CHROM. 20 582

HYDROPHOBIC INTERACTION OF ALCIAN BLUE WITH SOLUBLE AND ERYTHROCYTE MEMBRANE PROTEINS

GIAN MARCO GHIGGERI and GIOVANNI CANDIANO

Nephrology Section, Hospital of Lavagna, Lavagna (Italy)

FABRIZIO GINEVRI

Nephrology Department, G. Gaslini Institute, Via 5 Maggio 39, Genoa (Italy)

ANTONIO MUTTI, ENRICO BERGAMASCHI and ROSSELLA ALINOVI

Institute of Clinical Medicine and Nephrology, University of Parma, Via Gramsci, Parma (Italy)

and

PIER GIORGIO RIGHETTI*

Department of Biomedical Sciences and Technologies, University of Milano, Via Celoria 2, Milan 20133 (Italy)

SUMMARY

Alcian Blue (AB), a cationic dye widely employed for monitoring negative surface charge variations on red blood cell (RBC), platelet and glomerular membranes of patients with nephrotic syndromes, was found in fact to aggregate with itself and precipitate in the pH range 7.0–7.8, *i.e.*, at the physiological pH values used for performing the binding assay between the dye and cell surfaces. This aggregation appears to be essentially hydrophobic as it is insensitive to urea but fully prevented in presence of 2% zwitterionic detergent. In addition, AB binds to most RBC membrane proteins solubilized by urea–detergent extraction, again suggesting hydrophobic interaction. AB also interacts with freely soluble proteins such as haemoglobin and myoglobin; such binding is disrupted by ethylurea and/or 2% zwitterionic detergent, typical inhibitors of hydrophobic liaisons. AB also strongly binds to myoglobin with all the negative charges blocked by esterification of the carboxyl groups, again ruling out direct interaction via surface negative charges. It is concluded that AB binding to the RBC surface can hardly monitor variations in surface charge due to sialic acid residues but, at best, variations in surface hydrophobicity.

INTRODUCTION

Alcian Blue (AB), a cationic dye possessing a bulky aromatic region (four benzene and four imidazole rings, in a haeme-like structure coordinating a central copper ion) and four positive charges due to quaternary amino groups, has recently come to play an important role in monitoring the negative surface charge changes of red blood cells (RBCs), platelets and glomerular walls in normal individuals as

compared with patients with nephrotic syndrome and focal glomerulosclerosis. Today, it is an accepted physiological mechanism that the fixed negative charges (resulting from the presence of sialic acid residues and anionic glycosaminoglycans) on the walls of the nephron provide an electrostatic barrier to the filtration of a host of negatively charged (at physiological pH values) macromolecules present in plasma (as epitomized by albumin)[1]. Hence, in glomerular diseases, the enhanced excretion of albumin has been associated with the loss of such negative charges on the glomerular capillary walls, strongly diminishing such electrostatic repulsion[2]. Therefore, the monitoring of the charge density and variation on the capillary walls could be an important tool for assessing the glomerulus function in nephrotic syndromes. The major impediment to this goal, however, comes from the difficulty in obtaining enough renal tissue for analysis.

In a search for a substitute, simple chemical test, Levin *et al.*[3] noticed that AB binding to RBCs and platelets could be used as a valid alternative to direct monitoring of negative charges on the glomerulus capillary walls, as AB binding was significantly reduced in children with steroid-responsive nephrotic syndromes (SRNS). Their findings were subsequently confirmed by them[4] and in an independent study by Boulton-Jones *et al.*[5]. On the other hand, this method has been subjected to major criticism by Feehally *et al.*[6] and Sewell and Brenchley[7]. The first group, by repeating the same AB binding test to RBCs, was unable to reproduce the differences among control, neprotic syndromes, membranous nephropathy and uraemic (non-nephrotic) patients, all of the tests yielding an average 85 ng of AB bound per 10^6 RBCs. They concluded that no evidence could be found that RBC charge measurements should reliably mirror the glomerular capillary wall charge. Sewell and Brenchley[7] reported some direct chemical tests demonstrating that AB alone, in solution in phosphate-buffered saline (PBS) at physiological pH, tends to give precipitates in the first few hours after dissolution. In addition, the presence of albumin at 1 mg/ml causes an augmented, faster precipitation. Moreover, they noted that AB (originally a textile dye) was designed to be precipitable at neutral and slightly alkaline pH values, so that textiles dipped in AB solutions would retain a permanently insoluble precipitate within their fibres. Hence they too ruled out any correlation between AB binding to RBCs and surface charge.

In this work, we have undertaken an extensive physico-chemical evaluation of the behaviour of AB alone in different solvents and in the presence of membranaceous and cytoplasmic proteins. One of the major techniques used was "electrophoretic titration curves"[8,9], which simultaneously monitors the behaviour of AB and proteins, either alone or in a mixture, over the pH range 3–10. Our results indicate an extensive hydrophobic interaction, with little evidence for any ionic binding.

EXPERIMENTAL

Chemicals

Myoglobin (sperm whale) was obtained from Calbiochem (La Jolla, CA, U.S.A.), ribonuclease (bovine pancreas) and lysozyme (egg white) were obtained from Sigma (St. Louis, MO, U.S.A.), sphingomyelin and Coomassie Brilliant Blue R-250 from Serva (Heidelberg, F.R.G.), Alcian Blue BGX from BDH (Poole, U.K.), N,N,N′,N′-tetramethylethylenediamine (TEMED), N,N′-methylenebisacrylamide

(Bis), ammonium persulphate and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) from Bio-Rad Labs. (Richmond, CA, U.S.A.), carrier ampholytes and Silane A-174 (Bind Silane) from LKB (Bromma, Sweden), urea, ethylurea and Triton X-100 from Fluka (Buchs, Switzerland) and silica TLC plates and other chemicals of analytical-reagent grade from Merck (Darmstadt, F.R.G.).

Haemoglobin preparation

Normal human adult haemoglobin was prepared according to the International Committee for Standardization in Haematology[10]. Thus, one volume of blood collected in heparin or EDTA was washed three times with saline (8.5 g/l of sodium chloride), then the red cells were lysed with 0.5 volume of carbontetrachloride and 1 volume of distilled water by shaking for 10 min. Red cell debris was subsequently removed by centrifugation at 4°C for 30 min at 2500 g. To the haemolysate 100 μg/ml of potassium chloride were added.

Ghost's preparation

Human erythrocyte membranes were prepared from fresh blood according to Dodge et al.[11]. To obtain the electrophoretic titration curves for protein components, they were solubilized in 8 M urea and 2% Triton X-100.

Carboxyl group modification

The carboxyl groups of myoglobin were esterified by suspending the protein in methanol and adding hydrochloric acid to a final concentration of 0.1 M for 24 h at 25°C. The reaction was stopped by dilution with a large volume of ice-cold water and the excess of acid and methanol were removed by dialysis against 1 mM hydrochloric acid[12].

Electrophoretic titration curves

Electrophoretic titration curves[8,9] were obtained in polyacrylamide gels (0.75 mm thick) supported by silanized glass plates as described by Bianchi Bosisio et al.[13]. The polyacrylamide gel slab (12 × 12 cm) was cast to contain 7% T, 3% C matrix and 2% Ampholines (in the following percentage ratios: 45% pH 3–10, 15% pH 4–6, 15% pH 8–9.5 and 25% pH 9–11). In some instances 8 M urea, 3% Triton X-100 or 2% CHAPS were included in the polymerization mixture; in that event the samples were also equilibrated with the same additives. The first dimension (isoelectric focusing of carrier ampholytes) was run at a constant 10 W until the steady state (800 V, 11 mA) was reached (usually 1 h). The second dimension (electrophoresis perpendicular to the pH gradient) was then run at a constant 700 V for 20 min. In both dimensions the electrode strips were impregnated with 0.2 M sodium hydroxide (cathode) and 0.2 M orthophosphoric acid (anode). pH measurements were made by cutting gel strips (4 mm) and placing them in 0.1 M potassium chloride solution at room temperature. When necessary, the gels were stained in a colloidal dispersion of Coomassie Blue in 12% trichloroacetic acid.

Electrophoretic titration curves in rehydratable gels

Some experiments were performed with ethyl- or butylurea as disaggregating agents. In these instances, owing to the polymerization inhibition of the additive, the
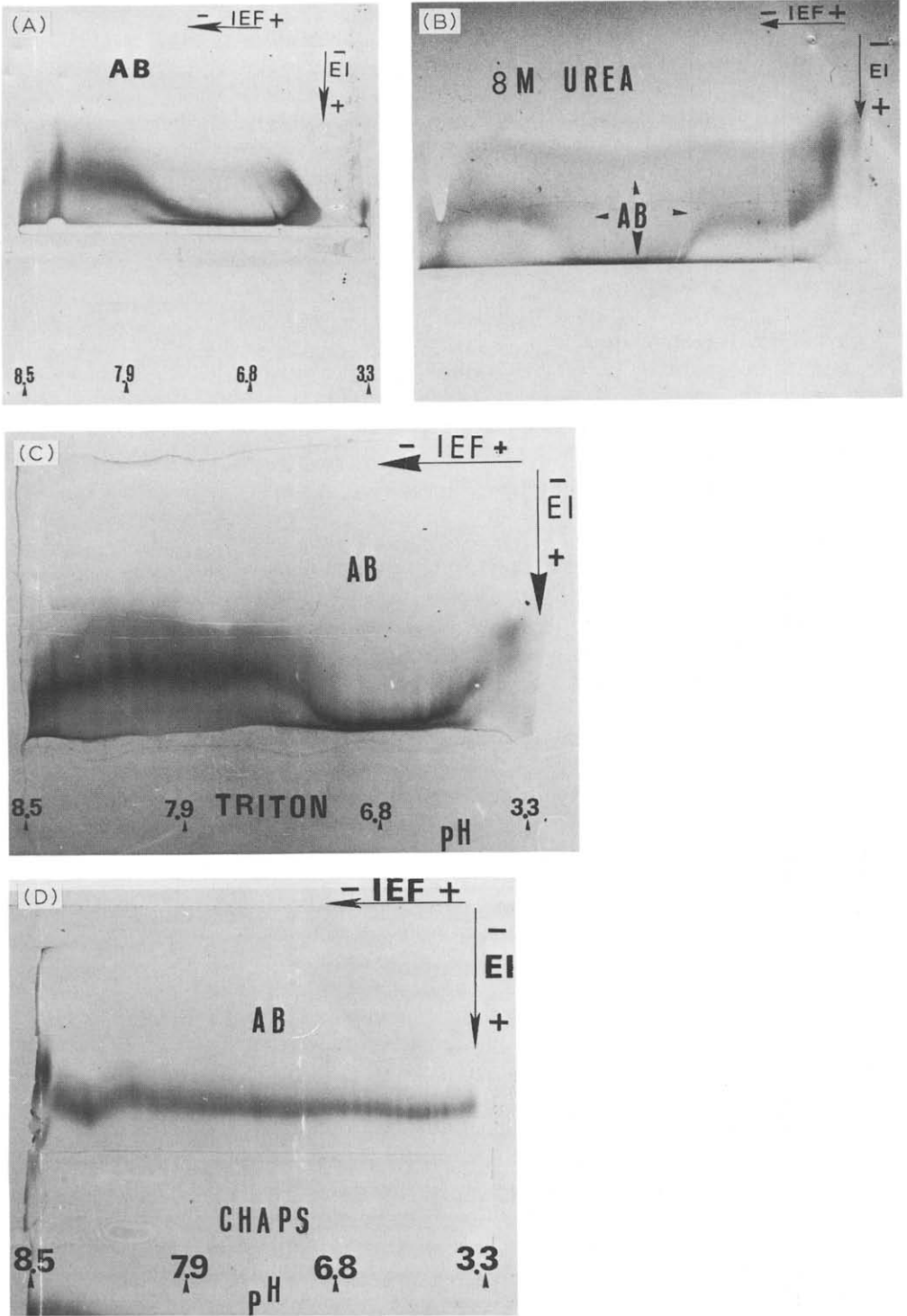
Fig. 1. Electrophoretic titration curves of AB. The gel was a 7%T, 3%C polyacrylamide containing 2% Ampholine in the pH range 3.5–10 (dimensions 12 × 12 cm, 0.75 mm thick). First dimension: focusing of carrier ampholytes alone (1 h at 800 V at the steady state). Second dimension: zone electrophoresis perpendicular to the pH gradient (constant 700 V for 20 min). (A) Titration curve of AB alone (1 mg/ml solution in 25 mM magnesium chloride; 200 μl loaded into the trench just before the second dimension run). (B) Same as A, except that the gel and sample contained 8 M urea. (C) Same as A, except that the gel and sample were equilibrated in 3% Triton X-100. (D) Same as A, except that the gel and sample contained 2% zwitterionic detergent (CHAPS).

gels were polymerized as above, but in the absence of carrier ampholytes, washed twice in distilled water and then dried overnight. Rehydration was performed by soaking the gels in a solution containing 6 M ethylurea (or 1 M butylurea) in 2% Ampholine for 48 h at room temperature.

Thin-layer chromatography (TLC)

TLC was performed on silica plates by using chloroform–methanol–water (75:22:3) as the mobile phase. Before TLC, sphingomyelin was preincubated with AB for 1 h, extracted four times with chloroform and dried under nitrogen.

Incubations

Unless specified otherwise, AB was incubated with proteins in the presence of 1 mM magnesium chloride at 37°C for 15 min. In all instances the additives (detergents, ureas) were admixed with AB before proteins and preincubated for 5 min.

RESULTS

Electrophoretic titration curves of AB alone

We first investigated the behaviour of AB alone or in solution with different disaggregating agents with the two-dimensional technique of "electrophoretic titration curves". Given the presence in the molecule of only quaternary and tertiary amino groups, the electrophoretic pattern of AB over the pH range 3–8 should be represented by a straight line parallel to the sample application trench in the cathodic direction, indicating constant surface charge along this titration interval. On the contrary (Fig. 1A), when run alone, AB produces a sigmoidal curve, with a flat portion (in reality a heavy precipitate against the edge of the trench) in the pH range 6.0–7.5. As AB was analysed alone, it can only be concluded that in the pH range 6–7.5 it aggregates with itself and precipitates out of solution. When the same experiment is repeated with gel and sample equilibrated in 8 M urea (Fig. 1B) a better behaviour is obtained, with two coloured lines moving out of the trench (the fastest migrating component probably representing a contaminant of AB), but there is still a heavy precipitate in the same pH range 6.0–7.5 and, in fact, additional precipitation throughout the pH range 3.0–8.5. This suggests that the AB precipitate is sparingly, if at all, hydrogen bonded. When the same experiment is repeated in the presence of non-ionic detergent (3% Triton X-100 in both sample and gel) (Fig. 1C) the migration behaviour is clearly improved, but still the precipitate around neutrality is not abolished. However, when the neutral is substituted by a zwitterionic (CHAPS) detergent (known to have a much stronger disaggregating power)[14], AB now migrates as a straight line parallel to the trench, all precipitates being completely abolished (Fig. 1D). This behaviour is a clear symptom of hydrophobic interaction.

Electrophoretic titration curves of AB and proteins

We next investigated the behaviour of AB in the presence of membrane and of soluble proteins. Erythrocyte ghost proteins, solubilized according to Dodge et al.[11], were run alone by the titration curve method. As the proteins solubilized are mostly loosely bound surface antigens, with a high density of negative charges, they migrate as lines parallel to the trench, but in the anodic direction (Fig. 2A). When, however, the
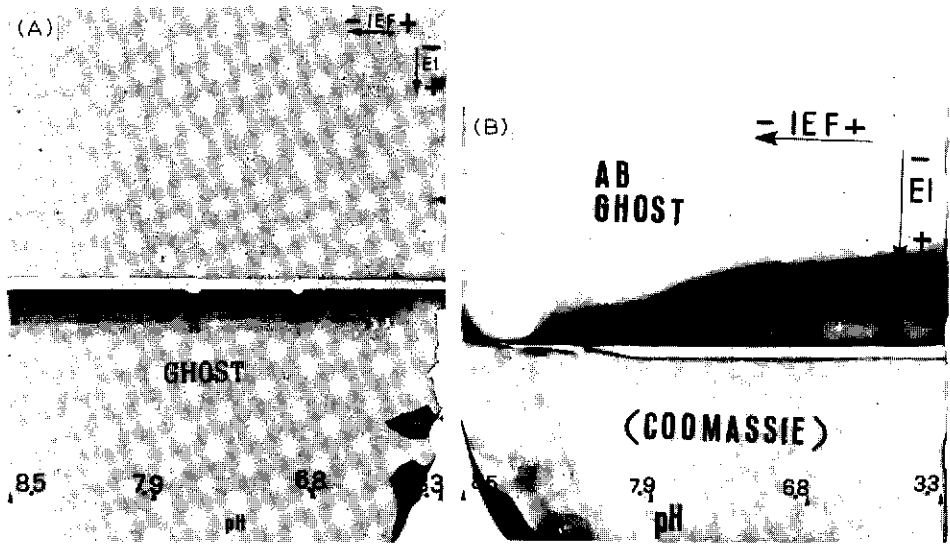
Fig. 2. Electrophoretic titration curves of erythrocyte ghost proteins in the absence and presence of AB. All other conditions as in Fig. 1. (A) 200 μl of solubilized ghost proteins (2 mg/ml) were applied to the trench. (B) Titration curves of a mixture of ghost proteins (2 mg/ml) and AB (1 mg/ml) mixed in a 1:1 ratio (200 μl loaded into the trench). Both gels stained with Coomassie Blue. Note that an identical pattern would be obtained in the absence of staining, indicating that all proteins are bound to AB.

same preparation is incubated with AB, this behaviour is completely reversed; all proteins seem to be coated by AB, and they all now migrate towards the catode, producing a heavy precipitate in the pH range 8–8.5, with additional precipitates throughout the titration interval (Fig. 2B). An identical pattern was obtained in the same gel prior to Coomassie staining (*i.e.*, by visualization of the AB colour), indicating that all proteins in the mixture were bound to AB throughout the pH range 3–9.

We next investigated the behaviour of AB with soluble, hydrohilic proteins, as epithomized by myoglobin (Myo) and haemoglobin (Hb). Fig.3 shows a typical titration curve for Myo alone in the pH range 3–9; the main component has a pI of 7.8 (trench cross-over point). When Myo is incubated with AB, massive precipitation occurs in the pH range 3.5–8, with only partial disaggregation above pH 8, where a blue cathodic line (free AB) and a brown anodic zone (free Myo) can be seen (in this pH range Myo begins to have a net negative charge yet, curiously, it does not bind to the positively charged AB; conversely, in the pH range 3–8, where massive coprecipitation occurs, both AB and Myo bear a net positive charge!) (Fig. 3B). Fig. 3C shows similar results with Hb, *i.e.*, strong precipitation in the pH range 3–8 and a smeared curtain of molecules in both the anodic and cathodic gel regions, due either to excess free molecules not engaged in the precipitation event or to a slowly solubilized precipitate under the electric field. Not much better results are obtained with the ternary mixture AB + Myo + Hb: the heavy Myo–Hb coprecipitate is still present in the pH range 3.5–8, with Hb tending to escape the precipitation unscathed, probably owing to a lack of free, excess AB (Fig. 3D). The situation can only be reversed in the presence of
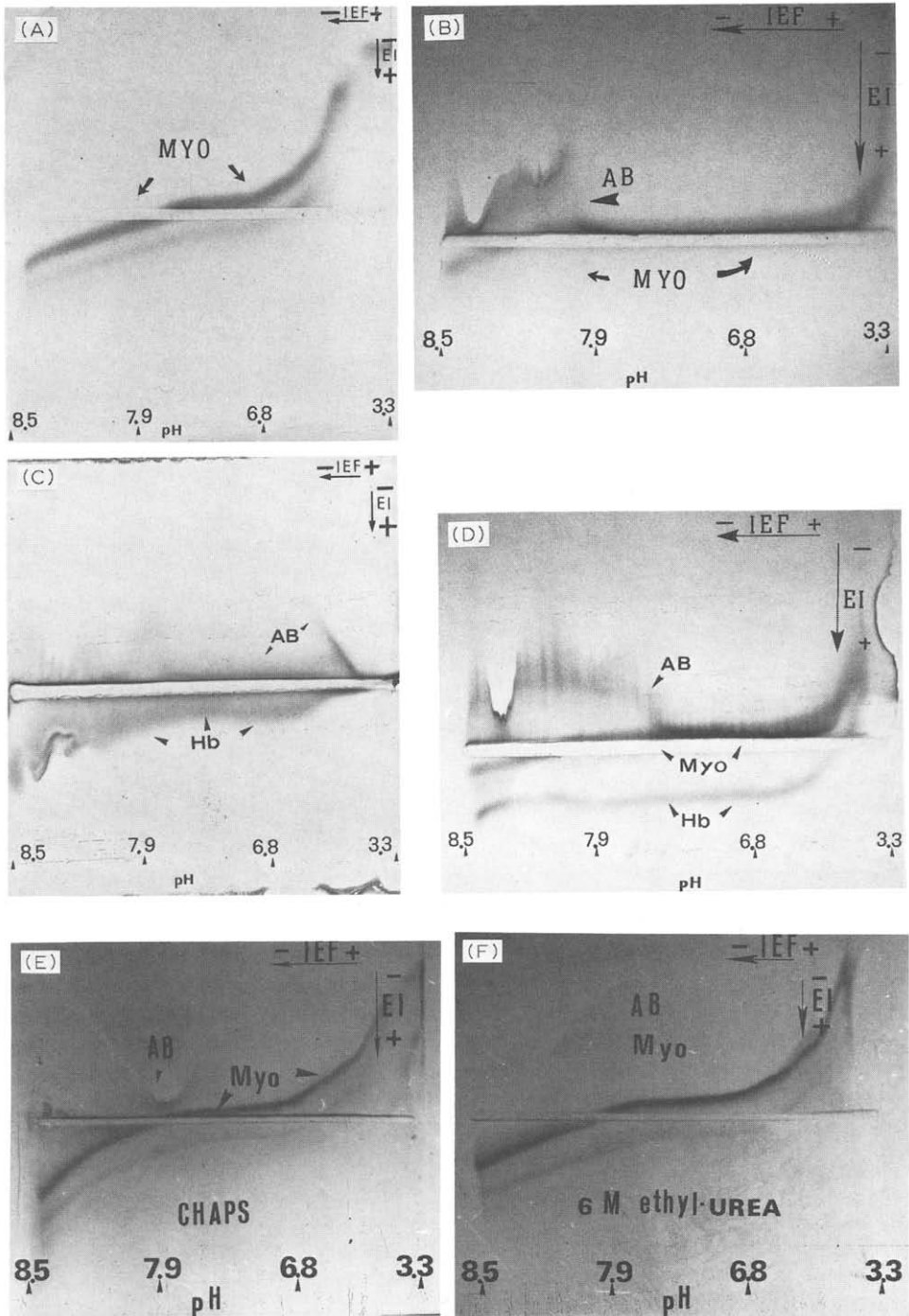
Fig. 3. Electrophoretic titration curves of myoglobin (Myo) and haemoglobin (Hb) in the absence and presence of AB and various additives. All other experimental conditions as in Fig. 1. (A) Titration curve of myoglobin alone (2 mg/ml; 200 μl loaded); unstained gel. (B) Titration curves of a mixture of Myo (2 mg/ml) and AB (1 mg/ml) mixed in a 1:1 volume ratio (200 μl applied); unstained gel. (C) Same as B, except that Hb was substituted for Myo. (D) Same as B and C, except that a 1:1 mixture of Myo and Hb was added to AB. (E) Same as B, except that the sample and gel were equilibrated with 2% CHAPS. Note the completely undisturbed titration curve of Myo, demonstrating complete lack of binding to AB. (F) Same as B, except that the sample and gel contained 6 M ethylurea. Note the complete disaggregation of the AB–Myo complexes.
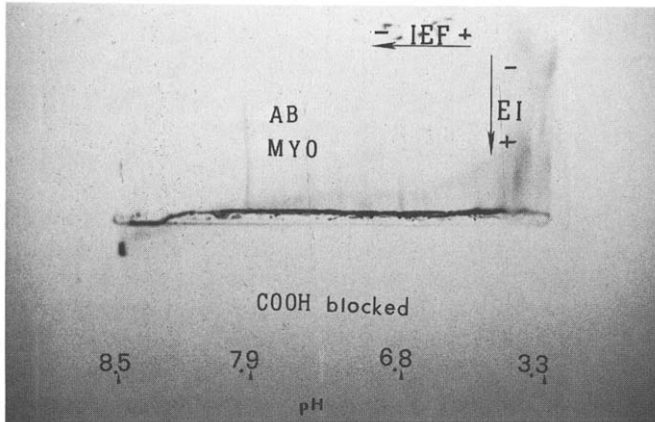
Fig. 4. Electrophoretic titration curve of a mixture of AB and myoglobin. All other conditions as in Fig.1. A 1:1 mixture of Myo (2 mg/ml) and AB (1 mg/ml) was analysed by isoelectric focusing coupled to electrophoresis at right-angles. Myo had been previously esterified on the carboxyl groups. Note the almost complete precipitation of the AB–Myo complex, suggesting strong hydrophobic binding and stacking of the dye on the polypeptide chain.

CHAPS (Fig. 3E); now Myo gives the expected titration curve, completely undisturbed by AB (compare with Fig. 3A) while AB is probably involved in a mixed micelle with CHAPS. This again indicates hydrophobic interaction among AB and proteins. Additional evidence comes from Fig. 3F; in the presence of 6 M ethylurea (another powerful disaggregating agent)[15], Myo reverts to the expected electrophoretic behaviour, completely unperturbed by the presence of AB.

As an additional check, we studied the behaviour of AB in the presence of carboxyl-esterified myoglobin. If it were true that AB interacts with negative surface charges in proteins, no binding should occur in this instance, as the protein now
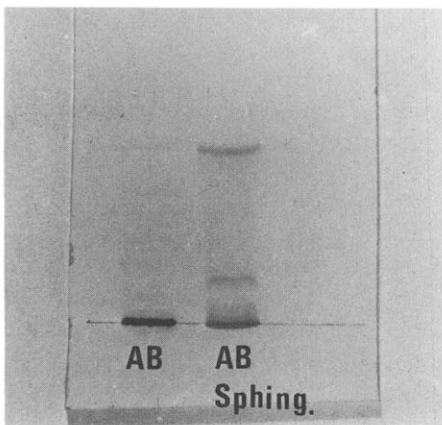

Fig. 5. TLC of AB and sphingomyelin. The chromatogram was developed with chloroform–methanol–water (75:22:3). AB, 10 μg spotted; AB–Sphing., 1 1:1 mixture of 10 μg each of AB and sphingomyelin. The TLC plate was not stained.

exhibits a purely positive charge. Here again, however, a mixture of AB and carboxyl blocked Myo produces a massive precipitate, even more intense than with untreated Myo (compare with Fig. 3B), extending over the entire titration interval (Fig. 4). As a final check, we studied the TLC migration of AB when admixed with a hydrophobic membrane component, sphingomyelin. In the elution system used, AB does not move from the application point, whereas in the presence of spingomyelin it co-migrates with all the components present in it (see Fig. 5; the bands developed are not stained and they exhibit the blue colour of AB). Given the structure of sphingomyelin, it is clear that the complex of AB with it can only occur via hydrophobic interaction.

DISCUSSION

Our data cast doubt on the validity of using AB as a probe of surface charge of RBC or glomerular capillary walls, as proposed[3–5]. Let us examine the available data.

Behaviour of AB alone in solution

As seen in all our titration curves (Fig. 1A–D), AB alone in solution tends to aggregate and precipitate at least over a 1.5 pH unit range (in general pH 6.0–7.5, in fact up to pH 8). This aggregation appears to be caused by hydrophobic interaction, as it is insensitive to 8 M urea but completely inhibited by a zwitterionic detergent (CHAPS). The fact that the the dye would precipitate was also noted by Sewell and Brenchley[7], who reported 50% precipitation simply on standing for only 2.5 h after dissolution in phoshate-buffered saline at pH 7.4. What is even more striking is that in our titration curves the ionic strength of focused carrier ampholytes is extremely low (possibly *ca.* 1 mequiv.l^{-1}) and this should, if anything, minimize hydrophobic interaction. Conversely, under the assay conditions of Levin and co-workers[3,4], the high ionic strength of the medium should amplify the phenomenon of AB precipitation, as hydrophobic bonds are favoured by high salt concentration.

Behaviour of AB in the presence of proteins

Although our data for AB in the presence of ghost components would appear to favour Levin and co-workers[3,4] hypothesis (binding to negative charges of surface proteins) (Fig. 2A and B), in reality the situation is different: the AB–ghost protein complexes completely precipitate in the pH range 8.0–8.5 and in fact give substantial precipitation throughout the titration interval (such precipitates intensify around pH 7). This is in agreement with Sewell and Brenchley[7], who reported increased precipitation of AB in the presence of 1 mg/ml of albumin (the latter is known to bind to many hydrophobic molecules). What is even more striking is the behaviour of soluble proteins, such as Myo and Hb. The interaction with Myo goes against any expectations: if it were true that AB binds via ionic bonds, the binding should be substantial above the pI of Myo (*i.e.*, above pH 7.8) and should not occur at all below the pI, where the protein acquires an excess of positive charges. However, experimentally, the opposite is found: massive precipitation and complexing between AB and Myo occur throughout the cationic segment of the Myo titration curve, where both AB and Myo carry a positive charge and therefore, in principle, should repel each other. The same (and even more pronounced) happens with carboxyl-blocked Myo (Fig. 4): here everything is extensively precipitated in the pocket. In this last instance

Myo should have essentially no negative charges, yet the interaction is more tenacious. However, owing to the esterification of the carboxyl groups, it is certain that the overall hydrophobicity of the surface of the macromolecule is increased, and the stronger binding of AB again suggests hydrophobic interaction.

Behaviour of the complexes in the presence of detergents and alkylureas

The fact that AB alone, when run in an electrophoretic titration curve, does not aggregate or precipitate in the presence of CHAPS is indicative of hydrophobic interaction on the bulky aromatic dye surface possibly with the formation of a stack of molecules that eventually precipitate. Masking of such surfaces by incorporation in the detergent micelles abolishes this phenomenon. With protein–AB complexes, not only CHAPS but also alkylureas (ethylurea) are able to disrupt such interactions. Interestingly, asymmetrically substituted alkylureas can be regarded as simple detergents, unable to aggregate into micelles. Recently, they were utilized to inhibit hydrophobic interaction between a large protein, ferritin, and the surface of a gel containing an immobilized pH gradient[16].

Behaviour of other dyes

In addition to the above data, additional evidence comes from the work of Sewell and Brenchley[7] on the behaviour of other positively charged dyes. They noted that many cationic dyes, such as Toluidine Blue, Methyl Green, Cupreolinic Blue, Maxilon Blue TL and Ruthenium Red, do not behave like AB with respect to RBC binding. In the light of our results this is not surprising: Toluidine Blue (or the very similar Basic Blue) is composed of only three condensed aromatic rings, flanked on the outer surface by a quaternary and a primary (Toluidine Blue) or a quaternary and a tertiary (Basic Blue) amino groups. The much reduced hydrophobic area is probably sufficient to minimize or abolish completely any hydrophobic interaction with proteins.

In conclusion, we believe that AB cannot be used as a reliable indicator of negative surface charge of cells or glomerular walls, but that perhaps it could be used to probe hydrophobic patches on such surfaces. In fact, we have recently demonstrated[17] that the RBC membrane of nephrotic children contains much reduced levels of phospholipids. Hence, the apparent differences in AB binding reported by Levin and co-workers[3,4] might well be attributed to variations in the hydrophobic domains of the membrane, rather than to surface charge alterations (they clearly stated that, notwithstanding the large differences in AB binding to the RBC membranes of normal and nephrotic children, the total amount of sialic acid residues on the cell surface was the same in the two populations).

REFERENCES

1 W. M. Deen, M. P. Bohrer, C. R. Robertson and B. M. Brenner, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 36 (1977) 2614–2618.

2 J. I. Kreisberg, D. B. Wayne and M. J. Tarnovsky, *Kidney Int.*, 16 (1979) 290–300.
3 M. Levin, C. Smith, M. D. S. Walter, P. Gascoine and T. M. Barratt, *Lancet*, ii (1985) 239–242.
4 M. Levin, D. Gibb, C. Smith, M. D. S. Walters and T. M. Barratt, *Lancet*, ii (1986) 929–929.
5 J. M. Boulton-Jones, G. McWilliams and L. Chandrachud, *Lancet*, ii (1986) 186–188.
6 J. Feehally, A. Samanta, H. Kinghorn, A. C. Burden and J. Walls, *Lancet*, ii (1986) 635–635.
7 R. F. Sewell and P. E. C. Brenchley, *Lancet*, ii (1986) 635–636.
8 P. G. Righetti, R. Krishnamoorthy, E. Gianazza and D. Labie, *J. Chromatogr.*, 166 (1978) 455–460.
9 P. G. Righetti, G. Gacon, E. Gianazza, D. Lostanlen and J. C. Kaplan, *Biochem. Biophys. Res. Commun.*, 85 (1978) 1575–1581.
10 W. F. Moo-Pen and R. M. Schmidt, *Br. J. Haematol.*, 35 (1977) 161–164.
11 J. T. Dodge, C. Mitchell and D. Hanahan, *Arch. Biochem. Biophys.*, 11 (1983) 119–136.
12 C. A. Broomfield, J. P. Roehem and H. A. Scheraga, *Biochemistry*, 4 (1965) 751–760.
13 A. Bianchi Bosisio, C. Loeherlein, R. S. Snyder and P. G. Righetti, *J. Chromatogr.*, 189 (1980) 317–330.
14 D. Satta, G. Schapira, P. Chafey, P. G. Righetti and J. P. Wharmann, *J. Chromatogr.*, 299 (1984) 57–72.
15 P. G. Righetti, C. Gelfi and M. L. Bossi, *J. Chromatogr.*, 392 (1987) 123–132.
16 T. Rabilloud, J.-J. Pernelle, J. P. Wahrmann, C. Gelfi and P. G. Righetti, *J. Chromatogr.*, 402 (1987) 105–113.
17 S. Ginevri, G. M. Ghiggeri, G. Candiano, R. Oleggini, R. Bertelli, M. Piccardo, S. Perfumo and R. Gusmano, *Pediatr. Nephrol.*, (1988) in press.