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Eosin Staining of Gelatine

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Summary. The mechanism of gelatine staining with four selected fluorone derivative dyes (eosin y, ethyl eosin, methyl eosin, uranin) was investigated. Gelatine films were stained in dye-buffer-ethanol solutions at varying pH and in the presence of NaCl and urea. Dye binding was recorded spectrophotometrically. Ionization constants of auxochromic phenolic groups were determined from pH-absorbance curves of dye-buffer-ethanol solutions. Dye-binding was greatest at pH below pK_{OH} and decreased with increasing pH. The addition of NaCl reduced dye binding slightly below pK_{OH} but markedly above pK_{OH}. The addition of 8 M urea decreased dyebinding regardless of pH. Comparing the pH dependence of dyebinding for eosin y and esterified eosins with ionization constants revealed that ionic bonding is unlikely to occur at the carboxyl group as well as at the phenolic group. Dye binding is intimately related to the presence of Br-groups. These results are discussed in conjunction with the functional structure of the dye ions and current concepts of dyebinding mechanisms.

Introduction

Staining with anionic dyes has frequently been attributed to ionic bonds (salt linkages) between negatively charged dye ions and positively charged sidechain groups in proteins (amino, guanidino) (Lillie, 1969). This theory and its implications with respect to ionization of tissue macromolecules has been treated by Pischinger (1926), Singer (1952) and more recently by Harms (1965) and Baker (1966). However, in many instances the interpretation of dyebinding results cannot be based exclusively on the classical salt linkage. Thus there has been a trend to leave or modify the ionic bonding model. According to Neale (1947) and Otto (1953a, 1953b, 1955) dye ions are attracted to macromolecules by long range electrostatic forces caused by opposite charges of dye and macromolecule, whereas dyebinding is established by specific short range forces (hydrogen bonds, ion-dipole attraction, dipole-dipole attraction, van der Waal's forces, hydrophobic bonds). The role of long range electrostatic forces has been doubted (Alexander and Kitchener, 1950), but the significance of short range forces has been demonstrated in several connections, most convincingly in the selective cationic staining of nucleic acids (Peacocke and Skerret, 1956; Lerman, 1961, 1969; Steiner and Beers, 1961; Gersch and Jordan, 1965, Drummond, Simpson-Gildemeister, and Peacocke, 1965; Scott, 1967; Galley, 1968). The operation of specific short range forces is connected with particular sterical requirements. Proposals for the fulfilment of such sterical requirements have been made for several selective dye-macromolecule interactions (Vercauteren, 1950; Otto, 1953a; Meloan, Valentine, and Puchtler, 1971; Scott, 1972).

The role of *hydrogen bonding* in protein chemistry is well known. In connection with protein staining the hydrogen bond has been dealt with in general by Neale

(1947) and Otto (1953b and 1955) and for particular staining procedures by Goldstein (1962) and Lillie (1964). The significance of *dipole attraction* and *van der Waal's forces* has been emphasized by Neale (1947), Otto (1953b and 1955), Scott (1967), Schmitz-Moormann (1968a) and recently by Horobin and co-workers (Horobin and James, 1970; Horobin and Murgatroyd, 1971; Horobin and Bennion, 1973). The latter authors in accordance with Derbyshire and Peters (1955) and Zollinger (1965) also stressed the role of *hydrophobic bonding* between dyes and macromolecules.

Eosin y is the most frequently used anionic dye, primarily due to its general and unselective binding to proteins, extracellular as well as intracellular. Though several reports deal with staining of tissues of various origin (animal, plant, bacterial) with eosin y and related compounds, little is known about the binding mechanism (Naylor, 1926; Conn and Holmes, 1928; Kölbel, 1948; Harris, 1949; Hairston, 1955; Kelly, 1956a and 1956b; Scharf, 1956a and 1956b; Goldstein, 1969).

It is the purpose of this study to examine the mechanism of the binding of eosin y and related compounds (methyl eosin, ethyl eosin, uranin) to protein (gelatine) by correlating dye binding results with structural and chemical characteristics of the dyes.

Materials and Methods

Dyes. Eosin y (Michrome no. 93, E. Gurr Ltd.), eosin gelblich (C.I. 45380, Merck), eosin ethyl (Michrome no. 129, E. Gurr Ltd.), eosin ethyl (G11100, G. T. Gurr Ltd.), eosin methyl (Michrome no. 303, E. Gurr Ltd.) and uranin (L. J. Pointing and Son Ltd.).

Spectrophotometry. pK_{OH}-values of the above dyes were estimated from pH-E_{max} curves (absorbance at absorption maximum as a function of pH). Absorbance of 0.8×10^{-5} M to 1.2×10^{-5} M solutions of dye in a mixture of buffer and ethanol (40% v/v final ethanol concentration) was recorded on a Beckman DB electron absorption spectrophotometer, using a 10 mm cyvette; (molar concentrations were calculated on the basis of molecular weight of pure dye). Buffer solutions were Walpole acetate buffer, Michaelis barbital-acetate-HCl-buffer, Sörensen phosphate buffer and Sörensen-Walbum glycine buffer. Experiments below pH 1.0 were carried out in HCl solutions. Further absorbance of 1.2×10^{-5} M solutions of eosin y in buffer/ethanol with the addition of gelatine (Bacto Gelatine, Difco) was recorded.

Staining. Gelatine covered polyester strips (Agfa Gevaert graphic gelatine film, 61101508) were stained for 30 min at room temperature in $6.0 \cdot 10^{-5}$ M to $6.0 \cdot 10^{-4}$ M solutions of eosin y, ethyl eosin, methyl eosin and uranin in buffer/ethanol as above. Staining experiments were performed at varying pH and ionic strength with or without the addition of urea. After rinsing two min. in buffer/ethanol, the strips were dried for 30–60 min at 60° C and left for two hours in NH_3 vapor to promote colorchange to basic color. After renewed drying, E_{max} was recorded spectrophotometrically by placing the stained strip in the sample cyvette. Strips left in buffer/ethanol without dye for 30 min were used as reference.

Finally it was controlled, that dissolution of gelatine from the strips and staining of the polyester base did not occur. Beer's law was checked for solutions of eosins and uranin within the concentration range used.

Results

Spectrophotometry. A plot of absorbance versus dye-concentration (5×10^{-7} M to 4×10^{-5} M) showed direct proportionality, indicating that Beer's law is obeyed within the concentration range used in the present study.

Characteristic absorption curves for eosin y, ethyl eosin, methyl eosin and uranin are shown Fig. 1. The absorption maximum lies in the range 520–540 nm for halogenated dyes and at shorter wave length (495 nm) for uranin. All of the

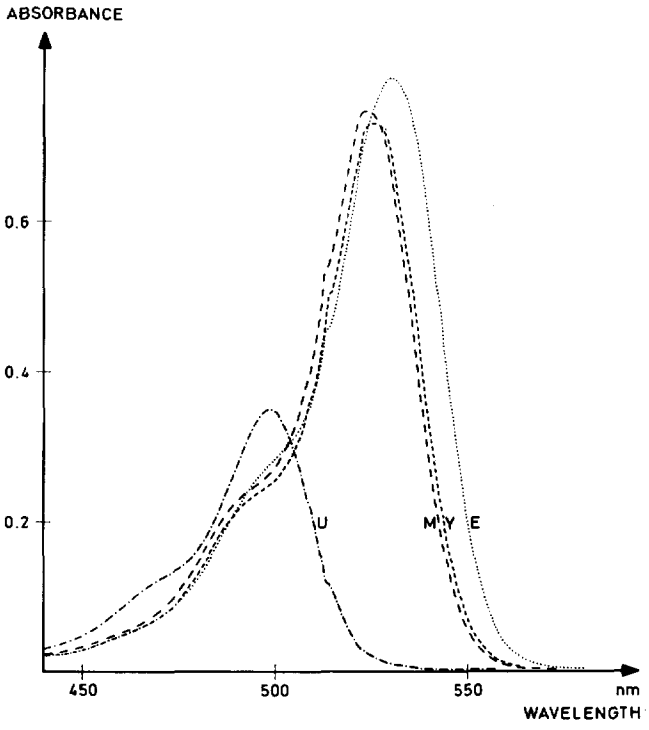

Fig. 1. Absorption curves of $1.2 \cdot 10^{-5}$ M uranin (U), eosin y (Y) methyl eosin (M) and ethyl eosin (E) in 40% ethanol at pH 9.0

dyes exhibit a characteristic shoulder approximately 50 nm from the maximum. The absorption curves of fluorones are significantly dependent on pH (Fig. 2). Upon lowering pH the main absorption band vanishes whereby the maximum is shifted to shorter wave length. Concomitantly the molar extinction coefficient is considerably decreased. Visually these changes are percepted as a colorchange from red to feeble yellow. As the spectral changes are caused by protonization of the phenolic group, the pK of this group (pK_{OH}) can be estimated from curves showing E_{max} as a function of pH (Fig. 3). For graphic purposes, the per cent of ionization has been depicted instead of E_{max}. Hereby all pK_{OH} values can be read immediately from the curves. The values are listed in Table 1.

Table 1. Absorption maxima and pK_{OH}-values for fluorone dyes in 40% ethanol

Dye	Abs. max. (nm)	pK_{OH}
Eosin y	524–530	3.0
Ethyl eosin	528–532	2.5
Methyl eosin	522–530	2.9
Uranin	440–445; 493–497	1.4; 6.9

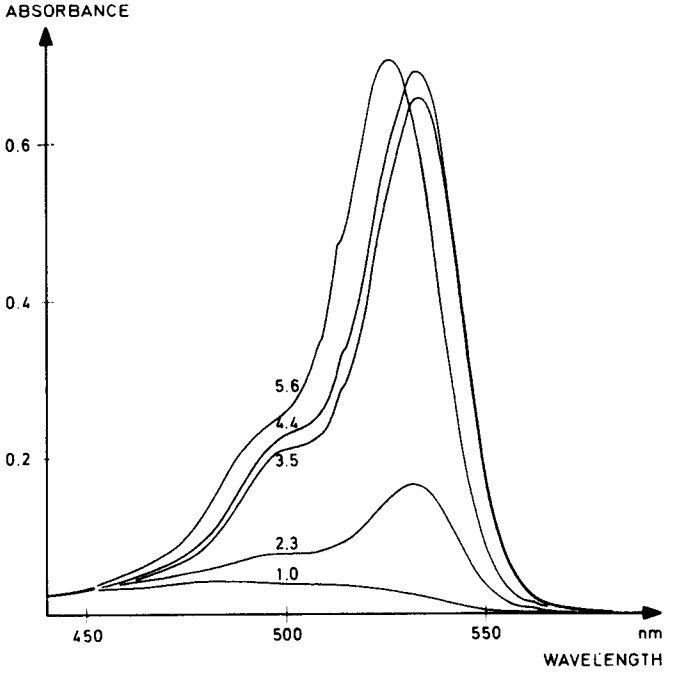

Fig. 2. Absorption curves of $1.2 \cdot 10^{-5}$ M eosin y in 40% ethanol at varying pH

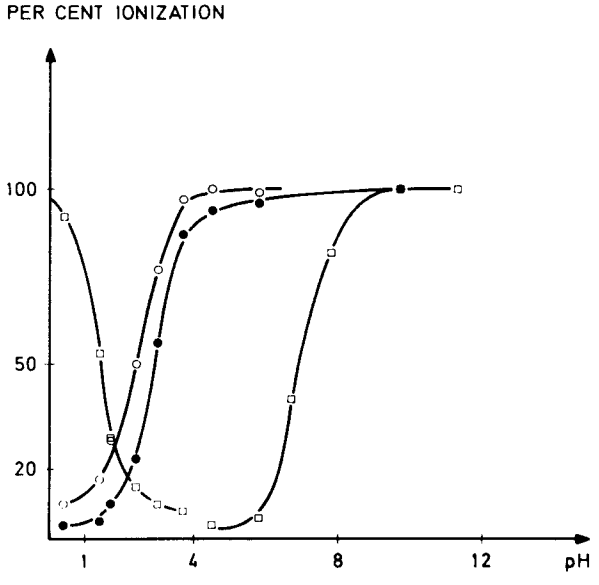

Fig. 3. Ionization curves of uranin (□), eosin y (●) and ethyl eosin (○) in 40% ethanol

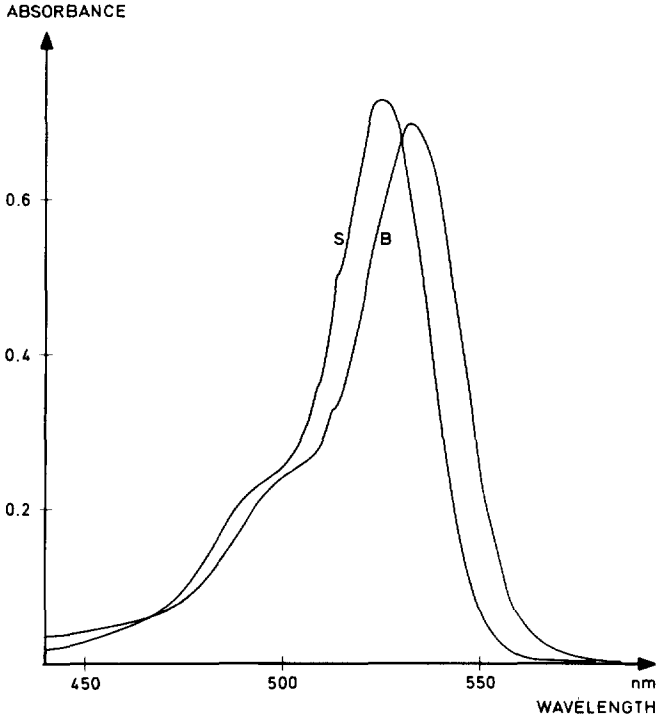

Fig. 4. Examples of absorption curves of eosin y in solution (*S*) and bound to gelatine (*B*). Note the difference in position of the absorption maximum

No change in the position of the absorption maximum was observed upon the addition of gelatine to dye solutions.

Staining of Gelatine. Absorption curves of stained strips showed the same general form as curves of dye solutions, but a 10 nm bathochromic shift of the absorption maximum was invariably seen (Fig. 4). The influence of pH on gelatine staining with eosins and uranin is shown Fig. 5. It is noticed, that the greatest amount of dye is bound at pH below pK_{OH}. Increasing pH leads to a decrease in dye binding. The results for the three eosins show great similarity, whereas uranin demonstrates a different dyebinding pattern. The binding of uranin is negligible regardless of pH. It seems that the binding of uranin increases until a maximum is reached and then decreases again. Simple calculations reveal, that the concentration of uranin, when dye uptake is maximal is of the same order of magnitude as the concentration in the dyebath, whereas eosin concentration at low pH (1.0) is approximately 25 times dyebath concentration. The influence of ionic strength on dyebinding is demonstrated Fig. 6. At low pH dyebinding is relatively insensitive to changes in ionic strength. At pH 4.2 dyebinding decreases significantly upon the addition of increasing amounts of NaCl. The addition of 8 M urea reduces dyebinding above as well as below pK_{OH}. However,

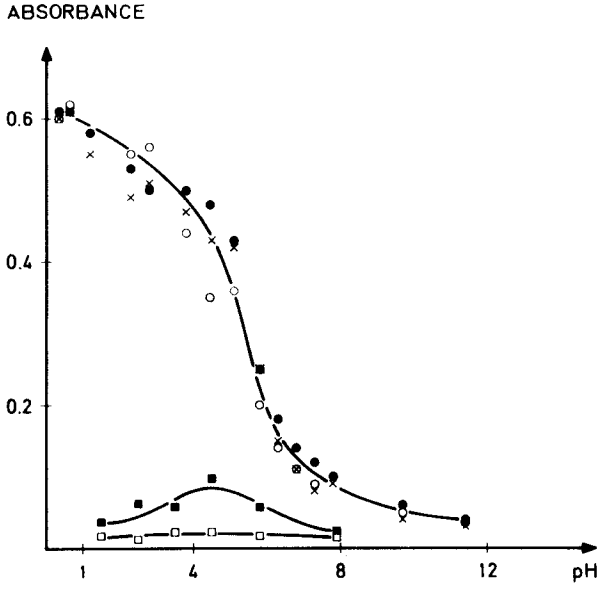

Fig. 5. Staining of gelatine with $6.0 \cdot 10^{-5}$ M eosin y (●), ethyl eosin (○), methyl eosin (×) and uranin (□), and $3.0 \cdot 10^{-4}$ M uranin (■) in 40% ethanol at varying pH

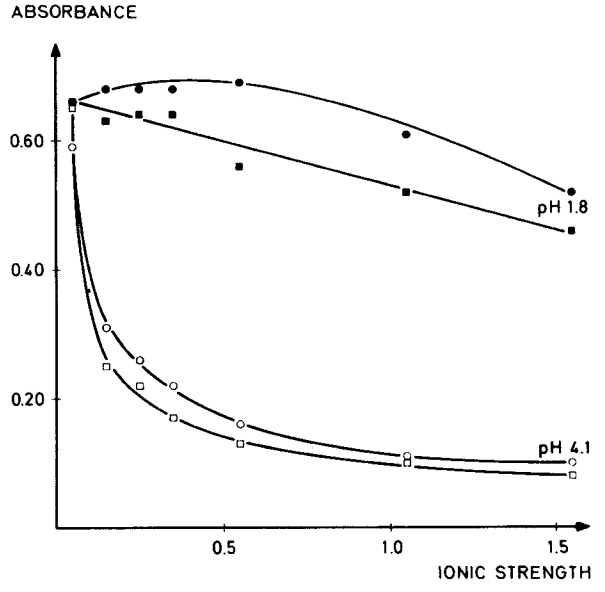

Fig. 6. Gelatine staining with eosin y (⚬/●) and ethyl eosin (▫/■) in 40% ethanol at pH 1.8 and 4.1 as a function of ionic strength

comparisons are difficult to accomplish at constant pH. Due to protonization urea acts as a buffer. Even in 8 M urea staining is not completely prevented.

Discussion

All experiments were carried out in buffer/ethanol mixtures in order to enhance the solubility of eosins and uranin. Due to the carboxyl group eosin y is easily soluble in water at pH above pK_{COOH}, (approximately 5 according to Scharf, 1956a). Uranin behaves similarly. Methyl eosin and ethyl eosins at any pH and uranin and eosin y at pH below pK_{COOH} are sparely soluble in hydrophilic solvents, whereas the solubility in ethanol is somewhat greater (Gurr, 1960; Lillie, 1969). In buffer solutions containing 40% ethanol all dyes are sufficiently soluble for the present experiments.

Spectrophotometry. The obeyance of Beer's law within the concentration range used justifies the estimation of pK_{OH} from the E_{max}-pH curves. The pK_{OH} of eosin y found in this study accords with values obtained by Kölbel (1948) and Scharf (1956a) — 3.0, whereas the ethyl eosin examined by Scharf was considerably more basic than the present one. The pK_{OH} of methyl eosin unexpectedly differed somewhat from that of ethyl eosin. Some variation in the pK-values might be caused by impurities in commercial samples of the dyes. Though Hanig and Koch (1959) found a low content of impurities in eosin y, Graichen and Molitor (1965) showed great variation in the content of impurities in commercial samples of a related fluorone dye. These impurities, noticed by several authors (Holmes, Melin, and Peterson, 1932; Scharf, 1956a; Rosenthal, Puchtler, and Sweat, 1965; Horobin, and Murgatroyd, 1967; Horobin, 1969) probably consist of fluorones of lower bromination than eosins (Hanig and Koch, 1963).

Functional Structure of Eosins and Uranin. The understanding of spectral properties and staining characteristics of individual dyes requires a detailed knowledge of the functional dye-structure. The fundamental work of Lewis and collaborators has made valuable contribution to the correlation of structural, physical and chemical properties of dyes (Lewis and Calvin, 1939; Lewis, 1945), and in several connections quantemechanic calculations on energetic characteristics and charge distribution of dyes have given promising results (Kuhn, 1949; Grinter and Heilbronner, 1962).

Eosin y (Fig. 7a) is a tetrabrominated derivative of uranin (fluorescein) (Fig. 7d), belonging as such to the phenylsubstituted hydroxyxanthenes (fluorones). According to classical color rules the parachinoid ring constitutes the chromophore. Newer concepts ascribe the chromophoric properties to the whole xanthene part of the molecule (Lewis and Calvin, 1939; Lewis, 1945). The three rings of the xanthene moiety lie in one plane, while the fourth ring is twisted out of the xanthene plane due to the sterically bulky carboxyl group. Lewis, Magel, and Lipkin (1942) later supported by Sharp and Sheppard (1957) and Gomes de Mesquita, MacGillawry, and Eriks (1965) have shown that even in unsubstituted triphenylmethane dyes, steric factors cause a twisting of the rings. The position of the carboxyl group and the fourth ring offering least energetic requirements is the one in which the ring is perpendicular to the xanthene plane. This configuration of the molecule sterically facilitates lactone formation of uranin

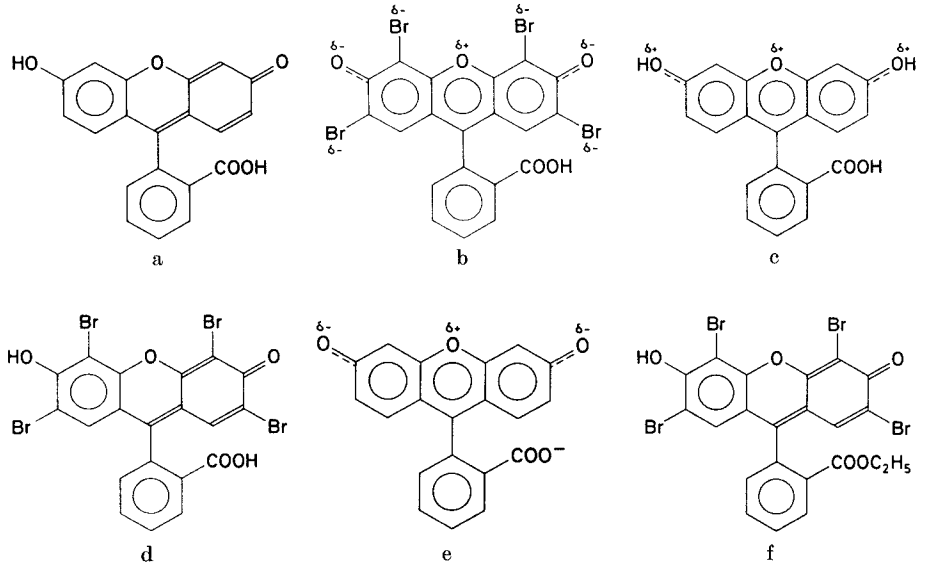

Fig. 7. *a* eosin y; $pH < pK_{OH}$. *b* eosin y; $pK_{COOH} > pH > pK_{OH}$. Distribution of main partial charges. *c* uranin; $pH < pK_{OH^+}$. Distribution of main partial charges. *d* uranin (fluorescein); $pK_{OH^+} < pH < pK_{COOH}$. Distribution of partial charges omitted. *e* uranin; $pH > pK_{OH}$. Distribution of main partial charges. *f* ethyl eosin. Distribution of partial charges omitted

and eosin y. Due to the twisting, the fourth ring is not part of the xanthene resonance system. It also follows, that ionization of the carboxyl group does not influence the absorption curve of the dye (Haring and Heller, 1941; Kölbel, 1948; Scharf, 1956a). In ethyl eosin and methyl eosin the carboxyl group is esterified with ethanol and methanol respectively (Fig. 7f). Hereby ionization and lactone formation is prevented (Scharf, 1956a). The auxochromic hydroxy-oxy groups lie in the same plane as the xanthene moiety and they are part of the xanthene resonance system. Ionization of the phenolic group therefore causes spectral changes by perturbing the xanthene π-electron cloud.

Tetrabromination of uranin to produce eosin y affects the spectral and chemical properties of the molecule. The absorption maximum undergoes a bathochromic shift from 495 to 525 nm. The acid strength of the auxochromes is increased, pK decreasing from approximately 7 to approximately 3 (Scharf, 1956a). Substitution of strongly electro-negative groups (Br) in the resonance system markedly alters the charge distribution in eosins. The decrease of pK_{OH} signifies a marked decrease of electron density in the —O—H bond. Ethyl eosin and methyl eosin behave like eosin y, as esterification of the carboxylic group does not influence the charge distribution in the resonance system to any significant degree (Kölbel, 1948; Scharf, 1956a).

The net charge carried by eosins is a function of pH. For eosin y $pK_{OH} = 3.0$ and $pK_{COOH} \simeq 5$. Thus below pH 3 eosin y is mainly unionized. Between pH 3

and 5 eosin y carries mainly one negative charge and above pH 5 eosin y aquires two negative charges. Ethyl eosin and methyl eosin behave similarly except for ionization of the carboxyl group. In uranin the acidity of the phenolic group is diminished, pK being ~ 7. Thus uranin is mainly unionized in the range from pH 2–4. It carries one negative charge above pH 5 due to ionization of the carboxyl group and two negative charges above pH 7. At very low pH the oxy-group is protonized (Fig. 7c), and hence uranin is positively charged (pK 1.4).

The indicator properties of eosins are well known (Kölbel, 1948; Scharf, 1956a; Lillie, 1969). The color change (Fig. 2) parallels a change in electronic distribution caused by ionization of the auxochrome(s). Upon ionization of the phenolic group, the molecule aquires a considerable resonance stabilization, resulting in a bathochromic shift of the absorption maximum and a considerable increase in the molar extinction coefficient. In the ionized state, a distinction between the auxochromes cannot be maintained. This was represented by Scharf (1956a) by two mesomeric forms. The equality of the auxochromes is however better demonstrated by assigning one partial negative charge to each auxochrome. According to Lewis (1945) the central heteroatom will tend to participate in the aromatic structure by more or less offering electrons to the π-electron cloud of the xanthene moity. The oxygen atom thus aquires a partial positive charge (Harms, 1965). In eosin y, the strongly electronegative Br-atoms attract electrons from the resonance system, aquiring partial negative charges. This probably increases the positive charge at the heteroatom but diminishes the charge at the auxochromes as judged from the acid strength. Without commenting on the magnitude of individual partial charges, the charge distribution in eosin y will be as shown in Fig. 7b. Due to bromination polarization is much more pregnant in eosins (Fig. 7b) than in uranin (Fig. 7e). The charge distribution is probably also—to a minor extent—influenced by the inductive effect of the ionized carboxyl group. This group tends to induce a partial positive charge at the central carbon atom, causing a slight increase of the electron density in the —O—H bond, whereby the acid strength should be slightly decreased. In ethyl eosin and methyl eosin this effect is not present, due to lacking ionization of the ester group. The inductive effect of the carboxyl group, however, is so small, that it is not conclusively demonstrated in pK values of different eosins. For uranin two color changes are known. The one at the higher pH is caused by ionization of the —OH group. It thus parallels the colorchange observed for eosins. The colorchange at the lower pH is caused by protonization of the parachinoid oxygen atom. This protonization results in a considerable resonance stabilization of the molecule (Fig. 7c), thus causing a bathochromic shift of the absorption maximum.

Theoretically possible binding sites in eosins and uranin can now be predicted. Uranin displayes one localized negative charge at the carboxyl group above pK_{COOH} and another negative charge distributed as two partial negative charges at the auxochromes and one partial positive charge at the heteroatom above pK_{OH} (Fig. 7e). In the unionized state (Fig. 7d), the auxochromes theoretically posses hydrogen bonding properties. Eosin y behaves similarly, but the distribution of partial charges is more pregnant due to four additional partial negative charges at the Br-atoms (Fig. 7b). Thus hydrogen bonding and electrostatic dipole attraction are possible below pK_{OH}, while ionic bonding and dipole at-

traction are possible above pK_{COOH}. This is true also for methyl eosin and ethyl eosin with the exception of ionic bonding involving the carboxyl group.

Dyebinding. Gelatine covered polyester strips proved satisfactory for the present staining experiments. The transparency of the strip in the dried state allowed spectrophotometric recording of the amount of dye bound in the gelatine film. The uncontrolable removal of bound dye during dehydration was avoided by omitting this procedure. The sorption of NH_3 vapor by the gelatine ensured spectrophotometric recording under comparable conditions. The method overcomes the problem mentioned by Deitch (1955) of comparing the amount of dye bound in the acid and the basic form of martius yellow and naphtol yellow s.

Significance of pH. The pH-dyebinding curves for different eosins demonstrate, that the carboxyl group does not participate — or at least is not essential for dyebinding. Maximal dye uptake and pH-dependence of dyebinding differ only insignificantly for eosin y and esterified eosins. This accords with the qualitative statement, that ethyl eosin can be used as a background stain as well as eosin y (Gurr, 1960; Harms, 1965; Lillie, 1969).

The present pH-dependence of dyebinding accords with results obtained for anionic staining of proteins in general (Pischinger, 1926; Drawert, 1937b; Singer and Morrison, 1948; Singer 1952; Deitch, 1955) and collagen and gelatine in particular (Drawert, 1937a; Otto, 1953a; Gustavson, 1956). Several authors have shown, that they apply also for water soluble eosins above pH 3 (Conn and Holmes, 1926; Kölbel, 1948; Harris, 1949; Hairston, 1955), whereas eosin binding below pH 3 was not included, probably due to the sharp decrease in solubility. The pH-dyebinding curves resemble protein titration curves (Fig. 5) and similar results have been found for the binding of simple acids to various proteins (Loeb, 1922; Elöd, 1933; Gilbert and Rideal, 1944; Otto, 1953a; Gustavson, 1956; Mathieson and Whewell, 1964), though the affinity of simple acids to protein is less than the affinity of acid dyes. Decreasing dyebinding with increasing pH is ascribed to decreasing electrostatic attraction between dye and macromolecule below IP and to electrostatic repulsion above IP (Pischinger, 1926; Neale, 1947; Singer, 1952; Harms, 1966; Lillie, 1969).

pH below pK_{OH}. The considerable eosin binding below pK_{OH} in the present study is not consistent with the ionic bond concept as eosins are mainly uncharged. Neither is the significance of the long range electrostatic attraction, emphazised by Neale (1947) and Otto (1955) corroborated below pK_{OH}. In accordance with the present dyebinding results Gustavson (1956) showed that collagen uptake of acetic acid was greater in the unionized than in the ionized form. Gustavson ascribed this to hydrogen bonds between collagen and unionized acetic acid. Further evidence of non-ionic bonding to collagen was given by Otto (1953b), who demonstrated that dyebinding *decreases* with increasing number of sulfonic groups in the dye. Contrarily, Deitch (1955) found that naphtol yellow s but not martius yellow was able to combine with proteins below pK of the auxochromic —OH group. This difference was attributed to the binding capacity of the $-SO_3$-group present in n.y.s. but not in m.y. However, it must be recognized, that collagen might differ from other proteins with respect to dyebinding due ot the high content of amino acids favouring hydrogen bonding (Lillie, 1969). Accordingly it appears from an early work of Fraenkel-Conrat and Cooper (1944)

that the stoichiometric anionic dyebinding demonstrated for several proteins, does not apply to gelatine.

Comparing the dyebinding curves for eosins and uranin respectively, hydrogen bonding is not likely to occur in the present study. Uranin structurally offers the same possibilities for hydrogen bonding as do eosins. Yet the binding of uranin to gelatine is negligible. The role of hydrophobic bonding in dyeing was considered by Zollinger (1965) and Horobin and Bennion (1973). In connection with eosins it was mentioned by Goldstein (1969). It is unlikely, however, that hydrophobic bonds play any role in the present experiments, as the marked distribution of partial charges in eosins, which would disfavour hydrophobic bonding, is seen to increase dyebinding considerably when compared with uranin. The only significant functional difference between eosins and uranin lies in the Br-atoms and the distribution of partial charges caused by bromination. Thus it is tempting to assume that the binding mechanism at low pH depends on these partial charges, i.e. either ion-dipole or dipole-dipole attraction. The significance of these forces in dyebinding has been stressed by several authors (Neale, 1947; Otto, 1953a, 1953b, 1955; Schmitz-Moorman, 1968a; Horobin and James, 1970; Horobin and Bennion, 1973).

pH above pK_{OH}. At pH above pK_{OH}, the one negative net charge aquired by ionization would be expected to cause a significant electrostatic attraction between dye and protein. For eosin staining of gelatine, this electrostatic attraction is apparently of minor importance, as no increase in dye binding is seen in connection with ionization. The *decrease* of dyebinding actually found in this range parallels the decrease of the net charge of the gelatine. It is probably caused by electrostatic repulsion between eosins and ionized gelatine carboxyl groups.

The existence of two different binding mechanism, as proposed by Gustavson (1956) for acetic acid binding to collagen, also must be considered, i.e. dipole attraction below pK_{OH} and another mechanism above pK_{OH}. Though ionic bonding involving the carboxyl group is insignificant, a salt linkage to one of the auxochromes is still possible. This requires, that the negative charge is concentrated at one auxochrome only. Hereby the energetic symmetry of the chromophoric system is lost. According to Lewis and Calvin (1939), McKay and Hillson (1965) and Hillson and McKay (1966) this will produce a shift of the absorption maximum and a marked reduction in the molar extinction coefficient. Such metachromatic effect in connection with eosin staining has been mentioned by Hairston (1955), Kelly (1956a) and was demonstrated clearly in the binding of eosin to protamine (Kelly, 1956b). Similar effects caused by the addition of neutral salts to solutions of eosins were reported by Goldstein (1969). Though metachromatic behaviour of dyes has often been attributed to aggregation (Rabinowitch and Epstein, 1941; Douglas, Spicer, and Bartels, 1966; Winkelman and Bradley, 1966; Goldstein, 1969), McKay and Hillson (1965) contended that these spectral changes can be explained on the bases on ionic interaction at the auxochromes. Eosin binding to protamine therefore *might* be ionic. The excessive content of basic amino acids in protamine would favour ionic binding. Gelatine staining with eosin in the present study was associated with a bathochromic shift of 10 nm (Fig. 4), but the general form of the absorption curve was unchanged. The significance of the bathrochromic shift is doubtfull, as the same effect could not be produced in gelatine-

eosin solutions. As eosin-binding to gelatine thus is established virtually without spectral changes, ionic bonding is unlikely to occur even above pK_{OH}. A hypothesis involving basically different binding mechanisms above and below pK_{OH} therefore is unlikely.

Significance of NaCl. Reduction of dyebinding in the presence of neutral electrolytes is generally found in connection with both cationic and anionic staining (Singer and Morrison, 1948) and complete prevention of staining is considered indicative for pure ionic bonding (Scott and Dorling, 1965; Scott and Willet, 1966). Accordingly the partial prevention of staining in the present study is in keeping with a non-ionic bonding concept. In disagreement with the present investigation, Goldstein (1969) found, that the uptake of eosin y was enhanced in salt solutions. Though a similar effect is often observed with the addition of very small amounts of salt (Scott and Dorling, 1965; Schmitz-Moormann, 1968b) it does not accord with the salt effect generally found. Neale (1946, 1947) ascribed the salt effect to reduction of long range electrostatic forces, while Alexander and Kitchener (1950), Scott and Dorling (1965) and Schmitz-Moormann (1968b) attributed the salt effect to competition for binding sites between dye ions and salt ions. Neales concept requires the existence of a long range electrostatic attraction, not indicated by the dyebinding-pH curves in the present study (Fig. 5). If the difference in salt effect below and above pK_{OH} (Fig. 6) is considered qualitative, Neales concept as well as the competition concept ultimately lead to the adoption of two different binding mechanisms, one below and one above pK. If, however, this difference in salt effect is considered quantitative rather than qualitative, it might be explained though only one binding mechanism is present. Similarly it has been shown that the salt effect on protein titration curves, which is pregnant at slightly acid pH, decreases or disappears at low pH (Alexander and Kitchener, 1950; Gustavson, 1956).

Significance of Urea. The results of adding 8 M urea to the staining solution were inconclusive. Dyebinding was reduced above as well as below pK_{OH}, but it was not completely prevented. Urea is a powerfull agent for breaking hydrogen bonds, but it might also reduce binding due to electrostatic forces of any kind because of its dipolar character (Goldstein, 1962).

Conclusion

The correlation of ionization and structural characteristics with dyebinding results indicates, that the binding of eosins to gelatine is established by polar bonds involving partial charges at the Br-atoms and partial charges created by bromination. It further appears, that the significance of ionic interaction is negligible at the carboxyl group as well as at the auxochromes.

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