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ISOELECTRIC FOCUSING AS A PUZZLE

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SUMMARY

The formation of strong complexes of most acidic dyes with Ampholine in isoelectric focusing has been demonstrated by altering the dye to Ampholine ratio, by re-running single dye bands and by measuring the UV–visible spectra of the complexes. These complexes can only be disaggregated by changing the dielectric constant of the solvent or by high temperatures, or both. On an equimolar basis, the order of the dissociating powers of the most commonly used disaggregating agents is dimethylformamide $\approx$ tetramethylurea $>$ dimethyl sulphoxide $>$ urea $>$ formamide. A two-step binding model is postulated: first a strong, polydentate and undissociated Ampholine–dye salt, followed by hydrophobic dye–dye interaction.

INTRODUCTION

In general, estimates of p*I* values determined by electrophoresis are lower than the p*I* values obtained by isoelectric focusing (IEF)[1,2], largely because of interactions of proteins and buffer ions during electrophoresis[3]. Conversely, it has been stated that the p*I* of a protein determined by IEF also represents its isoionic point[4]. This concept implies that there are no interactions among proteins and carrier ampholytes. While this might be generally true, there have nevertheless been reports of the interaction between Ampholine and several classes of amphoteric species, including proteins and nucleic acids.

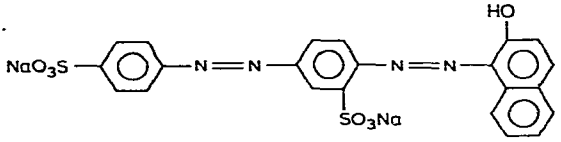
In the course of an extensive screening of dyes for their possible use as pH markers in IEF, some unusual and inexplicable results were obtained. This paper shows how dyes bind strongly to Ampholine and how it is possible to disaggregate these complexes.

EXPERIMENTAL

Materials

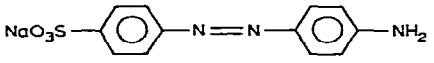
Acrylamide, N,N'-methylenebisacrylamide (Bis), tetramethylurea (TMU), dimethyl sulphoxide (DMSO), dimethylformamide (DMF) and formamide (FA) were obtained from Merck-Schuchardt (Munich, G.F.R.). Bis was recrystallized from acetone and acrylamide from chloroform, as described by Loening[5]. Ammonium persulphate and tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Labs., Richmond, Calif., U.S.A. Urea (ultrapure) was purchased from Mann Labs., New York, N.Y., U.S.A.

Of the several dyes investigated (more than 200, chosen from a list of 1500) we selected the following four: Biebrich Scarlet (I) (BDH, Poole, Dorset, Great Britain),



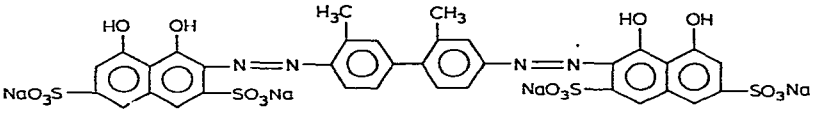
I

Sirius Supra Orange 7GL (Direct Orange 46) (II) (Bayer Italia S.p.A., Milan, Italy)



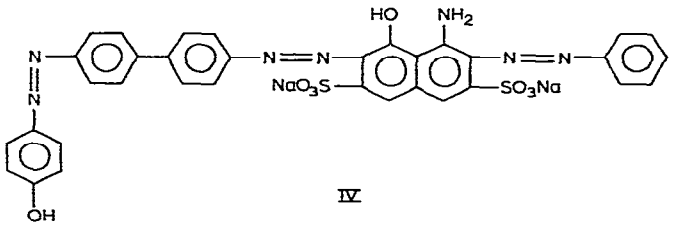
II

Benzo New Blue 5BS (Direct Blue 25) (III) (Bayer Italia) and



III

Benzo Dark Green B (Direct Green 1) (IV) (Bayer Italia).



IV

The rationale for choosing these four dyes is that two of them are amphoteric while the other two are not (and are strong acids). Moreover, two of them (Biebrich Scarlet and Benzo Dark Green B) have a strong affinity for proteins while the other two have a greater affinity for cellulose than for proteins. The criteria for choosing

the original 200 dyes were good solubility in water and absence of a metal as a ligand (because Ampholine binds to some metals[6]). Our results, however, apply to most of the acidic dyes we have investigated (which represent *ca.* 80% of the dyes tested). For the remaining *ca.* 20% (basic dyes), we can give no results at present as it is difficult to obtain good pH gradients in the pH range 9–11 in a gel slab.

Methods

IEF in gel slabs was performed in an LKB Multiphor 2117 chamber, with an ISCO Model 492 constant-wattage power supply[7]. The gels were prepared to contain 7.5% of acrylamide (the ratio of acrylamide to Bis being 25:1), 1% of Ampholine pH 2.5–4, 1% of Ampholine pH 3–6 and 0.1% of Ampholine pH 3.5–10. When additives were used, the gel was polymerized in two halves, as described by Hobart[8]. The additives used, and their final concentrations, were 8 *M* urea or 50% TMU or 50% DMF or 50% DMSO. The mechanical properties and optical clarity of the additive-containing gels were as follows: 8 *M* urea gels, same as control gels; 50% DMSO gels, transparency as good as the control gels, but softer and gluey; 50% DMF gels, completely opaque (milky appearance), very soft and sticky; 50% TMU gels, semi-liquid glue, which keeps losing fluid. With the last gels, the matrix is opaque and the Ampholines themselves are not completely soluble in this medium. Even when 10 or 12% of acrylamide was used, the situation was not much improved.

From the point of view of polymerization time, 8 *M* urea gels behave as control gels, while the 50% DMSO, 50% DMF and 50% TMU gels were poured in as the second layer and left to polymerize overnight (14–16 h). Usually, 1 *N* sodium hydroxide solution was used at the cathode and 1 *M* orthophosphoric acid at the anode. The gel was pre-run at 10 W for 1½ h, then the sample was applied and the run continued for 3½–5½ h at 14–15 W. The coolant temperature was usually 10–12°. Samples of 25 μl were applied to the cathodic site in pockets pre-cast in the gel, either at the original concentration (5 mg/ml in water) or at appropriate dilutions.

The apparent p*I* of each dye was measured by cutting out the coloured zone (*ca.* 60 μl of gel volume) and adding 0.25 ml of 10 m*M* potassium chloride solution. The pH was measured with a combined glass electrode with a Radiometer digital pH meter at 21°. No correction was made for the presence of additives. However, we verified experimentally that the pH increases by 0.05 pH unit per unit of urea molarity in the solution, while in the presence of DMF, TMU or DMSO the pH increases by 0.25 pH unit every 10% increments of concentration of any of the three additives in solution (all measurements at 21°).

UV–visible spectra were measured at room temperature (21°) with a Cary 118 spectrophotometer (Varian, Palo Alto, Calif., U.S.A.) in a 50 m*M* acetate buffer (pH 3.85) at a dye concentration of 30 μg/ml. This pH was the pH of a 1:1 mixture of Ampholines pH 2.5–4 and pH 3–6, so that increasing amounts of Ampholine in the solution did not alter the pH. This pH also happens to be the pH at which two of the dyes (Biebrich Scarlet and Sirius Supra Orange 7GL) show some of their apparent p*I*s in the absence of additives in the gel.

Melting curves (hyperchromic thermal transitions) at 585 nm were measured in a Gilford Model 2400 automatic spectrophotometer equipped with a K2R thermostat (Lauda) and a device programmed to increase the temperature of the cuvettes at a constant rate of 1.0°/min (ref. 9).

RESULTS

Evidence for complex formation

Originally, the 200 dyes selected were run over a wide pH range (pH 3.5–10) in order to be able to select those more suitable as pH markers. When the chosen dyes were subsequently run over narrow pH gradients, we observed non-reproducible and puzzling results, which prompted a more thorough investigation of their behavior in IEF.

As shown in Fig. 1, when the concentration of Biebrich Scarlet was varied over a 50-fold range, the pattern became increasingly complex, starting from one or two bands at low concentrations to six to eight at high concentrations. The apparent p*I* values of the four major components were 3.57, 3.76, 3.85 and 4.11. Similar results were obtained with the other dyes tested. This polydispersity, dependent on the ratio of Ampholine to sample, is currently interpreted in terms of the formation of complexes of the two species[10]. To check this interpretation, the bands of p*I* 3.76, 3.86 and 4.11 were cut out and re-run in a second gel. As shown in Fig. 1 (inset), they all behave as identical species and display a major component that does not occur in a band at any of the above positions, but at p*I* 3.57. Thus, reducing the sample load shifts the pattern towards lower p*I* species and reduces apparent microheterogeneity.

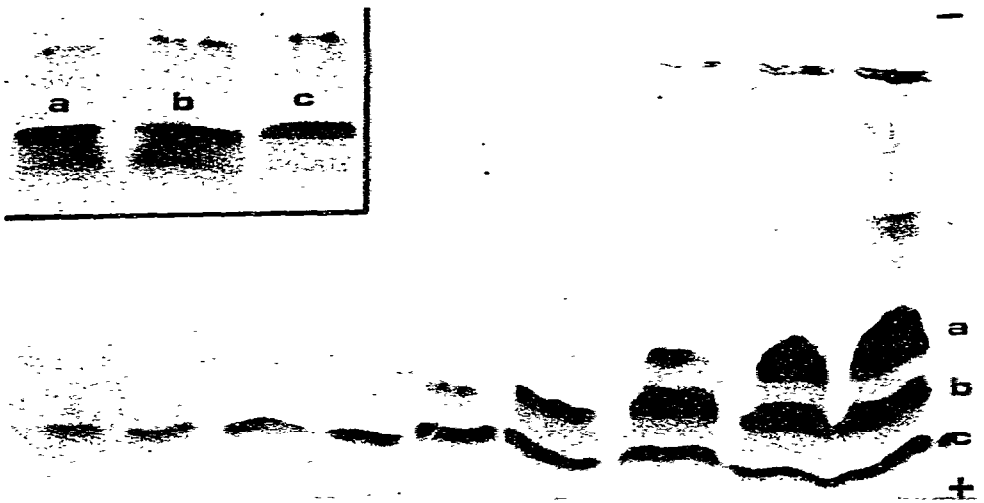


Fig. 1. IEF of Biebrich Scarlet in the pH range 2.5–6. Sample (25 μl) was applied to a pre-focused gel slab in pockets pre-cast in the gel at the cathodic side. The amounts applied were (from left to right) 2.5, 3.1, 4.2, 6.25, 12.5, 25, 50, 62.5 and 125 μg. The inset shows a re-run of bands (a), (b) and (c) in a second gel in the same pH range. The three bands behave identically and form bands in the same positions.

Additional evidence also comes from the detection of focused Ampholine patterns, on the basis of their different refractive indices[11]. As soon as focusing was completed, the gel was photographed against a black background with side illumination. Fig. 2 shows that all of the dye bands run on top of major Ampholine ridges, none being located in the valley between two adjacent species.

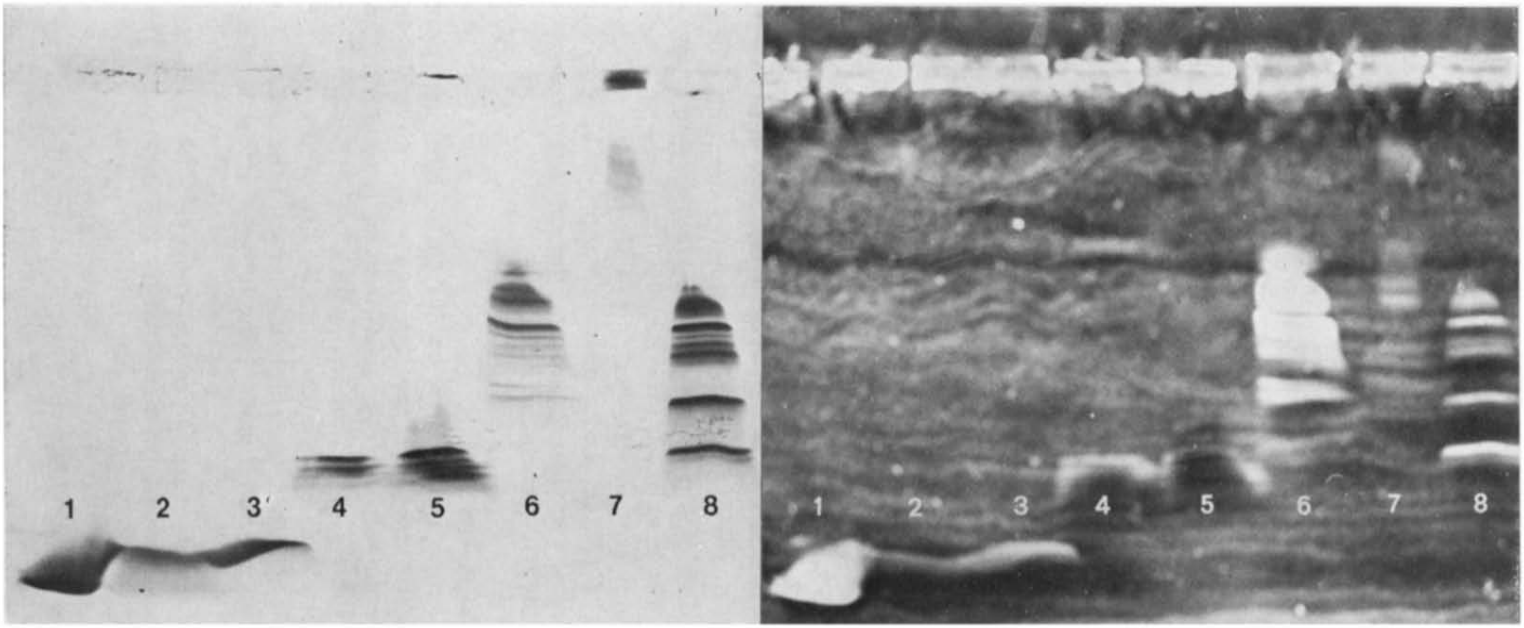


Fig. 2. IEF of dyes. Each dye was a single, major band cut from a previous run and re-run by applying directly the gel-trapped dye band to the surface of the gel slab, near to the cathodic (upper) side. Left: as soon as focusing was completed, the gel was photographed directly in transmitted light. Right: the same gel photographed in reflected light (against a black background with side illumination) to show up the refraction lines of focused Ampholine. The intense white lines are the focused dye bands (they are white because of light scattering and fluorescence, particularly with Nos. 1 and 6). The long, thin, white lines represent focused Ampholine species (*Ampholine ridges*); the long, thin, dark lines are the valleys in between. The white rectangles at the top are the pieces of gel used to apply the samples to the gel slab. Dyes: 1 = Reactone Red G (Geigy); 2 = Violet Supracen 4BF (BASF); 3 = Violet Supracen 3R (BASF); 4 = Sirius Violet 3B (Bayer); 5 = Diphenyl Brilliant Violet B (Geigy); 6 = Sirius Supra Orange 7GL; 7 = Benzo Red 8BS (Bayer); 8 = Biebrich Scarlet.

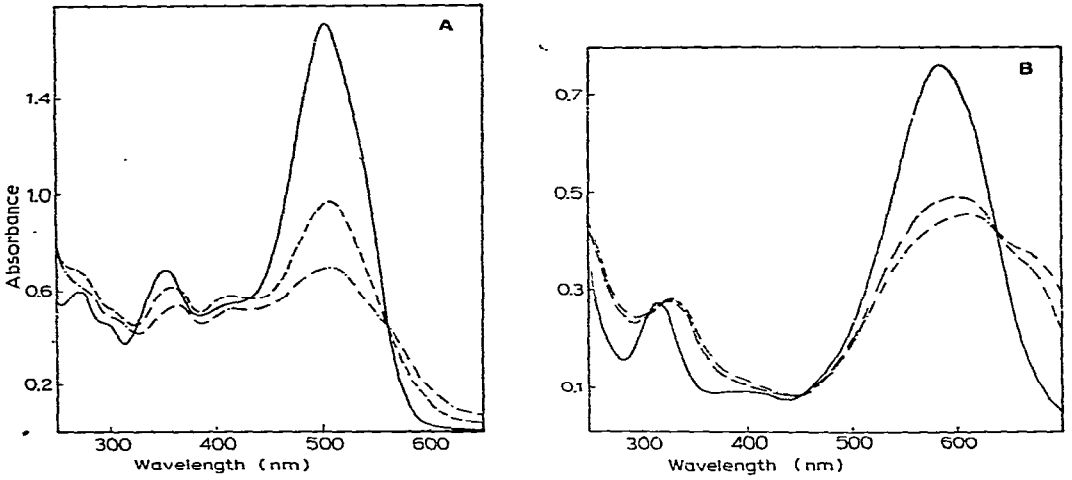


Fig. 3. UV–visible spectra. The spectra were measured with a Cary 118 spectrophotometer in 50 m*M* acetate buffer (pH 3.85) at a dye concentration of 30 μg/ml. ———, Spectrum of the dye alone; – – – –, with 1 μl of Ampholine pH 2.5–6 added; –·–·–, with 10 μl of Ampholine pH 2.5–6 added. A, Biebrich Scarlet; B, Benzo New Blue 5BS.

Perhaps the strongest evidence of dye–Ampholine binding comes from UV–visible spectra. When Biebrich Scarlet was analysed in the presence of increasing concentrations of Ampholine, all of the peaks and the minima in its spectrum were shifted towards the red end of the spectrum (bathochromic shift) by 7–10 nm (Fig.3A) and, in addition, a shoulder appeared at 562 nm. The absorption of the 503-nm peak was also extensively reduced; however, part of this reduction might have been due to light scattering by the finely dispersed precipitate formed (only in the case of this dye and at a high Ampholine concentration) by the Ampholine–dye complex. An even more dramatic red shift is apparent in the spectrum of Direct Blue 25, where the main 585-nm peak is shifted to 612 nm and a new chromophore appears at 672 nm (Fig. 3B). As more Ampholine is added to the system, the absorbance of the 612-nm peak decreases in favour of the 672-nm chromophore.

Partial disaggregation in urea

When increasing concentrations of Biebrich Scarlet were run in a control and in an 8 *M* urea gel, the apparent dye microheterogeneity was completely eliminated in the presence of the dissociating agent (Fig. 4). Moreover, the dye ran through to a much lower apparent p*I* value (2.84) compared with the p*I* spectrum in the pH range 3.57–4.11 for the control. This experiment was also followed kinetically. After an exponential rate for the first 15 min, the single dye band in the urea gel travelled at a constant linear rate of 0.66 mm/min up to 60 min. It took 3 h for the dye to reach an apparent p*I* value of 2.84. Conversely, the five major bands of Biebrich Scarlet in the control gel reached their apparent p*I* values (pH range 3.57–4.11) in 30–50 min. Their relative positions remained essentially unaltered for the duration of the experiment (Fig. 5). It is clear that the much slower rate in the absence of urea is due to interaction with Ampholine; were this is not the case, the sample in 8 *M* urea should travel at a slower rate, owing to the higher viscosity of this medium.

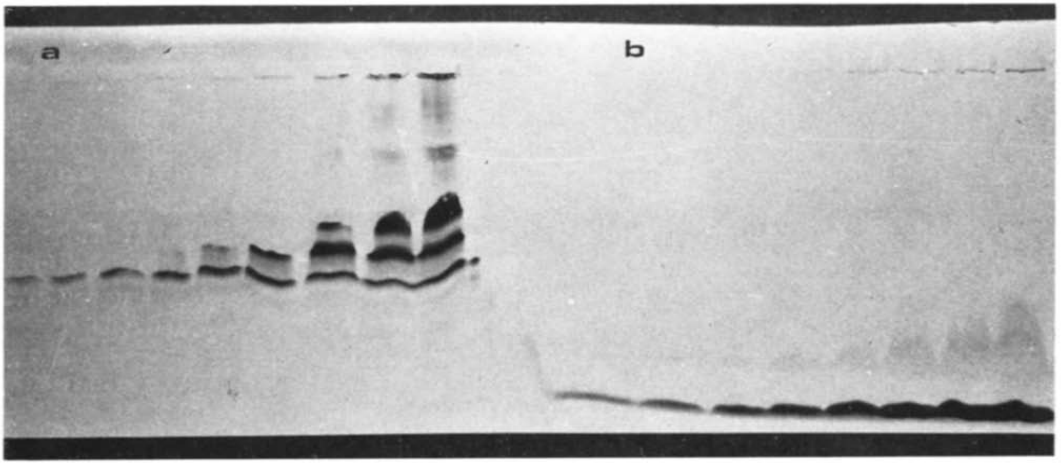


Fig. 4. IEF of Biebrich Scarlet (a) in a control gel and (b) 8 *M* urea gel. Sample loads in the two gels as in Fig. 1. The cathode is uppermost. The two black lines are the electrode filter-paper strips.

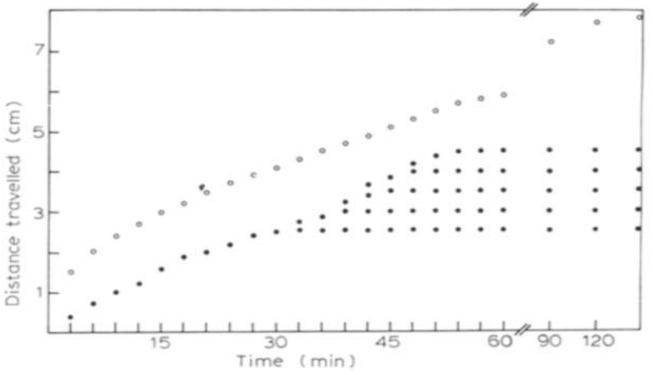


Fig. 5. IEF followed kinetically. The migration rates of the five major bands (closed circles) of Biebrich Scarlet in the control gel and of the single band (open circles) in the 8 *M* urea gel were measured. The migration distance was taken from the edge of the application pocket at the cathodic side.

The experiments were then repeated with all four dyes. The run was performed for a much longer time (6½ h) under a high voltage gradient (100 V/cm) so as to ensure ample time for equilibrium conditions to be attained. The apparent p*I* values reached were 2.84 (scarlet), 3.25 (orange), 3.53 (blue) and 3.99 (green) (Fig. 6). It is clear that, even under these drastic conditions, no complete disaggregation of the Ampholine–dye complex was achieved in 8 *M* urea.

Complete disaggregation in DMF, TMU and DMSO

When the experiments were run in DMF, TMU or DMSO, the dye–Ampholine complexes seemed to be effectively broken up. In fact, in DMF and TMU all of the dyes ran through the entire length of the gel and escaped in the anodic compartment, being slowly absorbed in the anodic filter-paper strip. With all three reagents, all of

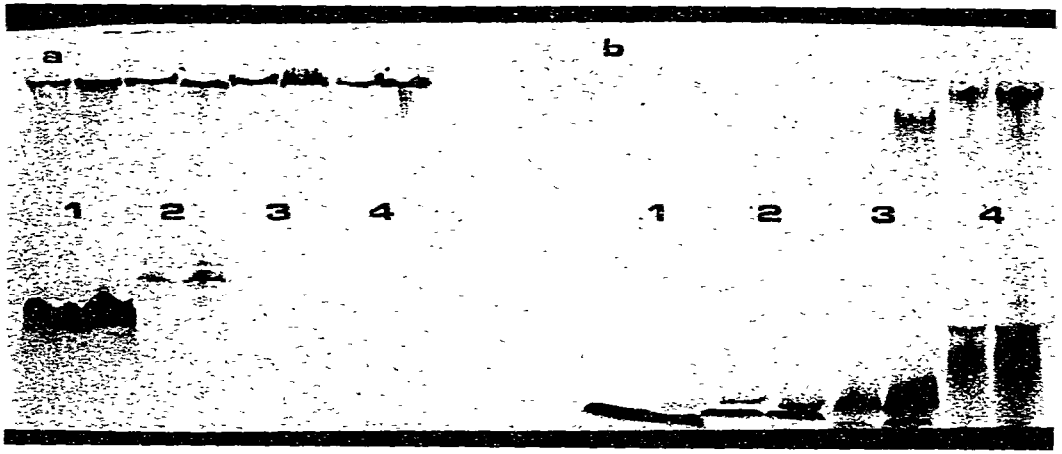


Fig. 6. IEF of dyes (a) in a control gel and (b) 8 *M* urea gel. Each sample was applied at two concentrations (62.5 and 125 μg). Samples: 1 = Biebrich Scarlet; 2 = Sirius Supra Orange 7GL; 3 = Benzo New Blue 5BS; 4 = Benzo Dark Green B.

the dyes reached an apparent p*I* value of *ca.* 2. Judging from the time needed for these conditions to be achieved in IEF and from the apparent p*I* values obtained, the order of the disaggregating power of these four reagents appears to be 50% DMF $\approx$ 50% (TMU $>$ 50% DMSO $>$ 8 *M* urea.

Melting curves

Another means of affecting the Ampholine–dye complexes, apart from altering the dielectric constant of the solvent, should be by changing the solvent temperature. Melting curves were therefore run in the presence of increasing concentrations of each of the four additives used, and the results are summarized in Fig. 7. In the case of Direct Blue 25, the complex has a melting point (T_m) of 87° in the absence of disso-

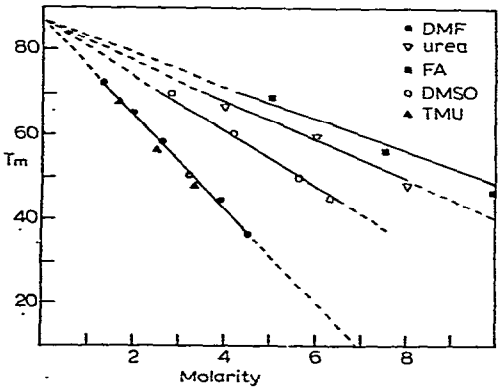


Fig. 7. Hyperchromic thermal transitions. Melting curves were measured with a Gilford Model 2400 automatic spectrophotometer with a programmed temperature increase of 1.0°/min at 585 nm. Benzo New Blue 5BS (30 μg/ml) was dissolved in 50 m*M* acetate buffer (pH 3.85) containing increasing molarities of each of the five dissociating agents tested. The melting point (T_m) of the Ampholine–dye complex, extrapolated to zero additive concentration, is 87°.

ciating agents. The relative slope of each of the curves obtained indicates the dissociating power of the additive concerned: the steeper the curve, the more powerful the dissociating agent. Hence it is clear that in 50% DMF (≡ 6.5 *M*) or 50% TMU (≡ 4.2 *M*) the complex is completely disaggregated at room temperature, while it is not completely inhibited in 50% DMSO (≡ 7.0 *M*). The situation is even worse in the presence of 8 *M* urea. These experiments are in excellent agreement with the empirical order of dissociating power for these four additives as obtained by IEF.

DISCUSSION

The results show that most acidic dyes form strong complexes with Ampholine, which are not dissociated during the process of focusing itself, by high voltages (100 V/cm), relatively high temperatures (15°) (it is customary to run focusing experiments at 2–4°) or prolonged focusing times (up to 8 h). These complexes can be disaggregated only by high temperatures (87° for Direct Blue 25), by drastically changing the dielectric constant of the solvent, or by a combination of the two. With dissociating agents, we have demonstrated that the most effective are DMF and TMU and the least effective are urea and formamide (on an equimolar basis). In IEF experiments we chose the limiting concentrations at which gel focusing can conveniently be carried out (50% TMU is in fact already too much). Higher levels of DMF, TMU and DMSO will either severely hamper gel polymerization or precipitate Ampholine, or do both. Urea gels could also be run in the presence of 10 or even 12 *M* urea[12], but this procedure would require much higher temperatures (55°) than are permissible for gel focusing in slabs.

Our results could be interpreted on the basis of a sequential binding model: first a polydentate salt bridge (with strong $R–NH_3^+$ $^-O_3S–R'$ and $R–NH_3^+$ $^-OOC–R'$ interactions), followed by hydrophobic dye–dye molecule interaction. Once the polydentate (and undissociated) Ampholine–dye complex is formed, the highly negative charge of the dye is decreased to such an extent that hydrophobic dye–dye interactions are made possible. This in turn could lead to an infinite stack of alternating aromatic rings among dye molecules, as demonstrated, for instance, in the case of riboflavin–adenosine molecules in aqueous solutions[13], reaching such a high molecular weight that the aggregate precipitates (all of the dyes tested are precipitated in the presence of Ampholine at 2–4°). It is symptomatic that these complexes are sensitive to both types of reagents, such as DMF and TMU, which mostly break hydrophobic interactions, and high temperatures, which mostly affect hydrogen bonds.

There have been hints in the literature about Ampholine–dye interaction[14]. Vesterberg[15] and Söderholm *et al.*[16] arrived empirically at a formulation of dissociating conditions by suggesting the staining of gels directly in the presence of Ampholine with Coomassie Brilliant Blue R 250 at high temperatures (60–65°) and at high alcohol concentrations (28% methanol).

Our results could have considerable biological implications. In fact, while Dean and Messer[17] and Baumann and Chrambach[18] excluded any protein–Ampholine interactions, the opposite results have been reported by other workers. Thus Frater[19] reported the binding of Ampholine to wool proteins and Kaplan and Foster[20] and Wallevik[21] complex formation between Ampholine and bovine serum albumin (BSA). These apparent discrepancies can be reconciled as follows: in general, in the pH

range 4–9 there is no Ampholine–protein interaction, but with strongly acidic (and possibily strongly basic) proteins or with unusual structures (BSA in known to bind to a multitude of ligands) interaction with Ampholine is strongly suspected. Another classical example is the focusing of tRNAs. Originally reported by Drysdale and Righetti[22], this was later demonstrated to be an artefact elicited by strong tRNA–Ampholine binding[9]. The two cases have strong common relationships: both tRNAs and the dyes used in this work are strong acids; the microheterogeneity is extensively reduced in both instances by 8 *M* urea; both classes of compounds exhibit apparent p*I*s in the pH range 3.5–4.5.

There are also other puzzling effects that could be interpreted in the light of our results, such as the focusing of soil humic substances[23], which has been challenged by Thornton[24]. Another interesting case is the focusing of heparin[25]. Given the known composition of heparin (a sulphated polysaccharide containing three SO_3^- groups per disaccharide unit), the finding that it is resolved by IEF into about 21 components with p*I* values in the pH range 3.2–4.2 seems to be as good as the finding that Biebrich Scarlet and Benzo New Blue 5BS (which are not even amphoteric) have p*I* values in the same pH range.

In conclusion, whenever complex formation between Ampholine and a substance under investigation is suspected, the experiment should be run at least in 8 *M* urea or, if possible, in 50% DMF and/or at high temperatures, *i.e.*, under conditions that ensure complete dissociation of the complex. We feel that we can now explain some of the puzzling effects that occur in isoelectric focusing.

ACKNOWLEDGEMENTS

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