionisation might be imagined to account in part for the circumstance that the spectrum of methylenblue-eosin is not compounded of those of methylenblue and eosin (sodium salt). This may be so, but the data given in the next section indicate that such an interpretation is insufficient to afford a satisfactory explanation of the difference in question. A more probable explanation is that the chromophore radicals in methylenblue-eosin mutually modify each other, though it is to be noted that no such modification is to

1. According to the formula $x = \sqrt{\frac{1}{c} \cdot \frac{k_w}{k_s}}$, where x is the degree of hydrolysis, c is the concentration, k_w is the water constant (1.2×10^{-14}), and k_s the dissociation constant of the free acid of eosin. This applies only if k_s is much larger than k_w . K_s is unknown, but since water soluble eosin in aqueous solution is not precipitated by carbonic acid, it follows that k_s is not less than the dissociation constant of the latter (3×10^{-7}), so that the formula holds good for eosin.

2. The relation is $\left(\frac{1-x}{x}\right)^2 = \frac{k_b k_s}{k_w}$, where x is the degree of hydrolysis, k_b the dissociation constant of the free base of methylenblue, k_s that of the free acid of eosin, and k_w the water constant.

be observed in alcoholic solution. The presence or absence of ionisation is not of much importance in deciding the problem at issue, for the possibility of a physical as opposed to a chemical process occurring in methylenblue-eosin staining turns upon the occurrence of hydrolysis. It is interesting to observe that the addition of chlorine ions (*i.e.* of 0.13 N NaCl) to an aqueous solution of methylenblue ($6 \times 10^{-5}\text{ N}$) the effect of which would be to diminish ionisation of methylenblue, causes the second faint band in the red to become more distinct.¹ When the concentration of sodium ions is increased by adding sodium iodide to water soluble eosin in aqueous (or alcoholic) solution, the colour and spectrum undergo no immediate change (subsequently a slow change occurs requiring several weeks for its full development).

To sum up we may say that methylenblue-eosin in alcoholic solution is not perceptibly hydrolysed, and that its staining act is therefore a chemical, not a physical, process. The data at present available, however, do not enable a conclusion to be arrived at as to the nature of the staining act in aqueous solution.

Although direct proof of the nature of methylenblue-eosin staining in aqueous solution is not available, nevertheless the fact that the staining act in alcoholic solution is a chemical reaction implies, though it does not prove, that the same holds for aqueous solution. The circumstance that the blue staining of nuclear protoplasm is effected not only by methylenblue-eosin in both alcoholic and aqueous solution, no matter whether in the form of chloride or of free base, is not easy to explain as a physical process due to adsorption or selective solubility of the dye, because the physical conditions in the four cases are dissimilar; on the other hand on the theory of stoichiometric reaction no such difficulty is experienced, the process being in each case the same. The same may be said of the staining of the oxyphile granules of leucocytes by methylenblue-eosin in alcoholic and in watery

1. In alcoholic solution of methylenblue the addition of CaCl_2 in $2 \times 10^{-2}\text{ N}$ concentration or of HCl in $4 \times 10^{-2}\text{ N}$ concentration does not alter the spectrum. In each of these cases advantage is taken of the relation
$$\frac{\text{concentration of anion} \times \text{concentration of kation}}{\text{concentration of undissociated salt}} = \text{constant.}$$
 If the colourless ion is increased in amount the amount of coloured ion becomes reduced to insignificant dimensions and does not appreciably modify the colour of the undissociated dye.

solution, by alcohol soluble eosin in alcoholic solution, and by water soluble eosin in watery solution, all three dyes being in the same molecular concentration.

It is very desirable that the condition of the dyes employed should be further defined in respect of osmotic pressure and of ionisation. The latter cannot at present be determined with sufficient accuracy to be of much value owing to the impossibility of ascertaining with accuracy the fraction of the conductivity which is to be attributed to impurities in the form of electrolytes. Osmotic pressure will be dealt with in the next section, but before passing to this subject some further details respecting the colour and spectra of the dyes employed, in various conditions, will be given.

It has long been known that when an acid, mineral or organic, is added to an alcoholic solution of methylenblue-eosin the solution assumes the colour of methylenblue and on spectroscopic examination shows the spectrum of methylenblue (Fig. 1, A) unchanged while the eosin bands are more or less obliterated (as in Fig. 2, s, t), and when the mixed liquid is used for staining sections only the eosin colouration appears. If, instead of acid, an alkali is added, the solution turns reddish violet in colour and shows the eosin (Fig. 1, B) spectrum, while the methylenblue spectrum becomes modified (as in Fig. 2, v); on using the liquid for staining sections only blue staining is obtained. If this observation is performed in a test tube, 2×10^{-5} N will be found a convenient concentration for the dye, and the acid and alkali should be employed in 1×10^{-3} N concentration; if the latter are employed in 5×10^{-5} N concentration the acid changes the colour of the liquid, but no change is produced by the alkali. In aqueous solution, contrary to what would be expected, no change of colour occurs. Now the action of acid in alcoholic solution is obviously directed to the acid radical of methylenblue-eosin, for the addition of acid to methylenblue in solution does not alter the colour or spectrum of the solution. Similarly alkalies in alcoholic solution act upon the basic radical of methylenblue-eosin, for the addition of alkalies to the sodium salt of eosin produces no change of colour (only after prolonged boiling with concentrated alkali is a change of colour producible).

Fig. 2. Spectra of various solutions viewed in a layer one centimetre thick, except when otherwise stated. The following spectra are each obtained from two liquids apposed in separate cells (Mb = methylenblue, Na_2E water soluble eosin, H_2E alcohol soluble eosin) :

a. Mb 2.7×10^{-5} N + Na_2E 2.4×10^{-5} N. Solvent—Absolute alcohol.

b. " 2.6 " + H_2E 2.5 " " "

c. " 2.7 " + Na_2E 2.4 " " Water.

d. " 2.6 " + H_2E 2.5 " " "

The remaining spectra are each obtained from a single solution :

e. Mb 2.7×10^{-5} N + Na_2E 2.4×10^{-5} N. Solvent—Absolute alcohol.

f. " 2.6 " + H_2E 2.5 " " "

g. " 2.7 " + Na_2E 2.4 " " Water.

h. " 2.6 " + H_2E 3.0 " " "

i. Na_2E 2.5 " + Na I .7 N. " Absolute alcohol.

j. " " " + Na I .7 N. " "

k. H_2E 2.5 " + Na I .7 N. " "

l. " " " + Na I .7 N. " ,¹

m. Na_2E 2.3 " + Na I .1 N. " Water.¹

n. H_2E " " + Na I .1 N. " ,¹

o. Na_2E " " " " "

p. " " " + NaCl 1.5 N. " "

q. H_2E " " " " "

r. " " " + NaCl 1.5 N. " "

s. " " " + H_2SO_4 1×10^{-3} " Absolute alcohol.²

t. " " " + H_2SO_4 " " Water.²

u. Mb 2.7 " " " Absolute alcohol.

v. " " " + KOH 1×10^{-3} " "

w. " " " " Water.

x. " " " + KOH 1×10^{-3} " "

1. Spectrum observed after the liquid had been mixed for a week.

2. In a layer three centimetres thick.

If to an alcoholic solution of methylenblue (the most convenient concentration being that of 1×10^{-4} N to 1×10^{-5} N) sodium or potassium hydrate is added in the proportion of one molecule to one of methylene-blue, the liquid preserves its colour and shows no alteration of its spectrum; if alkali is added in greater quantity, e.g. in the proportion of twenty molecules to one of methylenblue, the colour quickly changes to reddish-purple subsequently becoming purple-red, the methylenblue band in the spectrum showing less absorptive power between C and D while the dark band retains its position and intensity

undiminished, as in Fig. 2, *v*. In watery solution no change is produced either in colour or spectrum by the addition of alkali.

If to a dilute alcoholic or watery solution of eosin in the form of the sodium salt hydrochloric or sulphuric acid is added in equimolecular amount, the colour of the liquid changes and inclines to that of the acid eosin, the spectrum changing also to that of acid eosin, but the colours and spectrum are not quite pure for the further change next to be described is easily produced if too much acid is added.

Excess of acid (five to twenty molecules of acid to one molecule of eosin) almost completely decolourises eosin solutions of 2.5×10^{-5} N concentration, producing in alcohol a faint light brownish colour with an exceedingly faint fluorescence and the spectrum shown in Fig. 2, *s*., while in water a light reddish-brown colour with a very slight olive-green fluorescence is seen, and the faint absorption spectrum, shown in Fig. 2, *t*., is obtained. To observe these spectra a layer of fluid three or more centimetres in thickness is necessary.

If to a 2.5×10^{-5} N solution of water soluble eosin sodium iodide in .1 N or greater concentration is added, the colour in alcoholic solution changes slowly to a slightly brownish pink and the spectral bands at E and F are slightly displaced towards the blue end, while a faint band appears beyond G; at the end of a week the solution has become of a darker yellowish brown colour and the latter band is deeper (Fig. 2, *i* and *j*). In water, the dye, after the addition of iodide of sodium retains its colour unchanged but loses its fluorescence, the bands at E and F being unaltered, but a dark band has appeared extending to the right of G.

If alcohol soluble eosin is employed, the addition of sodium iodide produces in alcoholic solution a slightly brighter and more violet-pink colouration, but no recognisable alteration of the spectrum is at first noted; at the end of a week a yellowish-brown colour is produced, and the spectrum is that shown in Fig. 2, *l*. In watery solution the colour does not change on adding sodium iodide, but fluorescence is abolished, the spectrum remaining unchanged; at the end of a week the liquid has become of a reddish-brown colour and exhibits the spectrum shown in Fig. 2, *n*.

If to an aqueous solution of water soluble eosin, sodium chloride in 0.1 N concentration is added, no change in colour or spectrum occurs (Fig. 2, *p*). If eosin in the form of free acid is employed the colour becomes of a lighter and purer violet pink and the absorption bands become lighter, without, however, changing their position (Fig. 2, *r*).

OSMOTIC PRESSURE

Further data are necessary to elucidate the nature of the staining act, which forms the subject of this investigation. In particular it is desirable to ascertain the osmotic pressure exerted by the dyes. Observations of this kind made with aniline dyes appear to be limited to some determinations made by Krafft,¹ who found that the molecular weight of perfectly dry rosaniline chloride, methylenblue and methylviolet, ascertained by observing the raising of the boiling-point of absolute ethyl alcohol, corresponded to the accepted formulae of these dyes, but that when care was not taken to exclude the presence of traces of water, or when water was used as the solvent, the raising of the boiling-point was less than that required by theory and an apparent molecular weight, perhaps twice as large, was obtained. The latter observation is of considerable significance, for, taken in conjunction with the well known property which such dyes exhibit to separate out from their solvents not in the crystalline, but in the amorphous or globomorphous state, it indicates that these substances are in the latter case present in the colloidal form. Whether the dye is present in an imperfectly developed colloidal state still capable of exerting a definite osmotic pressure, or whether it is present in two different states, being partly in colloidal, and partly in true, solution, is not yet determined, but the latter view is probably correct since it is found that, when the dye is added in successive amounts to the solvent, the raising of the boiling point or lowering of the freezing point is most marked after the first addition, and each subsequent addition of the dye is attended with less effect than the preceding

1. 'Ueber colloïdale Salze als Membranbildner beim Färbeprocess,' *Ber. d. deut. chem. Gesellschaft*, 1899, 32 Jahrg., Bd. II, S. 1608.

It is difficult to explain this circumstance except on the assumption that the first portion added passes largely into true solution, the solvent becoming rapidly saturated by the quantities of the dye subsequently added, so that the only form in which further solution can take place is the colloidal form. It is interesting to note that Krafft found that fuchsin, methylenblue and methylviolet diffused through parchment paper, while benzopurpurin, benzazurin and azobblue showed no trace of diffusion.

In Tables 3 and 4 some determinations¹ are given of the influence of methylenblue-eosin, methylenblue and alcohol soluble eosin upon the boiling point of methyl alcohol, and that of methylenblue and water soluble eosin upon the freezing point of water. In these determinations no attempt was made to obtain the dyes in a perfectly dry condition,² since it was desired to investigate their condition under circumstances similar to those obtaining in the staining liquids, alcoholic and watery, used in histological technique, in the preparation of which no attempt is made to exclude the presence of traces of water from the dyes employed.

The results obtained in Tables 3 and 4 show that methylenblue-eosin, as also methylenblue and both forms of eosin, dissolved in methyl alcohol or water, exist in the colloidal state, thus resembling fuchsin, methylviolet, tannin and soap. The last column of these Tables gives the amount of dye which may be regarded as present in true solution, calculated on the assumption that the dyes exist in a diphasic condition. These results are, however, in all probability, except in the case of Exp. 6, Table 3, too high owing to the difficulty of completely removing from the dyes slight impurities, which may exert osmotic pressure. The disturbing influence of even mere traces of such impurities is readily understood, when it is borne in mind that a 0.01 N concentration of methylenblue-eosin, for example,

1. With Beckmann's apparatus, *Zeitschr. f. physikal. Chemie*, 1902, Bd. XL, S. 129.

2. The dyes employed contained 7% to 12% of water. If methylenblue-eosin is dried to constant weight at 100° C., it becomes very slightly soluble in alcohol, requiring repeated renewal of the solvent in order to obtain complete solution; the spectrum of the dye is not altered. The same is true of alcohol soluble eosin. In neither case is the solubility improved by soaking the dried dye again in water. On the other hand pastilles of the freshly precipitated dye, incompletely dried in air, dissolve at once in methyl alcohol.

would be represented by a 1·2% solution, while the same concentration of sodium chloride would be represented by a ·06% solution. Alcohol soluble eosin can readily be obtained in a pure condition by adding an equivalent amount of sulphuric acid to water soluble eosin in aqueous solution; Exp. 6, Table 3, was made with eosin so precipitated,¹ and subsequently thoroughly washed and incompletely dried in air, while Exp. 5, Table 3, was made with a sample obtained from Grübler. The purification of the remaining dyes, in particular the separation of small quantities of salt, is attended with great difficulty. Sodium chloride, for example, is formed when methylenblue and water soluble eosin are mixed in the required proportion; if the bulky precipitate thus formed is washed with large quantities of distilled water serious loss from solution of the dye, which is highly insoluble only in saline liquids, occurs. In Exp. 3, Table 4, a sample of eosin puriss.² was employed. In Exp. 4, the dye was prepared by dissolving freshly precipitated and carefully washed alcohol soluble eosin in the calculated amount of a solution of pure sodium hydrate and evaporating to dryness³; a lower degree of osmotic pressure was noted. The methylenblue employed was supplied by Grübler in a specially purified form suitable for injection *intra vitam*. The sparing solubility of methylenblue-eosin in alcohol renders difficult accurate ebullioscopic observations, the degree of alteration of the boiling point obtained being inconveniently small; with water as solvent no such observations are possible.⁴

The difficulty of ensuring the absence of traces of electrolytes from the dyes investigated renders determinations of the conductivity of these solutions of little value in affording knowledge of the degree of ionisation present.

1. For analysis, see p. 409.

2. For analyses of these dyes see pp. 407-411.

3. Commercial water soluble eosin may exhibit more than twice the osmotic pressure required by theory for the pure salt.

4. It is interesting to note that Michaelis (Dent. medicin. Wochenschr., 1904, No. 42; Virchow's Archiv, 1905, Bd. 179, S. 195) finds that watery solutions of eosin and methylenblue, though optically inhomogeneous, are not resolvable into submicroscopic particles. Michaelis and also Zsigmondy (Zur Erkenntniss der Kolloide, 1905, S. 160) found, however, that a watery solution of fuchsin was partially resolvable into ultramicroscopic particles, whence both observers conclude that this dye is diphasic in such solution.

Although the osmotic pressure of methylenblue-eosin in aqueous solution cannot be determined, the marked tendency of such solutions to form films and amorphous precipitates of the dye, especially on the addition of a trace of neutral salt, such as sodium chloride, shows the highly colloidal nature of such solutions. Alcohol soluble eosin in aqueous solution can be equally readily precipitated by neutral salts ; methylenblue is similarly precipitated, but stronger concentration of salt is required. Water soluble eosin is not precipitated by sodium chloride or iodide.

It appears, therefore, that all the stains dealt with in this paper exhibit colloidal characters in alcoholic and aqueous solutions.

The occurrence of the colloidal state probably explains why it is that no recognisable degree of hydrolysis could be recognised by the method of observation adopted in the preceding section. It would appear that in the colloidal state the dyes in question are shielded from the dissociative influence of the solvent.

SUMMARY

The main conclusions resulting from the present investigation are the following :—

1. The staining act of methylenblue-eosin in alcoholic solution is a chemical reaction.
2. Methylenblue-eosin in alcoholic and aqueous solution exhibits colloidal characters, as do also methylenblue and eosin in the water soluble and alcohol soluble forms.

TABLE I. COLOUR AND FLUORESCENCE OF DYES EMPLOYED

DYE	CONCENTRATION	COLOUR BY TRANSMITTED LIGHT		FLUORESCENCE	
		In Alcohol	In Water	In Alcohol	In Water
Methylenblue, $2C_{16}H_{18}N_3SCl + H_2O$	·001 N ·00001 N	Dark blue Blue	Dark blue Blue	None None	None None
Methylenblue, to which potassium hydrate has been added ¹	·001 N ·00001 N	Dark purple blue Purple blue	Dark blue Blue	None None	None None
"	·001 N	Yellowish red	Brownish red	Strong light yellowish green	Slight olive green
Water soluble eosin, $C_{20}H_6Br_4O_5Na_2$	·00001 N	Bright pink	Slightly brownish pink	Strong light yellowish green	"
"	·0003 N ²	Deep pink	Deeper brownish pink	Slight yellowish green	"
Alcohol soluble eosin, $C_{20}H_8Br_4O_5$	·00001 N	Brilliant slightly violet pink	Violet pink with slight brownish tint	Very slight yellowish green	Very slight olive green
"	·001 N	Dark purple	—	Strong dark yellowish green	—
Methylenblue-eosin, $C_{62}H_{40}N_6S_2Br_4O_6$	·00001 N	Bluish purple	Reddish purple	Strong dark yellowish green	Very slight dark olive green

1. In the proportion of six molecules of potassium hydrate to one molecule of methylenblue.
2. In ·001N concentration, alcohol soluble eosin is of a darker red colour than water soluble eosin in the same concentration.

TABLE II. DETERMINATION OF THE AMOUNTS OF METHYLENBLUE AND OF EOSIN REQUIRED TO PRODUCE EQUALITY OF COLOUR WITH A SOLUTION OF METHYLENBLUE-EOSIN

No. of Exp.	CONCENTRATION OF		SOLVENT	MODE OF OBSERVATION	RESULT	REMARKS
	Methylenblue-eosin	Methylenblue				
1	·56 × 10 ⁻⁵ N	·54 × 10 ⁻⁵ N	Water sol. ·48 × 10 ⁻⁶ N	{Methylenblue-eosin placed in one test-tube; methylenblue and eosin put in a second test-tube of the same diameter as the first	Complete equality of colour	Cp. spectra A, B and D, fig. 1 ¹
2	"	·53 "	Alc. sol. ·50 "		Very nearly complete equality of colour	Cp. spectrum C, fig. 1 ¹
3	"	·54 "	Water sol. ·48 "	{Methylenblue-eosin, methylenblue and eosin placed separately in glass cells, the two latter being apposed and comparison made with the first	Complete equality of colour	Cp. spectra E, F and H, fig. 1 ¹
4	"	·53 "	Alc. sol. ·50 "		Very nearly complete equality of colour	
5	"	·54 "	Water sol. ·48 "	{In test-tubes, as in 1 and 2	Complete equality of colour	Cp. spectrum G, fig. 1 ¹
6	"	·53 "	Alc. sol. ·60 "		Very nearly complete equality of colour immediately after mixing	
7	"	·54 "	Water sol. ·48 "	In glass cells, as in 3 and 4	Methylenblue + eosin: too blue	
7	"	·54 "	"		Nearly complete equality of colour	
8	"	·53 "	Alc. sol. ·50 "		Methylenblue + eosin: too blue	
8	"	·53 "	"		Nearly complete equality of colour	

1. The concentration employed for the spectra given in fig. 1 is five times that employed in this table.

TABLE III. RAISING OF THE BOILING POINT OF METHYL ALCOHOL, BROUGHT ABOUT BY METHYLENBLUE-EOSIN, METHYLENBLUE AND ALCOHOL SOLUBLE EOSIN. AMOUNT OF SOLVENT EMPLOYED 15.78 g. (MOLECULAR RAISING OF B.P. = 8.8° C.).

No. of Experiment	Dye	Amount of Dye employed	Molecular Concentration of Dye	Observed Raising of Boiling Point	Corresponding Molecular Concentration (calculated)	Observed Raising of Boiling Point in Percentage of Calculated Amount
1	Methylenblue-eosin, 1st sample...	0.098 g.	0.0052 N.	+ 0.010° C.	0.0011 N.	22
2	" " 2nd "	0.100 g.	0.0053 N.	+ 0.030° C.	0.0037 N.	71
3	Methylenblue ... "	0.352 g.	0.0283 N.	+ 0.091° C.	0.0104 N.	37
4	" ... "	0.101 g.	0.0081 N.	+ 0.013° C.	0.0015 N.	18
5	Eosin (alcohol soluble) 1st sample	0.154 g.	0.0152 N.	+ 0.016° C.	0.0018 N.	12
6	" " 2nd "	0.146 g.	0.0144 N.	+ 0.000° C.	—	0

TABLE IV. LOWERING OF THE FREEZING POINT OF WATER, BROUGHT ABOUT BY METHYLENBLUE AND WATER SOLUBLE EOSIN (SODIUM SALT). AMOUNT OF SOLVENT EMPLOYED 13 g. (MOLECULAR LOWERING OF F.P. = 19° C.).

No. of Experiment	Dye	Amount of Dye employed	Molecular Concentration of Dye	Observed Lowering of Freezing Point	Corresponding Molecular Concentration (calculated)	Observed Lowering of Freezing Point in Percentage of Calculated Amount
1	Methylenblue ... "	0.358 g.	0.035 N.	— 0.102° C.	0.0054 N.	15
2	" ... "	0.031 g.	0.003 N.	— 0.010° C.	0.0005 N.	19
3	Eosin (water soluble) 1st sample	0.447 g.	0.050 N.	— 0.781° C.	0.0412 N.	82
4	" " 2nd "	0.143 g.	0.016 N.	— 0.106° C.	0.0050 N.	31