Journal of Chromatography, 265 (1983) 27–35
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,819

TRIAZINE DYE BINDING OF HUMAN α-FETOPROTEIN AND ALBUMIN

G. BIRKENMEIER*, E. USBECK, L. SARO and G. KOPPERSCHLÄGER
Institute of Physiological Chemistry, Karl-Marx-University, Liebigstrasse 16, 7010 Leipzig (G.D.R.)
(Received December 29th, 1982)

SUMMARY

Column chromatography was used to investigate the interaction of human α-fetoprotein and albumin with different immobilized dyes. Binding of α-fetoprotein to the dye conjugates was studied at pH 7.0. Between 56 and 93% of the total α-fetoprotein applied to the column was recovered in the break-through fractions of the respective runs. Of all the dyes, Cibacron Blue F3G-A adsorbs α-fetoprotein most strongly. This interaction clearly depends on the degree of dye substitution of the gel. A relatively weak interaction exists between α-fetoprotein and immobilized Procion Red HE-3B. This is used in the purification of α-fetoprotein by negative chromatography resulting in a 16.6-fold enrichment of this protein.

Human albumin binds tightly to immobilized Cibacron Blue F3G-A as well as to Cibacron Brilliant Blue FBR-P and shows a lower affinity to Procion Blue MX-R. Procion Red dyes, which are structurally different from Cibacron Blue F3G-A are also capable of interacting with serum albumin. The results obtained are discussed in terms of the present theoretical conceptions about dye-protein interactions.

INTRODUCTION

The general similarity of the chemical and functional properties of α-fetoprotein and albumin has led to the assumption that both proteins are evolutionary related[1]. Both α-fetoprotein and albumin have been shown to bind steroids[2], bilirubin[3,4], fatty acids[5] and different dyes[6].

The binding of human serum albumin to immobilized Cibacron Blue F3G-A was first reported by Travis and Pannell[7]. Since then, several studies have been carried out to elucidate the binding mechanism between albumin and other serum proteins and this immobilized dye[8–12]. The original assumption that Cibacron Blue F3G-A may occupy the bilirubin binding site in human albumin could not be confirmed by a later report[9]. Other organic compounds such as fatty acids were found to compete with the dye for binding to albumin[10].

Some reports dealt with the interaction of α-fetoprotein obtained from different sources to immobilized Cibacron Blue F3G-A[13–15]. However, the interaction of human α-fetoprotein with this dye is still controversial[16,17].

0021-9673/83/$03.00

In the present paper a comparative study of the interaction of α-fetoprotein and albumin from human cord serum with Cibacron Blue F3G-A and other structurally similar and dissimilar dyes of the triazine class is described. In particular, the applicability of the different dye conjugates for the purification of α-fetoprotein is assessed.

The results are discussed in terms of the present theoretical assumptions about dye–protein interactions.

EXPERIMENTAL

Materials

Cibacron Blue F3G-A and Cibacron Brilliant Blue FBR-P were obtained from Ciba Geigy (Basle, Switzerland). All Procion dyes were purchased from ICI Organics Division (Blackley, Manchester, Great Britain). Sephadex G-100 was from Pharmacia (Uppsala, Sweden). Anti-human α-fetoprotein from rabbit, α-fetoprotein standard and M-partigen immunodiffusion plates for albumin were obtained from Behringwerke (Marburg, G.F.R.). Agarose was a product from Serva (Heidelberg, G.F.R.).

Methods

Cibacron Blue F3G-A, Cibacron Brilliant Blue FBR-P and Procion dye derivatives of Sephadex G-100 were prepared according to the method of Boehme *et al.*[18] For the determination of the ligand concentration the dye conjugates and appropriate dye standards were incubated in 50% acetic acid for 2 h in a boiling water-bath. The absorbance of the samples and of the standards was measured at the wavelength of the absorption maximum of the respective dyes in acetic acid solution. The degree of dye substitution of the gels is expressed in μmol of dye per g of dried gel.

Human serum was obtained from the umbilical cord of newborn infants. The serum samples (0.5 ml) were dialysed against 400 ml of starting buffer at 4°C for 24 h prior to their application to the column.

The chromatographic runs were performed after equilibration of 20 ml of the dye conjugates with 10 m*M* sodium phosphate buffer (pH 6.0 or 7.0) in glass columns (12 × 1.5 cm). The flow-rate was 10 ml/h and 2.5-ml fractions were collected. The columns were regenerated by exhaustive washing with 0.01 *M* sodium hydroxide and distilled water followed by equilibration with buffer.

Protein solutions were concentrated by ultrafiltration employing an Amicon UM-10 membrane filter.

Human α-fetoprotein was measured by rocket electroimmunodiffusion[19] using anti-human α-fetoprotein from rabbit and α-fetoprotein standard serum. Quantitation of human albumin was carried out by single radial immunodiffusion[20] using M-partigen immunodiffusion plates.

The protein content of the samples was determined according to the method of Janatova *et al.*[21] using bovine serum albumin as standard.

Pore gradient disc electrophoresis was performed as previously described[22].

RESULTS

Binding properties of the dye conjugates

The interaction of human α-fetoprotein and albumin with different immobilized triazine dyes whose structures are presented in Fig. 1 was investigated by means of chromatographic analysis. When cord serum which contains α-fetoprotein in an average concentration of 30 μg/ml was subjected to chromatography on the dye-liganded Sephadex G-100 gels, different elution diagrams were obtained as demonstrated in Fig. 2. In the cases of immobilized Cibacron Blue F3G-A (I-F3G-A) and immobilized Cibacron Brilliant Blue FBR-P (I-FBR-P), a number of serum proteins including α-1-proteinase inhibitor, α-1-acid glycoprotein, α-2-macroglobulin, and haptoglobins failed to be bound to the gels and appeared in the first protein peak of the eluates (Fig. 2A and B). A small fraction containing preferentially transferrin and immunoglobulins was eluted in either case by applying a linear salt gradient. Albumin was tightly bound to I-F3G-A and I-FBR-P and was desorbed by 1 *M* sodium chloride as an approximately pure protein (as checked by immunodiffusion and disc electrophoresis). Albumin was also found to be adsorbed selectively on immobilized

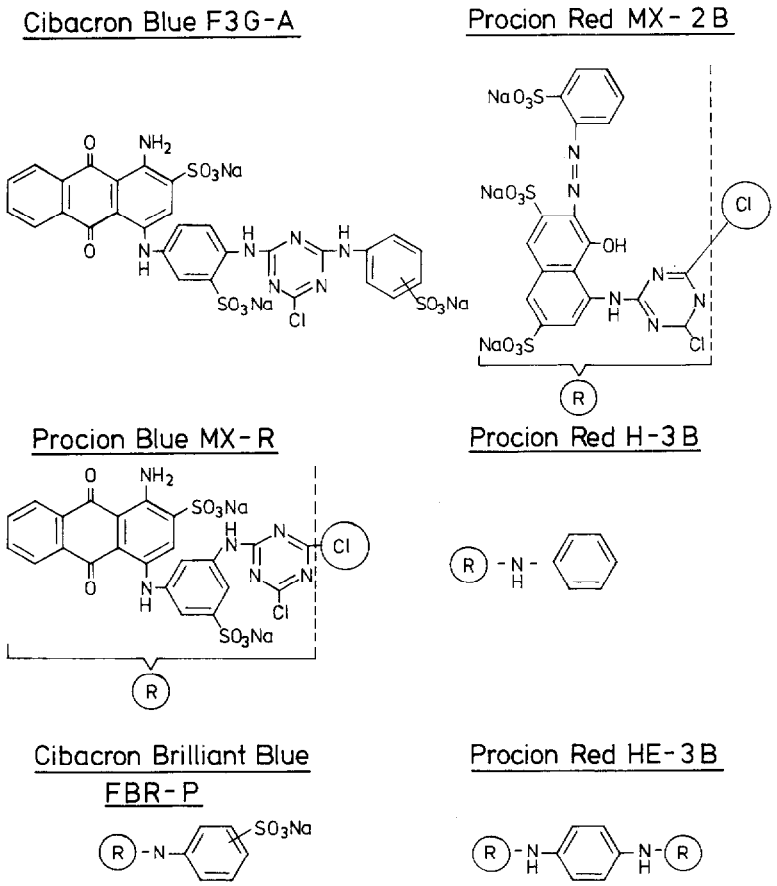

Fig. 1. Structures of the triazine dyes investigated in this work.

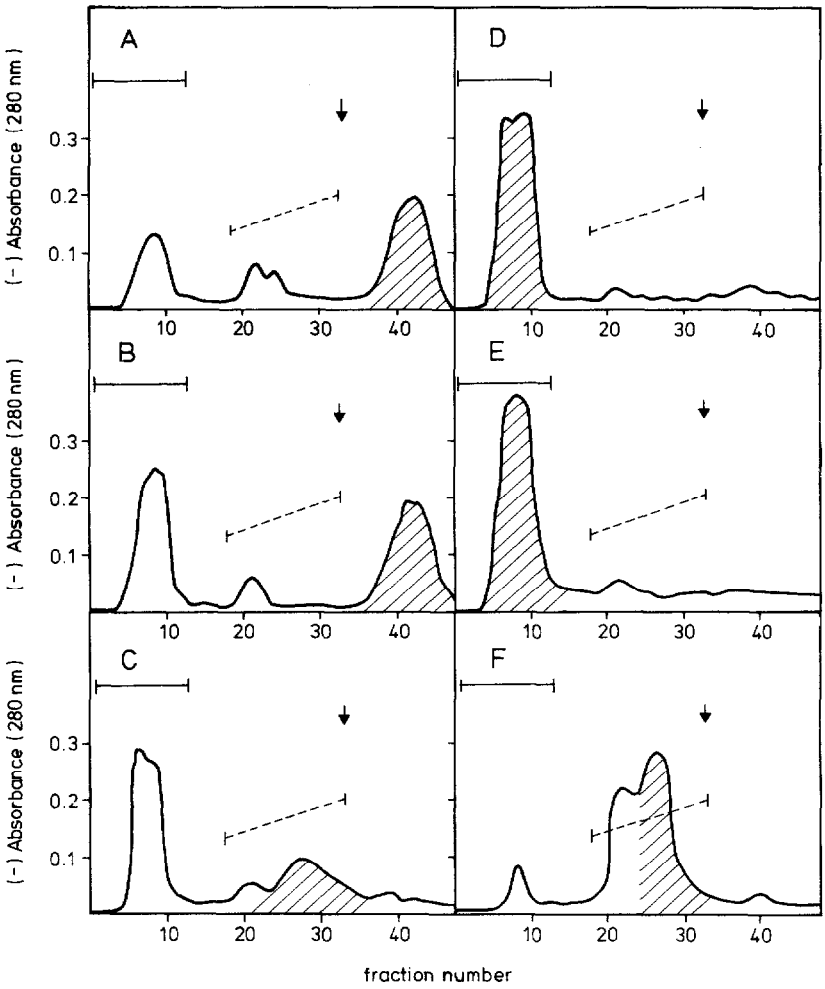

Fig. 2. Chromatography of cord serum on different dye conjugates. The columns (12 × 1.5 cm) were equilibrated with 10 m*M* sodium phosphate buffer (pH 7.0), and 0.5 ml of cord serum, previously dialysed against this buffer, was applied to the gels. The columns were first eluted with equilibration buffer followed by a salt gradient from 0 to 0.5 *M* sodium chloride and finally by 1 *M* sodium chloride. The flow-rate was 10 ml/h and 2.5-ml fractions were collected. The unbound fraction of each run (horizontal bars) was pooled, concentrated by ultrafiltration to 0.5 ml and then tested for total protein, albumin and α-fetoprotein. The broken lines indicate the salt gradient from 0 to 0.5 *M* sodium chloride, while the arrows indicate the starting point for the elution with 1 *M* sodium chloride. The shaded areas label the fractions containing albumin. The gels and their ligand concentrations (μmol/g) are as follows: A, I-F3G-A (11.7); B, I-FBR-P (11.0); C, I-MX-R (14.2); D, I-MX-2B (13.1); E, I-H-3B (14.0); F, I-HE-3B (32.0).

Procion Blue MX-R (I-MX-R) (Fig. 2C). However, in contrast to the other dye conjugates, I-MX-R bound albumin less tightly, as indicated by the lower salt concentration of the eluting buffer.

Immobilized Procion Red MX-2B (I-MX-2B) and immobilized Procion Red H-3B (I-H-3B) practically failed to bind the proteins of the cord serum which consequently appeared in the break-through fraction (Fig. 2D and E). On the other hand

immobilized Procion Red HE-3B (I-HE-3B) was found to have the highest binding capacity for serum proteins. The bound proteins including albumin could be eluted and divided into two fractions when a gradient of sodium chloride was applied (Fig. 2F). This indicates that albumin also binds to I-HE-3B but with a lower affinity than to I-F3G-A and I-FBR-P. With respect to the binding capacities of the different dyes for albumin the binding increased in the order I-H-3B < I-MX-2B < I-MX-R < I-HE-3B < I-FBR-P = I-F3G-A (Table I).

In order to assess the ability of the different dyes for binding α-fetoprotein, the break-through fractions of the chromatographic runs presented in Fig. 2 were analysed by rocket electroimmunodiffusion. The simultaneous determination of albumin and the total protein content allowed us to evaluate the applicability of different dye conjugates for the purification of α-fetoprotein. Table I shows that at low ionic strength and low ligand concentration of the gels, the triazine dye-conjugates bind human α-fetoprotein to different extents. The amount of α-fetoprotein recovered in the break-through fractions of the individual runs ranged between 56 and 93% of the total α-fetoprotein applied. Considering the purification of α-fetoprotein it becomes obvious from this table that the best result was obtained with I-HE-3B. The 16.6-fold enrichment results from the binding of most of the contaminating proteins including albumin and the appearance of α-fetoprotein in the break-through fraction with 89% recovery.

Effect of ligand concentration

Among all the dyes investigated, I-F3G-A was found to retard human α-fetoprotein most strongly. This interaction of α-fetoprotein with I-F3G-A is influenced by the degree of dye substitution of the gel, as demonstrated in Fig. 3. When a gel

TABLE I

BINDING CAPACITIES OF TRIAZINE DYE CONJUGATES FOR HUMAN α-FETOPROTEIN AND ALBUMIN

The break-through fractions of the chromatographic runs depicted in Fig. 2 were pooled and concentrated to 0.5 ml. Albumin and α-fetoprotein were determined by radial immunodiffusion and rocket electroimmunodiffusion, respectively. The purification of α-fetoprotein was determined by calculation of the α-fetoprotein (AFP)/protein ratios.

Immobilized dye	*Sample*	*Protein (mg/ml)*	*Albumin (mg/ml)*	*AFP (μg/ml)*	*AFP/albumin*	*AFP/protein*	*Purification of AFP*
	Cord serum	53	32	30	0.9	0.56	–
I-F3G-A	Break-through fractions	5.6	n.d.*	17	∞	3.0	5.35
I-FPR-P	Break-through fractions	13	n.d.*	28	∞	2.1	3.75
I-MX-R	Break-through fractions	20.3	3.0	27	9.0	1.33	2.37
I-MX-2B	Break-through fractions	38.4	23	26	1.1	0.67	1.19
I-H-3B	Break-through fractions	34.5	25	26	1.0	0.75	1.33
I-HE-3B	Break-through fractions	2.9	0.64	27	42.1	9.31	16.62

* n.d. = Not detectable.

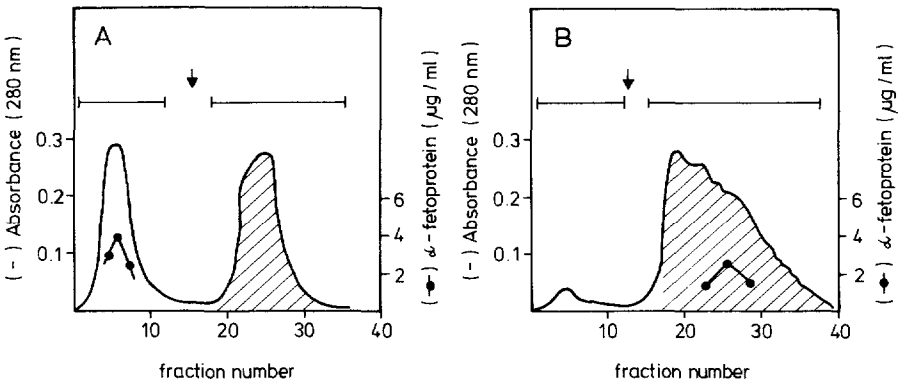

Fig. 3. Chromatography of human cord serum on differently substituted Cibacron Blue F3G-A Sephadex G-100 gels. A 0.5-ml sample of cord serum was dialysed and applied to the column containing the dye conjugate which was previously equilibrated with 10 m*M* sodium phosphate buffer (pH 7.0) at 4°C. After washing the column with equilibration buffer, the bound proteins were eluted by 2 *M* sodium chloride (arrows). The content of α-fetoprotein in the pools (horizontal bars) and of the single fractions was checked by rocket electroimmunodiffusion. The shaded areas indicate the fractions containing albumin. A, I-F3G-A (3.0 µmol/g); B, I-F3G-A (143 µmol/g).

with a dye substitution of 3 µmol/g was applied, *ca.* 90% of α-fetoprotein became detectable in the break-through fraction and no α-fetoprotein remained bound (Fig. 3A). This column permitted a complete separation of α-fetoprotein from albumin, which was exclusively confined to the second protein peak. Rechromatography of the unbound protein fraction (first peak) under the same conditions revealed that α-fetoprotein was eluted again at the same position with high recovery. The latter finding excludes the possibility that unbinding of α-fetoprotein to I-F3G-A is due to an overloading of the column with protein especially with albumin.

When the ligand concentration of the gel was increased to 143 µmol/g, 93% of α-fetoprotein was bound in addition to other proteins (Fig. 3B). By employing

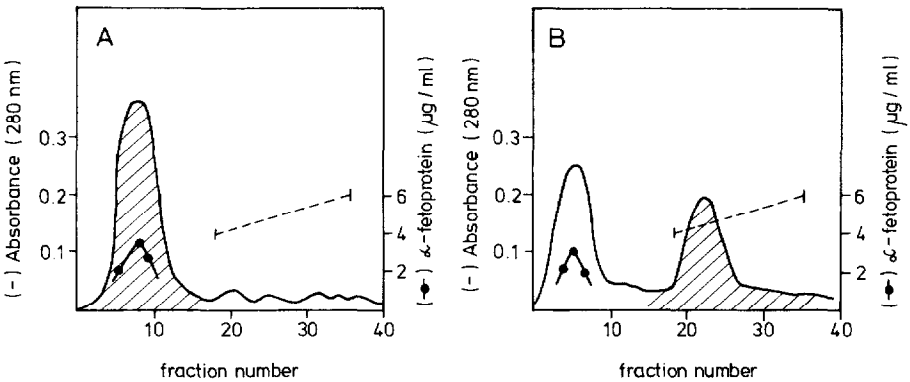

Fig. 4. Chromatography of cord serum on Procion Red dye conjugates at pH 6.0. A 0.5-ml sample of cord serum was loaded on to the column containing the dye conjugate which was previously equilibrated with 10 m*M* sodium phosphate buffer (pH 6.0). The unbound proteins were washed out with equilibration buffer and the bound proteins were eluted by a salt gradient from 0 to 0.5 *M* sodium chloride (broken line). The shaded areas indicate the fractions containing albumin. A, I-MX-2B (13.1 µmol/g); B, I-H-3B (14.0 µmol/g).

such a highly substituted gel, the chromatographic separation of human α-fetoprotein from albumin was not attainable.

Effect of pH value

Since electrostatic interactions are probably dominating in the dye–protein interaction, an increase in protein protonation should facilitate their binding to the anionic dyes. Indeed, when cord serum was chromatographed on various dye conjugates at pH 6.0, complete adsorption of α-fetoprotein onto I-F3G-A, I-FBR-P, I-MX-R and I-HE-3B was found (not shown). On the other hand, I-MX-2B as well as I-H-3B fail to bind α-fetoprotein at pH 6.0 (Fig. 4A and 4B). However, these two dyes differ at this pH in their interaction with albumin. I-H-3B is then capable of removing albumin from cord serum by adsorption which can be eluted in a purified form by a salt gradient. It is interesting to note that none of the other serum proteins share this property of albumin.

DISCUSSION

Binding of α-fetoprotein to triazine dyes might be deduced from its similarity to albumin because of the sequence homology of both proteins[1], and of the binding of fatty acids[5], various dyes[6], oestrogens[2] and bilirubin[3,4]. However, binding of human α-fetoprotein to Cibacron Blue F3G-A is still controversial. Lai *et al.*[16] recently reported an adsorption of human α-fetoprotein on a Cibacron Blue column. This results disagree with those of Gold *et al.*[17] and Young and Webb[15] who found that human α-fetoprotein does not exert any affinity to this dye.

It seems reasonable to assume that these discrepancies might be due to variations in the preparation of the dye conjugates and to different chromatographic conditions. As shown earlier[23], the degree of dye substitution is important in judging the binding of serum proteins to immobilized Cibacron Blue. Also, the binding of human α-fetoprotein to I-F3G-A rises drastically when the dye ligand concentration of the gel is increased. In this respect the behaviour of α-fetoprotein resembles that of other serum proteins such as transferrin, immunoglobulins and haptoglobins[23]. Clear differences, however, exist between α-fetoprotein and albumin in their affinity to I-F3G-A, which become most prominent at very low ligand concentrations.

In the search for further triazine dyes as affinity ligands for α-fetoprotein, different compounds were tested (Fig. 1). At neutral pH α-fetoprotein does not exhibit remarkable binding either to chromogens structurally related to Cibacron Blue or to triazine dyes structurally different to Cibacron Blue such as Procion Red dyes. In respect to the applicability of immobilized triazine dyes for the purification of α-fetoprotein, three dyes deserve particular interest. I-HE-3B appears well suited in the separation of α-fetoprotein from other serum proteins by negative chromatography, resulting in a 16.6-fold purification. In comparison with other conventional purification methods, a similar effective enrichment could be obtained only after two chromatographic steps[14]. It should be noted that the α-fetoprotein preparation obtained after chromatography on I-HE-3B still contains small amounts of albumin. Therefore, in order to yield α-fetoprotein free from contaminating albumin, I-F3G-A and also I-FBR-P should preferentially be used[24].

At present, no explanation can be offered for the fact that human α-fetoprotein

behaves significantly different to albumin with respect to its binding to Cibacron Blue, despite the many similarities in chemical structure and functional properties. The reason for this difference could be that α-fetoprotein, in contrast to albumin, is glycosylated. The oligosaccharide structure might be thought to cover the dye-binding site and thus it may diminish the accessibility of this site to the dye ligand. It was shown by Gianazza and Arnaud[11,12] that differences in the carbohydrate component of serum proteins are apparently of significance to the elution order of proteins from immobilized Cibacron Blue.

All studies published so far[8,9,11,12,23] point out that complex interactions are involved in the binding of serum proteins to Cibacron Blue; however the chemical mechanism of the dye-protein interaction is still a matter of debate. The hypothesis of Stellwagen *et al.*[25] that binding of Cibacron Blue to proteins is necessarily symptomatic for the presence of a nucleotide-binding domain in proteins can no longer be maintained. In seeking a more general interpretation for dye-protein interaction we recently proposed that the dye-binding site is an apolar region on the surface of the protein molecule surrounded by hydrophilic amino acid residues. Hydrophobic as well as electrostatic forces are apparently involved in the binding of the dye to the protein. The specificity of the dye–protein interaction is assumed to depend primarily on the hydrophobic interaction, whereas the electrostatic forces mainly contribute to the stability of the complex[26].

In the following, the results of this study should be discussed in terms of this hypothesis. As shown in Fig. 2, albumin binds to I-F3G-A, I-FBR-P and I-MX-R with comparable specificity but displays lower affinity to I-MX-R than to the other two dyes. This is probably due to the lack of the terminal sulphonated aminophenyl ring in Procion Blue MX-R, indicating the importance of charged groups in one moiety of the dye for the binding to albumin. Furthermore, albumin can be displaced from I-F3G-A by competing bromaminic acid (unpublished). These findings agree with the results of Leatherbarrow and Dean[9] who stressed the importance of the anthraquinone moiety of this dye for the interaction with albumin. This large hydrophobic ring system confers upon the albumin dye complex an apparent specificity.

It is noteworthy that other triazine dyes dissimilar to Cibacron Blue are also capable of binding albumin (Figs. 2 and 4). Procion Red MX-2B and Procion Red H-3B are naphthalene triazine dyes which differ only in the substitution of one chlorine group by an aminophenyl ring. Both dyes fail to bind albumin and α-fetoprotein at pH 7.0. However, by lowering the pH to 6.0 I-H-3B selectively adsorbs albumin (Fig. 4). We suppose that in this case the apparent binding specificity is caused by hydrophobic forces between the unsubstituted aminophenyl ring of Procion Red H-3B and an apolar pocket on the surface of the protein molecule. The stability of the albumin dye complex favoured by lowering the pH is caused by electrostatic forces between the ionic groups of the dye and corresponding functional groups of the albumin adjacent to the hydrophobic pocket. Since histidine is preferentially titrated in the pH range 7.0–6.0, this amino acid is assumed to be involved in the formation of the Procion Red H-3B dye-binding site in albumin.

ACKNOWLEDGEMENTS

We are grateful to Professor E. Hofmann for many helpful discussions and for

critically reading the manuscript. We thank also the VEB Chemiekombinat Bitterfeld, Coloristisches Labor, Karl-Marx-Stadt, G.D.R., for the generous gift of the triazine dyes.

REFERENCES

1 E. Ruoslathi and W. D. Terry, *Nature (London)*, 206 (1976) 804.
2 C. J. P. Smith and P. C. Kelleher, *Biochim. Biophys. Acta*, 605 (1980) 1.
3 E. Ruoslathi, T. Estes and M. Sepälä, *Biochim. Biophys. Acta*,578 (1976) 511.
4 C. B. Berde, M. Nagai and H. F. Deutsch, *J. Biol. Chem.* 254 (1979) 12609.
5 N. K. Carlsson, T. Estes, J. Degroot, J. T. Holden and E. Ruoslathi, *Biochem. J.*, 190 (1980) 301.
6 Y. Endo, K. Kanai, S. Lino and T. Oda, in R. Masseyeff (Editor), *L'Alpha-Foeto-Proteine*, INSERM, Paris, 1974, p. 47.
7 J. Travis and R. Pannell, *Clin. Chim. Acta*, 49 (1973) 49.
8 S. Angal and P. D. G. Dean, *FEBS Lett.*, 96 (1978) 346.
9 J. Leatherbarrow and P. D. G. Dean, *Biochem. J.*, 189 (1980) 27.
10 E. C. Metcalf, B. Crow and P. D. G. Dean, *Biochem. J.*, 199 (1981) 465.
11 E. Gianazza and P. Arnaud, *Biochem. J.*, 201 (1982) 129.
12 E. Gianazza and P. Arnaud, *Biochem. J.*, 203 (1982) 637.
13 P. C. Kelleher, C. J. Smith and R. Pannell, *J. Chromatogr.*, 173 (1979) 415.
14 J. T. Wu, L. H. Wu and A. C. Madsen, *Biochem. Med.*, 23 (1980) 336.
15 J. L. Young and B. H. Webb, *Anal. Biochem.*, 88 (1978) 619.
16 P. C. W. Lai, A. J. Luft, L. L. Huang and F. L. Lorscheider, *Comp. Biochem. Physiol.*, 68 (1981) 107.
17 P. Gold, H. Labitau, H. C. G. Wong, S. O. Friedman, J. Krupey and J. Shuster, *Cancer Res.*, 38 (1978) 6.
18 H.-J. Böhme, G. Kopperschläger, J. Schulz and E. Hofmann, *J. Chromatogr.*, 69 (1972) 209.
19 C.-B. Laurell, *Anal. Biochem.*, 15 (1966) 45.
20 G. Mancini, A. O. Carbonara and J. F. Heremans, *Immunochemistry*, 2 (1965) 235.
21 J. Janatova, J. K. Fuller and M. J. Hunter, *J. Biol. Chem.*, 243 (1968) 3612.
22 G. Kopperschläger, H. Storch and G. Birkenmeier, *Z. Med. Labor.-Diagn.*, 18 (1977) 300.
23 G. Birkenmeier and G. Kopperschläger, *J. Chromatogr.*, 235 (1982) 237.
24 K. Huse, M. Himmel, G. Birkenmeier, M. Bohla and G. Kopperschläger, submitted for publication.
25 E. Stellwagen, R. Cass, S. T. Thompson and M. Woddy, *Nature (London)*, 257 (1975) 716.
26 G. Kopperschläger, H.-J. Boehme and E. Hofmann, in A. Fiechter (Editor), *Advances in Biochemical Engineering*, Springer, Berlin, Heidelberg, New York, 1982, p. 101.