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PROTEIN PRECIPITATION INDUCED BY A TEXTILE DYE

PRECIPITATION OF HUMAN PLASMINOGEN IN THE PRESENCE OF PROCION RED HE3B

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SUMMARY

It was demonstrated that human purified plasminogen was precipitated in the presence of the textile dye Procion Red HE3B. The amount of precipitated plasminogen was dependent upon the molar ratio between the dye and protein and seemed to be independent of the protein concentration. A certain amount of dye was coprecipitated with the protein; this was shown also to be related to the dye-to-protein molar ratio. The precipitation of plasminogen induced by the dye was shown to have a pH optimum and to involve ionic and hydrophobic interactions. Procedures were devised which enabled the recovery of precipitated plasminogen in a soluble and non-denatured form totally free from dye. However, the precipitation of plasminogen by Procion Red HE3B could not be used as a single-step purification procedure from a heterogeneous starting material like plasma because of coprecipitation of other proteins. Nevertheless it is suggested that frequently there is a chance that a given protein will be precipitated by a given dye and therefore that protein precipitation by dyes could be a useful complementary method for the purification of proteins.

INTRODUCTION

The interaction of plasminogen with the dye Procion Red HE3B was first demonstrated by Harris and Byfield[1] who discovered that a column of this dye immobilized on agarose gel could extract plasminogen from serum. We wanted to study in more detail the interaction between the dye and protein. We observed that Procion Red HE3B could induce plasminogen precipitation. The present paper deals with a thorough study of the phenomenon and of its potential for the purification of proteins.

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Protein precipitation induced by dyes has already been described: for instance, fibrinogen can readily be precipitated with nitroblue tetrazolium[2,3]. Polylysine can be precipitated with methyl orange[4] or trypan blue[5]; this effect forms the basis of a sensitive assay of this homopolyamino acid. Precipitation by the acridine dye rivanol was described more than 30 years ago[6,7] and has since become widely used for protein fractionation, even on an industrial scale[8].

Other workers have induced protein precipitation by using chemically synthesized derivatives of dyes: for instance, Haeckel *et al.*[9], in one of the earliest studies to recognize Cibacron Blue as a pseudo affinity ligand, noted that pyruvate kinase from yeast could be precipitated by Dextran Blue. More recently, dyes were applied to the elegant principle of affinity precipitation[10]: by grafting Cibacron F3G A at both ends of a spacer, Lowe and Pearson[11] obtained a bifunctional ligand which was shown to precipitate lactate dehydrogenase.

MATERIALS AND METHODS

Most chemicals used in this study were bought from Merck (Darmstadt, R.F.G.) or Carlo Erba (Milano, Italy). The ion exchangers were from IBF (Gennevilliers, France), DEAE-Trisacryl, or from Whatman (Maidstone, U.K.), DE 23; lysine agarose and Cibacron Blue agarose were prepared according to standard methods.

Procion Red HE3B was a gift from ICI France (Clamart). Its purity was tested by thin-layer chromatography on silica gel plates in the solvent system butanol–propanol–water (20:40:40, v/v/v) derived from Small *et al.*[12]; one major spot (R_F 0.55) was seen. The dye was also subjected to analytical high-performance liquid chromatography (HPLC) on a LiChrosorb RP-18 column (25 × 0.46 cm, Merck). The conditions for this separation were essentially those given in ref. 13. After injection the column was developed at 1 ml/min with a 60-min linear gradient between the initial buffer (10 ml of acetic acid and 5 ml of triethylamine made up to 1 l with water) and the final buffer (same composition as that of the initial one except that the adjustment of the volume was made with a mixture of equal parts of water and acetonitrile). The major peak represented more than 97% of the optical density, and was eluted at 85% of the gradient. These results prompted us to use the dye as supplied by the manufacturer for most of the described study. The concentrations of dye stock solutions were assayed by appropriate absorbance measurements. Spectra (recorded on a Beckman DU8 instrument against appropriate blanks) and extinction coefficient measurements were however obtained for a sample of lyophilized dye which had been filtered on a LH 20 column (Pharmacia, Uppsala, Sweden) equilibrated with water. It had been confirmed that this chromatography would ensure an efficient desalting of the dye, a precaution suggested in ref. 13.

Human plasminogen was purified from human fresh frozen plasma according to an automatic version[14] of the standard affinity chromatography technique described by Deutsch and Mertz[15]. The purified protein comprised only the non-proteolyzed Glu form of plasminogen, as confirmed by polyacrylamide gel electrophoresis[16]. Plasminogen concentrations were determined by measuring the absorbance of solutions at 280 nm using $\varepsilon^{1\%}_{1cm} = 17$[17], and occasionally by immunodiffusion using immunoplate (Behring, Marburg, F.R.G.).

Purified plasminogen was iodinated with 1,3,4,6-tetrachloro-3,6-diphenylglycoluril (Iodogen)[18] as recommended by the manufacturer (Pierce, Rockford, IL, U.S.A.): 1.4 ml of Iodogen solution (1 mg/ml) in methylene chloride were placed in a screw-cap tube (100 × 18 mm); the solvent was evaporated in a gentle stream of nitrogen. The dry tube was then rinsed with buffer (50 m*M* Tris–chloride pH 7.00 containing 0.2 *M* sodium chloride) Plasminogen (1.4 mg in 1 ml of the same buffer) was added to the tube and thereafter 0.5 mCi of $Na^{125}I$ (CEA, Orsay, France). The tube was then left standing in an ice-bath for 1 h; the protein solution was then removed by pipette and deposited onto a column of lysine agarose. The retained plasminogen was eluted with ε-aminocaproate and finally freed from the latter by gel filtration on Sephadex G-25. The recovered iodinated plasminogen (specific activity 0.12 mCi/mg) was pooled and stored frozen in aliquots.

Quantitative study of the precipitation of plasminogen induced by Procion Red HE3B

In order to study easily the precipitation reaction, it was conducted with plasminogen solutions that were appropriately spiked with iodinated plasminogen. The reactions were conducted in small polypropylene tubes by adding Procion Red HE3B (as a concentrated stock solution) to the plasminogen solution (usually 0.5 ml), mixing on a Vortex and letting the mixture stand at 4°C for 1 h. It was checked several times that this duration was sufficient; in any case, no more precipitate could be formed after this period had elapsed even when the precipitation conditions were not optimal, *e.g.*, at alkaline pH. The whole tube was then counted in a gamma counter (Intertechnique GC 3000) and then centrifuged at 10 000 *g* for 5 min in an Eppendorf centrifuge. The supernatant was carefully withdrawn with a fine Pasteur pipette. Gamma counting of the supernatant and/or precipitate gave a precise quantitative evaluation of the precipitated protein: the amount of dye in the precipitate was usu-

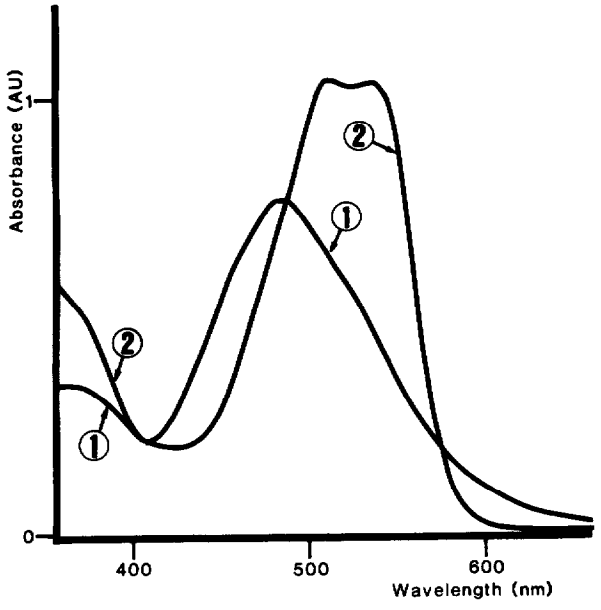

Fig. 1. Absorbance spectra of Procion Red HE3B dissolved in 50 m*M* sodium acetate pH 4.5 containing 0.2 *M* sodium chloride (curve 2) and dissolved in 1.25 *M* sodium hydroxide containing 8 *M* urea (curve 1).

ally determined by measuring the optical density of the precipitate redissolved in 1.25 *M* sodium hydroxide containing 8 *M* urea.

RESULTS AND DISCUSSION

Absorbance spectra of Procion Red HE3B

The absorbance spectrum of Procion Red HE3B dissolved in 50 m*M* sodium acetate buffer pH 4.5 containing 0.2 *M* sodium chloride is shown in Fig. 1 (curve 2). Extinction coefficient was measured at the wavelength of 530 nm. A value of 32 500 l mol^{-1} cm^{-1} was found which is in reasonable agreement with other published values[13]. The corresponding spectrum of Procion Red HE3B dissolved in 1.25 *M* sodium hydroxide containing 8 *M* urea is also shown in Fig. 1 (curve 1): the absorbance maximum is at 486 nm; the extinction coefficient at this wavelength was found to be 20 500 l mol^{-1} cm^{-1}. We confirmed that the presence of plasminogen along with dye in 1.25 *M* sodium hydroxide containing 8 *M* urea had no significant effect either on the position of the absorbance maximum or on the extinction coefficient value; also that the absorbance of the dye dissolved in the different solvents and at the concentrations used followed the Beer Lambert law[19].

Effect of pH

The effect of the pH on the reaction was studied with two different dye-to-protein molar ratios in a buffer containing 100 mmol/l of Tris base, glycine, disodium

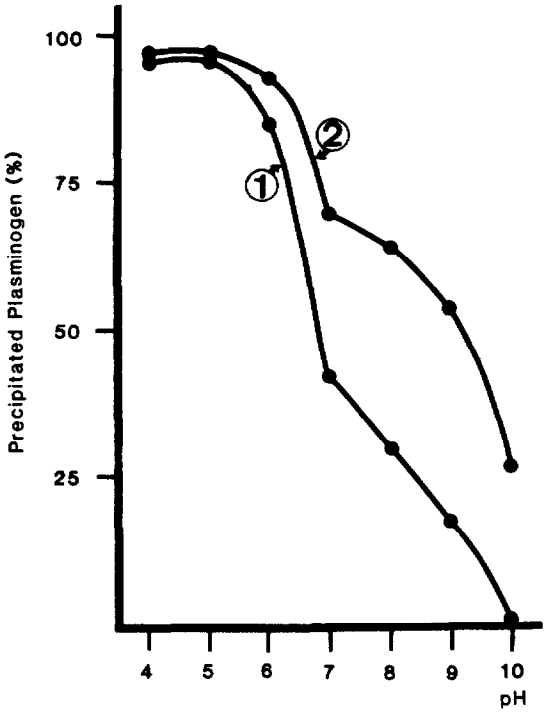

Fig. 2. Influence of pH on the precipitation of plasminogen induced by Procion Red HE3B. The buffer is given in the text. Two different dye-to-protein molar ratios were used: 13, curve 1; 33, curve 2.

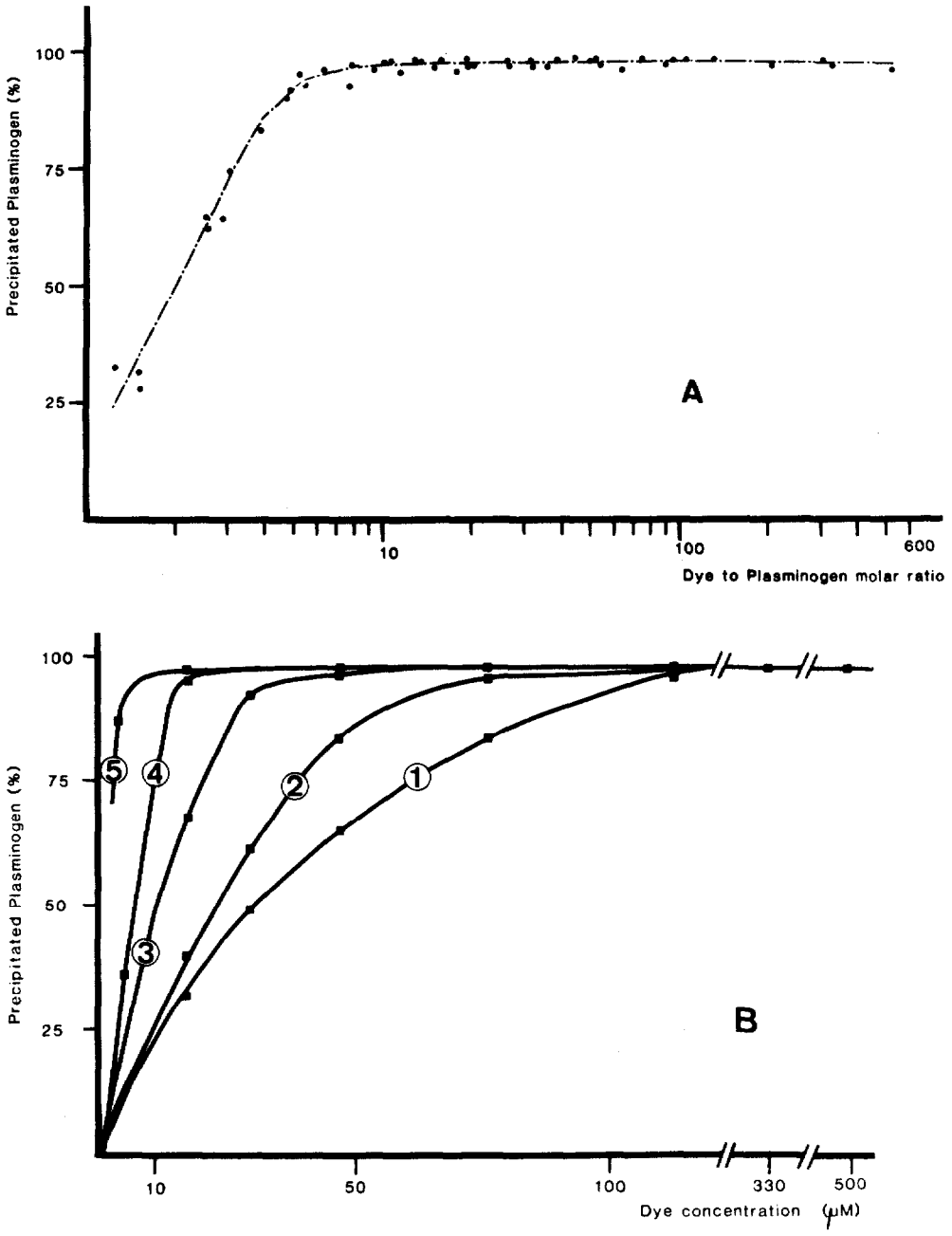

Fig. 3. A, Effect of the dye-to-plasminogen molar ratio on precipitation of the protein induced by the dye. Precipitation reactions were conducted in standard buffer as described in Materials and methods. B, variation of plasminogen precipitation with the concentration of the dye. Final concentrations of plasminogen in the precipitation medium were 45, 109, 218, 436 and 855 μg/ml for curves 5–1, respectively. In A the same experimental data as in B, as well as data obtained at final plasminogen concentrations of 1.35 and 0.020 mg/ml, were used.

hydrogenphosphate and sodium chloride which had been adjusted to the desired pH by use of hydrochloric acid or sodium hydroxide. Fig. 2 shows that the optimum pH for precipitation is situated between 4 and 5. Hence most of the quantitative studies described later were done in 50 m*M* sodium acetate pH 4.5 buffer containing 0.2 *M* sodium chloride, which will be referred to hereafter as the standard buffer. The sulphate groups of Procion Red are likely to carry a negative charge at pH $\geqslant 4.00$; thus, because of a pH optimum, it seems tempting to think that the interaction of the dye with plasminogen which ends in precipitation is at least partly due to interactions between the negative charges of the dye and positive charges on the plasminogen molecule, positive charges which do progressively disappear when the pH is increased. It has to be pointed out that when the molar ratio of the dye to plasminogen is increased, a shoulder appears on the descending limb of the precipitation *versus* pH curve; such a shoulder could well be explained by the formation at high dye-to-protein molar ratios of new interacting positive charges of higher p*K* than the former ones.

Effect of the molar ratio of dye to plasminogen on precipitation

Fig. 3B shows a family of curves illustrating the variation of the amount of precipitated plasminogen with the dye concentration at several different plasminogen concentrations. The curve in Fig. 3A shows the corresponding variation with the molar ratio of dye to plasminogen. This curve demonstrates that when the molar

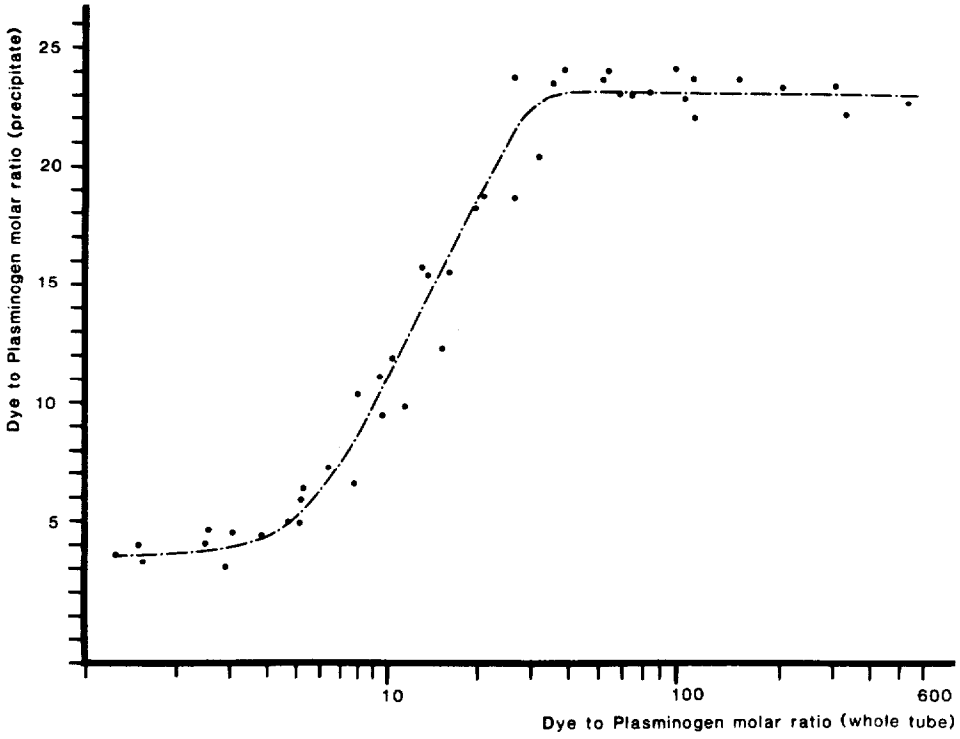

Fig. 4. Dye-to-protein molar ratio found in the precipitate as a function of the corresponding ratio used for the precipitation reaction. The experiments used were the same as those in Fig. 3.

ratio is equal or greater than 6 practically all the plasminogen is quantitatively precipitated.

Also, by mere visual inspection of the tubes in which the precipitations were conducted it was obvious that if plasminogen was indeed precipitated by the dye, the dye was also precipitated by the protein; the supernatants (at least for low molar ratios of dye to protein) were evidently much less coloured than the tube contents at the onset of the reaction. In order to be able to describe quantitatively the phenomenon, the amount of Procion Red HE3B present in the precipitate was evaluated as described in Materials and Methods.

Fig. 4 shows the variations of the molar ratio of dye to protein found in the precipitate with the dye/protein molar ratio used for the reaction. It is seen that at low molar ratios approximately four molecules of Procion Red are present in the precipitate, together with one molecule of plasminogen. When the molar ratio of Procion Red to plasminogen is increased, additional sites of the plasminogen molecule are involved up to an upper limit of 24 sites per molecule.

Influence of ionic strength on precipitation induced by the dye

The study of the optimum pH for precipitation of plasminogen by the dye had suggested that electrostatic interactions between charges of opposite signs played a rôle in the precipitation reaction. Hence an influence of the ionic strength was expected.

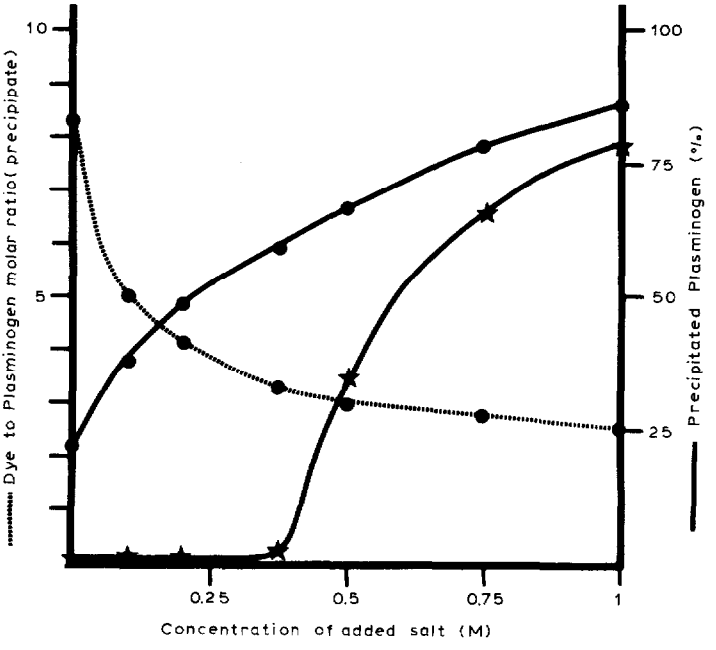

Fig. 5. Effect of sodium chloride molarity on the precipitation of plasminogen induced by the dye. The buffer was 50 m*M* sodium acetate pH 4.5. Filled circles correspond to experimental values which were obtained with a protein concentration of 0.9 mg/ml and a dye-to-protein molar ratio of 2.2. The dotted line shows the dye-to-plasminogen molar ratio found in the precipitate. Plasminogen was also partially precipitated in the absence of the dye (★) when the sodium chloride molarity was sufficiently high. Plasminogen concentration 0.9 mg/ml.

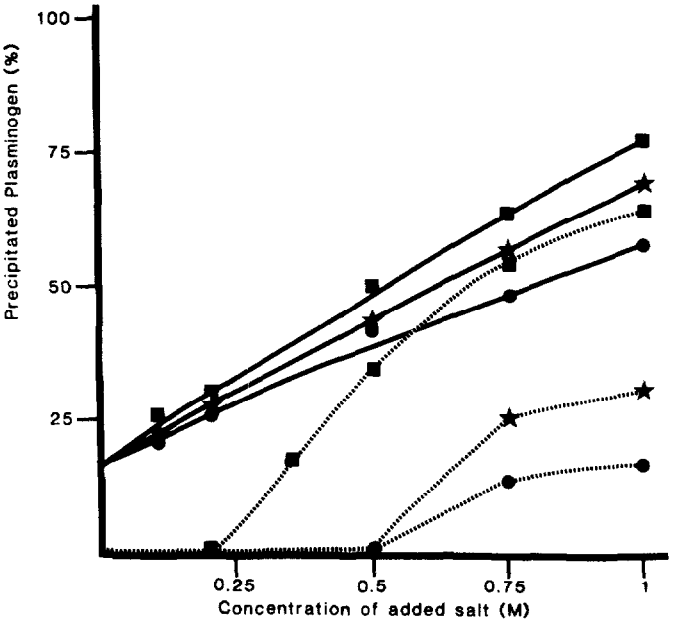

Fig. 6. Effect of different salts on the precipitation of plasminogen induced by Procion Red HE3B. The buffer was as in Fig. 5. The plasminogen concentration was 0.6 mg/ml and the dye-to-protein molar ratio was 1.4. Observed values in the presence of the dye are shown by the continuous lines and in the absence of the dye by the dotted lines. The experimental points are for potassium chloride (■), sodium chloride (★) and lithium chloride (●).

Fig. 5 shows the experimental variation of the amount of plasminogen precipitated when the molarity of sodium chloride in the precipitation medium was varied from 0 to 1 *M*. Clearly, precipitation of plasminogen by the dye increases with increasing molarity of sodium chloride. Noteworthy also is the fact that at higher than 0.3 *M* sodium chloride significant amounts of plasminogen are precipitated even in the absence of dye, due to the salting-out effect of sodium chloride. If one looks at the dye-to-protein molar ratio found in the precipitate, it is clear that this diminishes with increasing molarity of salt, this being more significant as long as no salting out of plasminogen (without dye) is likely to occur. Hence it could be tentatively concluded that an increasing concentration of sodium chloride, on the one hand, impairs fixation of the dye on the plasminogen molecule, in accord with a weakening of electrostatic bonds with increasing ionic strength, but on the other hand, a salting-out effect results in easier precipitation of plasminogen which has bound dye molecules.

The existence of a salting-out mechanism is substantiated by experiments with different salts, the results of which are shown in Fig. 6; clearly, the effect of a salt on plasminogen precipitation is correlated with its position in the lyotropic series[20].

Effect of ε-aminocaproate and related compounds on precipitation induced by dye

Plasminogen is known to bind ε-aminocaproate (EACA): both the number of binding sites and the dissociation constant have been established, as well as their location in the plasminogen molecule[21,22]. This ligand is used to elute plasminogen

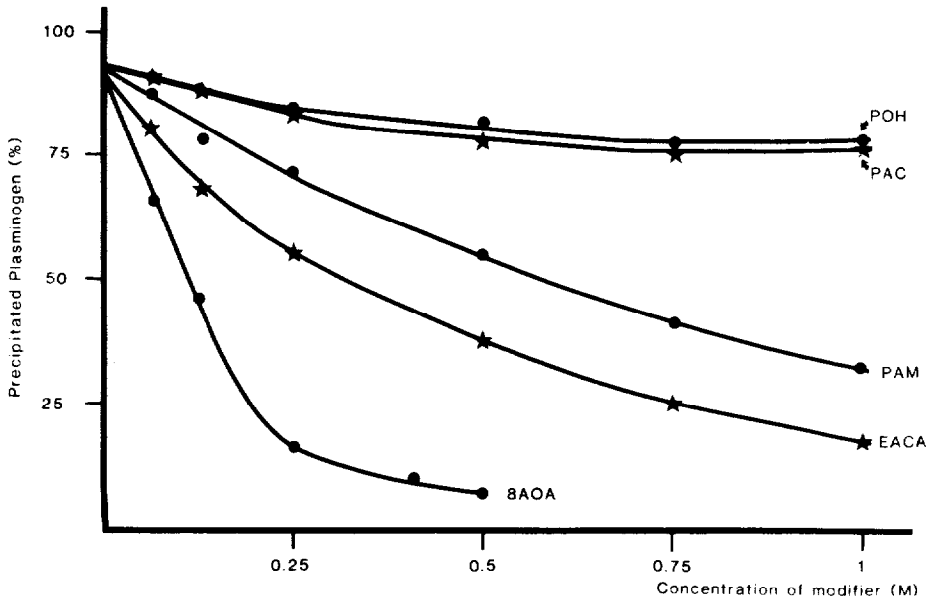

Fig. 7. Effect of varying the concentration of several organic components added to the precipitation mixture. Precipitation was conducted in the standard buffer with a dye-to-protein molar ratio of 14 (plasminogen concentration 0.44 mg/ml). Modifiers: EACA = ε-aminocaproate; POH = propanol; PAC = propionic acid; PAM = propylamine; 8AOA = 8-amino-1-octanoic acid.

from lysine agarose affinity chromatography columns[15]; it can be used also for desorption of plasminogen bound to fibrin[23]. It was therefore interesting to determine whether there was an effect on precipitation induced by the dye. The results are shown in Fig. 7: obviously EACA interacts with and reduces plasminogen precipitation. This was somewhat unexpected because it had been noted by Harris and Byfield[1] that EACA could not be used for elution of bound plasminogen from columns of immobilized Procion Red HE3B. However, it has to be pointed out that the concentrations of EACA which had a definite effect on plasminogen precipitation by the dye were much higher than those used for desorption of plasminogen from lysine agarose columns[15] or from fibrin[23]. Hence it could be postulated that the effect of EACA on precipitation was not mediated by its specific interaction on the plasminogen surface at its dedicated place[22] but was less specific: moreover it was found that 8-amino-1-octanoic acid, which is a very poor ligand for plasminogen[21], is more effective than EACA for reducing the amount of precipitation induced by the dye. It could therefore be thought, for instance, that EACA or 8-amino-1-octanoic acid interferes with the precipitation reaction because their carboxylic group could compete with the dye for the postulated positive sites on the surface of the plasminogen molecule, or their positive amino groups could pair with sulphonate groups on the Procion Red molecule and so hinder interaction of the dye with plasminogen. In order to verify these hypotheses we have looked at the effects of propionic acid, propanol and propylamine on the precipitation. The experiments show definitely (see Fig. 7) that precipitation is more effectively prevented if the organic modifier bears an amine group. This suggests that at least part of the effect is mediated through the onset of ion-pair interactions with the sulphonate groups of the dye.

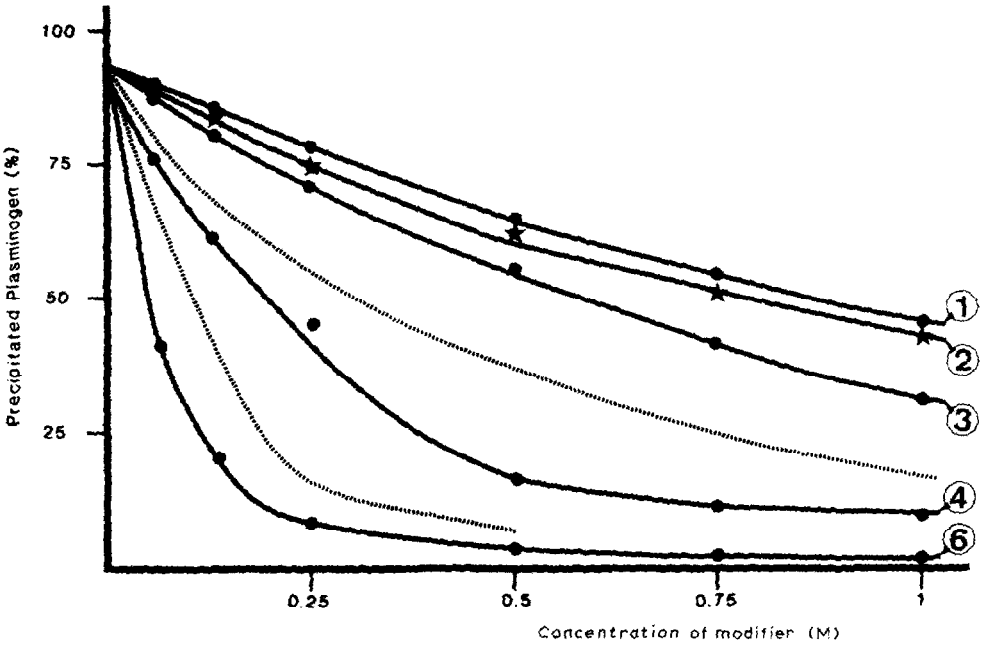

Fig. 8. Effect of several aliphatic amines on the precipitation of plasminogen induced by Procion Red HE3B. The precipitation conditions were as in Fig. 7. The number of carbon atoms in the amines are shown on the curves. Experimental curves (but not the experimental points) obtained with EACA and 8-amino-1-octanoic acid, already shown in Fig. 7, are also reported (dotted lines).

Noteworthy also is the fact that aliphatic amines are more effective in preventing precipitation the larger is the aliphatic chain (Fig. 8). In fact, if one uses the sets of hydrophobic fragmental constants defined by Rekker[24] to compute a hydrophobicity index for each of the amine-bearing organics, it is clear that the ability to prevent precipitation increases with increasing hydrophobicity of the modifier. This highlights the rôle played by hydrophobic interactions in the dye-induced precipitation of plasminogen.

Precipitation of plasminogen from plasma by dye

The mixing of dye and plasma at pH 4.5 resulted in easily visible precipitates even at a low dye-to-plasminogen molar ratio. However, the spiking of plasma with radioactive plasminogen showed that the formed precipitate did not contain plasminogen unless the dye-to-plasminogen molar ratio was $\geqslant 6000$ (see Fig. 9). This was attributed above all to the large amounts of albumin present in the plasma: examination by sodium dodecyl sulphate (SDS) gel electrophoresis of the precipitates obtained when the dye-to-plasminogen molar ratio was lower than 3000 showed the presence of albumin as the major protein (albumin was the only visible band after Coomassie blue staining when 30 μg of protein had been loaded onto the gel); also when human serum albumin was added to purified plasminogen at a final concentration similar to the one in the experiment with plasma, it was obvious that high dye-to-plasminogen molar ratios were also needed to precipitate plasminogen quan-

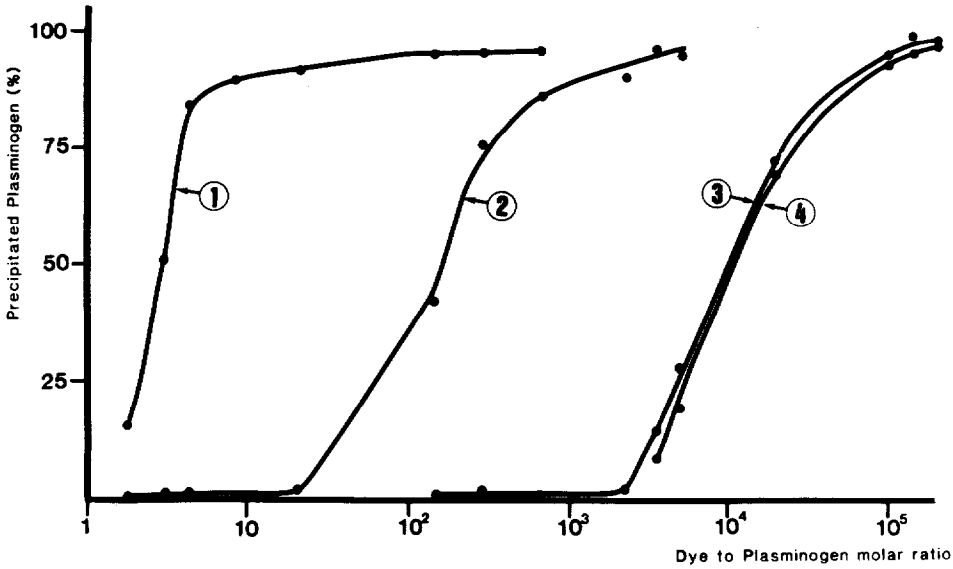

Fig. 9. Precipitation of plasmatic plasminogen induced by Procion Red HE3B. Curves: 1, purified plasminogen dissolved in standard buffer; 2, albumin-depleted plasma (20 ml of plasma filtered through a column, 40 × 3.8 cm, filled with Cibacron Blue agarose equilibrated in 50 m*M* Tris–chloride buffer pH 8.5; breakthrough peak containing plasminogen adjusted to pH 4.5 after addition of sodium acetate); 3, plasma equilibrated in standard buffer by gel filtration on a Sephadex G-25 column of appropriate volume; 4, purified plasminogen mixed in standard buffer with human serum albumin (45 mg/ml). The plasminogen concentrations were adjusted by appropriate dilutions to the same value, 0.021 mg/ml.

titatively. On the other hand, by depleting the plasma of albumin using chromatography on Cibacron Blue agarose, it was possible to precipitate plasminogen quantitatively with lower dye-to-plasminogen molar ratios (see Fig. 9).

Here one can recall that the interference of albumin in the interaction between a dye and another protein has already been observed: for instance, it was noted that the presaturation of Cibacron Blue agarose columns with albumin increased the purification of nitrate reductase from chlorella[25], and the presence of albumin weakened the interactions of interleukin 2 with Cibacron Blue agarose[26]. The presence of albumin was shown to influence heavily the partitioning of prealbumin to an upper phase containing Remazol Yellow covalently linked to polyethylene glycol[27].

Obviously the fact that albumin is precipitated from the plasma by Procion Red HE3B sooner than plasminogen suggests that the latter protein could be substantially purified by fractional precipitation. Indeed preliminary experiments have shown that this is possible. Nevertheless, for reasons which will be discussed later, it doesn't appear that this could become a practical purification method for this protein.

Conditions for disruption of dye binding to plasminogen

The complete solubilization of the precipitated plasminogen was easily obtained as described in Materials and Methods by using 1.25 *M* sodium hydroxide containing 8 *M* urea. However this mixture is likely to denaturate protein and cannot

be used for preparative purposes. The complete and rapid dissolution of the precipitate was also obtained by using hexylamine titrated with hydrochloric acid to pH 8.3 at concentrations of 0.5 *M* or higher. It is noteworthy that when the tube containing the redissolved precipitate was left in the cold a new precipitate formed. This second precipitate was shown not to contain protein (provided that the final protein concentration was lower than 0.5 mg/ml), but only dye driven out of its interaction with plasminogen and finally out of the solution, presumably because of ion pairing with the organic base.

Gel filtration of the supernatant of this second precipitate (conducted on a Sephadex G-25 column of appropriate volume, equilibrated in 0.5 *M* hexylamine adjusted to pH 8.3 with hydrochloric acid) was ineffective in disrupting tight binding of the dye to plasminogen: dye and protein were eluted together at the void volume of the column. In contrast, by depositing the "second supernatant" on columns of DEAE-Trisacryl or DEAE-cellulose it was possible to obtain an effective separation of the dye and protein; plasminogen did not bind to the column but the dye was quantitatively retained. A 1-ml column of DEAE-Trisacryl or DE-23 equilibrated in the previously mentioned 0.5 *M* hexylamine buffer could bind more than 6 μmol of dye before the dye appeared in the eluate at a concentration of $0.6 \cdot 10^{-6}$ *M*. It is worth mentioning also that plasminogen could be freed from dye by chromatography on lysine agarose: when the second supernatant was deposited on a lysine agarose column both the dye and protein were retained; plasminogen was selectively eluted by EACA and recovered totally free from dye as shown by absorbance measurements at 530 nm. It has to be pointed out that when such chromatography was applied to a precipitate prepared from plasma, the plasminogen obtained at the end of the procedure was not only totally free from dye but was also purified from the other proteins which had been coprecipitated by the dye.

It is worth mentioning that plasminogen samples which had been precipitated and redissolved according to the previously described procedure were found to be fully activatable with urokinase or streptokinase in the same way as were samples of purified plasminogen which had not been submitted to the precipitation–redissolution procedure.

CONCLUSIONS

The experiments described demonstrate that plasminogen is precipitated by Procion Red HE3B. The precipitation is thought to involve ionic interactions between charges of opposite signs, and interactions of an hydrophobic character. The exact molecular mechanism of the precipitation is however obscure. It is worth noting that Procion Red HE3B is a relatively wide molecule bearing six sulphonate groups. One could postulate that precipitation of plasminogen is induced because several dye molecules bridge together a large number of plasminogen molecules. A similar explanation was presented for precipitation induced by charged polymers (for instance, precipitation of proteases by polyacrylic acid[28]), but if this explanation were correct an influence of the plasminogen concentration would be observed: a decrease in the plasminogen concentration would probably induce a diminution of the precipitation induced by the dye; experimentally, at least in the range of plasminogen concentrations used (20 μg/ml to 1.35 mg/ml), the plasminogen concentration had no effect on

the percentage of precipitation, the important parameter being the dye-to-plasminogen molar ratio. Moreover it was possible to precipitate plasminogen with Procion Red H3B[29], another dye which can be described as a half Procion Red HE3B (structures of the two dyes are described in ref. 30). The H3B Red bears only three sulphonate groups which are clustered together and therefore it does not seem capable of "bridging together" a large number of plasminogen molecules.

The most likely explanation of the ability of the dye to precipitate plasminogen seems to lie in the fact that once a sufficient number of dye molecules are linked to one plasminogen molecule the surface hydrophobicity of the latter is modified, aggregation with other molecules can occur and precipitation follows. The precipitation itself depends also on the salt composition of the surrounding medium and/or the presence of organic modifiers.

We have seen that the dye precipitates not only plasminogen but also albumin from plasma and that moreover because of a tight binding of the dye and plasminogen a subsequent chromatographic step is necessary to free the protein from the dye. Therefore we cannot confidently claim that precipitation by Procion Red HE3B will be of practical benefit for the large scale purification of plasminogen.

Nevertheless it is anticipated that more or less selective protein precipitation induced by dyes could be more than a biochemical curiosity and could be established as an authentic purification method. Indeed from the work of Lowe and Pearson[11] it can be anticipated that every protein which has at least two affinity sites for one dye can be precipitated by grafting this dye at both ends of a spacer; our results and other work[2–8] have shown that precipitation can be induced in a simpler way by use of unmodified pure dyes.

Here it has to be emphasized that most dyes (and not only Procion Red HE3B and some other dyes[2–8] can be seen to be endowed with a "precipitating" potential; recently, Scopes[31] had written that a "wide variety of organic compounds possessing dual functions of hydrophobicity and polarity could cause precipitation". Indeed most textile dyes possess these "dual functions of hydrophobicity and polarity".

In the light of experience with pseudo affinity chromatography on immobilized dyes, one can infer that finding a useful dye for precipitating a given protein will be essentially a matter of trial and error[13]. Maybe it will be useful[32] to find a pair of dyes, one of which precipitates most proteins of the crude extract but not the target protein and the other which precipitates some of them including the protein of interest.

It should be emphasized that the ease of disruption of the interaction between the precipitant and protein will have to be considered; the existence of too tight a binding between the precipitant and the target protein (as is the case between plasminogen and Procion Red HE3B) will probably be a disadvantage for preparative applications of the method.

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