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Purification of Human Serum Albumin by Dye-Ligand Affinity Chromatography

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ABSTRACT

In this paper, four of the eight synthetic reactive dyes studied were used to isolate human serum albumin after these ligands were immobilized to cross-linked agarose gels. UV spectrophotometric methods were used to study the absorbing capacity of the eight dyes.

The relation between the adsorbing capacity or specificity of the adsorbents and the concentration of the adsorbents or the pH of the 10 mM phosphate buffers was studied; six different matrices (Xianfen beads, cross-linked dextran, polyvinyl alcohol fibre, silanized silica, cross-linked agarose and agarose beads) were evaluated. The best conditions for isolating human serum albumin were ascertained.

A larger scale dye-ligand chromatography system was used to purify human serum albumin and the cost factors involved were evaluated.

1 INTRODUCTION

Dye-ligand chromatography has been used as a technique to isolate proteins. Dye-ligand adsorbents offer many advantages, viz. they are avalilable as commodity chemicals in large quantities and at low cost, they are easily coupled directly to various matrices via their reactive functions and they are resistant to chemical and biological degradation. Furthermore, dye gels display high capacities for a wide range of proteins which can subsequently be desorbed in mild conditions and good yields.[1]

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Human serum is a complex mixture containing more than 100 different proteins. The isolation of human serum globulin is difficult, because more than 50% of the human serum content consists of albumin and this albumin has physical properties similar to those of other proteins having very low levels in human serum. It is therefore advantageous to develop effective methods to purify the albumin. In this respect, dye-ligand affinity chromatography is a very effective method.

Tavis & Pannell[2] proposed the use of the commercially available Cibacron Blue F3G-A as an affinity agent for human serum albumin. Several studies have been carried out to elucidate the binding mechanism between albumin and other serum proteins and immobilized dyes.[3-7]

Recently, a new method has been described to purify IgGs and albumin on a large scale by ion-exchange and affinity chromatography with Blue Trisacry.[8]

In this paper, some reactive dyes were used as affinity ligands to purify human serum albumin. UV spectrophotometric methods (using a circulating attachment) were used and different matrices were studied. The relations between the adsorbing capacity or specificity of the adsorbents for human serum albumin and the concentration of the adsorbents and pH value of 10 mM sodium phosphate buffers were studied quantitatively. Larger scale chromatography was used and its cost evaluated.

2 EXPERIMENTAL

2.1 Material

The reactive dyes used were synthesized by the authors. Agarose, dextran and human serum albumin were obtained from Shanghai Biochemical Material Shop and human serum from Shanghai Blood Center.

2.2 Methods

Long narrow pieces of Xianfen (obtained from bean starch) were crushed into beads, having 40–80 mesh.

Silica (60–100 mesh) (1 g) was mixed with 25 ml acetone containing 3-aminopropyltriethoxysilane at a concentration of 1% (v/v). The mixture was refluxed at 70°C for 2 h, the acetone evaporated and the silanized silica washed with water and 3M NaCl and then dried at 115°C for 6 h.

Agarose beads were prepared by mixing a hot aqueous solution of agarose at a concentration of 6% (w/w); the agarose solution was rapidly stirred until it cooled and hardened and the hardened agarose was then squeezed through an 80 sieve.

Agarose beads (1 litre) were mixed at room temperature with 1 litre of 1 M NaOH containing 5 g of $NaBH_4$, and to the stirred mixture was added 20 ml of 1-chloro-2,3-epoxypropane; the mixture was then heated at 60°C for 1 h. The cross-linked agarose beads were then washed on a coarse-glass Buchner filter with hot water till the washings were neutral.

Agarose (200 ml) at a concentration 6% (w/w) was dissolved in a pressure pan, and the solution rapidly stirred into *n*-butyl acetate (400 ml containing 2 g span 80) at 60°C for 15 min, cooled to 20°C, stirred for 15 min, filtered and washed with water, ether and water in sequence, and finally screened through an 80 sieve.

2.3 Preparation of dye-cross-linked agarose beads (dye linked other matrices with similar methods)

Cross-linked agarose beads (30 ml) were suspended in an equal volume of water and the mixture heated to 60°C. The dye (20 mg, 50 mg, 100 mg, 500 mg and 1000 mg in 30 ml of water, respectively) was added dropwise with vigorous stirring and after 10 min, 2–6 g of NaCl was added. The mixture was heated to 80°C and 1–2 g of Na_2CO_3 added. After 30 min, the dye beads were washed with hot water, followed by 10 mM sodium phosphate buffer (pH 4·7–8·5) until the washings were colourless.

2.4 Determination of immobilized dye concentrations

Immobilized dye concentrations were measured by spectrophotometric measurements of the dye released by digesting the matrix with 50% (v/v) acetic acid.[9]

2.5 Isolation of human serum albumin

A column (1 cm × 16 cm) of the dye beads was equilibrated with 10 mM sodium phosphate buffer (pH 4·9–8·5) and a sample of human serum (0·4 ml) was applied. The chromatographic experiment was run at 0°C, with a flow rate of 4 ml/24 min. All eluted proteins were collected as a single fraction until the E_{280} was less than 0·01, and the eluate was then concentrated with Visking Dialysis Tubing for polyacrylamide disk electrophoresis.

The bound proteins were desorbed from the column with 1 M NaCl. The total protein amount was measured by ultraviolet absorption and the eluted proteins were then concentrated for polyacrylamide disk electrophoresis.

The column could be regenerated for 5 M urea followed by an equilibration buffer.

2.6 Polyacrylamide disk electrophoresis

In order to determine the effect of albumin removal on serum fractionation, whole serum and serum after chromatography were subjected to polyacrylamide disk electrophoresis.[10]

Polyacrylamide disk electrophoresis was run under the following conditions: separating gel (7%, pH 8·0), stacking gel (2·5%, pH 6·7), buffer (pH 8·3), tracking dye (Cresol Red), fixing solution (12·5, trichloroacetic acid), specimen stain (Coomassie Brilliant Blue G250).

The percentage content of albumin in the eluted proteins was measured by the elution method; the eluent was 50% (v/v) acetic acid.

2.7 Spectroscopy in a study of the adsorbing capacity of eight dyes

Into the sample entrance (Fig. 1) was inserted 50 ml human serum albumin solution (25 mg albumin in 50 ml of 10 mM sodium phosphate buffer (pH 4·9–8·5)). The pump was turned on and the solution circulated several times, and the ABS value of the solution (λ 280 nm) then measured. Then, 15 g dye-cross-linked agarose gels was added to the solution, the mixture stirred and the ABS value of the mixture recorded every 30 s.

2.8 The making of the standard chart of dyes and human serum albumin

For every dye or human serum albumin, five 100 ml solutions of different concentration were made. The ABS value of each solution was measured and a standard chart drawn (see Fig. 2).

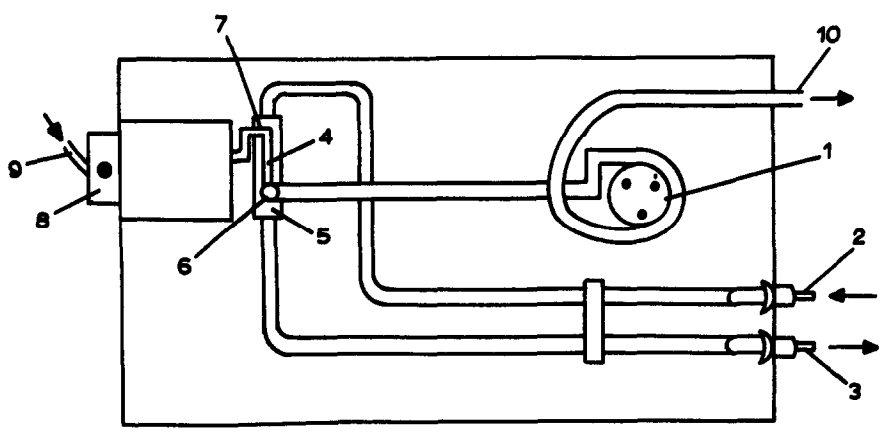


Fig. 1. Circulating device (1, pump; 2, constant temperature water entrance; 3, constant temperature water exit; 4, cell; 5, clip cover; 6, cell exit; 7, cell entrance; 8, pump switch; 9, sample entrance; 10, sample exit).

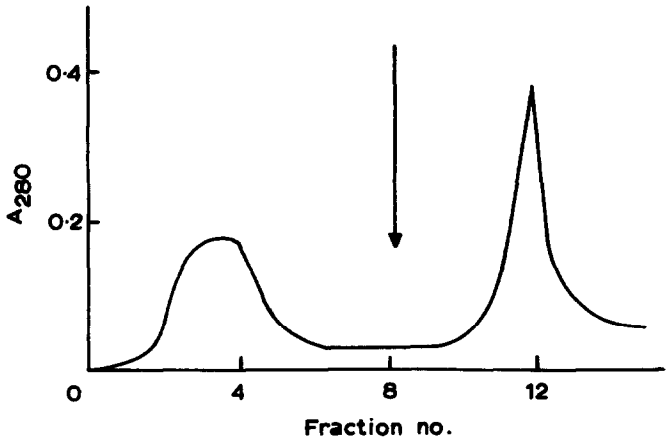


Fig. 2. Chromatography of human serum. Dye-ligand and its concentration: No. 1 dye (0·1442 μmol/ml). The column (1 cm × 16 cm) was equilibrated with 10 mM sodium phosphate buffer (pH 6·5), and 0·4 ml of human serum was applied to the gels. The column was first eluted with equilibration buffer, followed by 1 M NaCl. The flow rate was 4 ml/24 min. The arrow indicates the starting point for the elution with 1 M NaCl.

3 RESULTS/DISCUSSION

3.1 Results of affinity chromatography

3.1.1 Effect of dye-ligand concentration

The elution profile of the chromatography of human serum on dye No. 1 cross-linked agarose gels is shown in Fig. 2, using dye-ligand No. 1 (Table 1) at a concentration of 0·1442 μmol/ml.

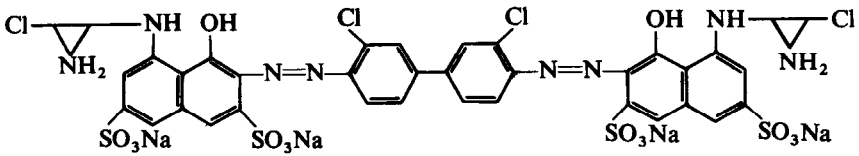
The results are shown in Tables 2 and 3. After chromatography of human serum on dye No. 1 linked agarose gels, 84·5% of human serum albumin was recovered, its purity was 86·3% (pH of 10 mM sodium phosphate buffer was 6·5).

The concentration of the immobilized ligand had a distinct influence on the efficiency of the bioaffined support. It was not necessary to prepare a support with a high concentration of the ligand. With increasing ligand density, the binding strength and non-specific interaction increased. At the same time, the efficiency of the matrix decreased owing to steric hindrance. These conclusions are apparent from Table 3, Fig. 2 and Fig. 3.

From Table 3, it is apparent that as the concentration of dye-ligand increases from 0·0121 to 0·4492 μmol/ml, the binding capacity of albumin increases from 12·27 to 29·53 mg, but purity decreases from 87·7% to 74·9%.

Figure 4 shows the relation between the binding capacity of human serum or albumin and the concentration of the dye-ligand. In the 0·0–0·15 μmol concentration range, as the concentration of dye-ligand increases, the

TABLE 1
Structures of Reactive Dyes



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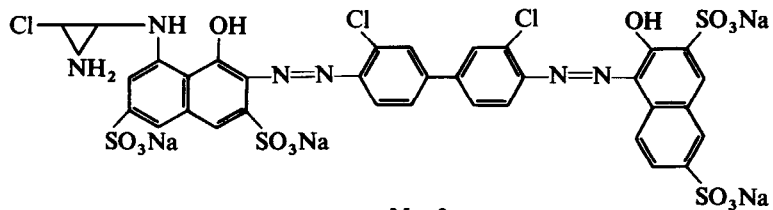

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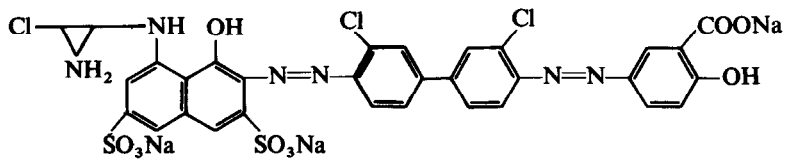

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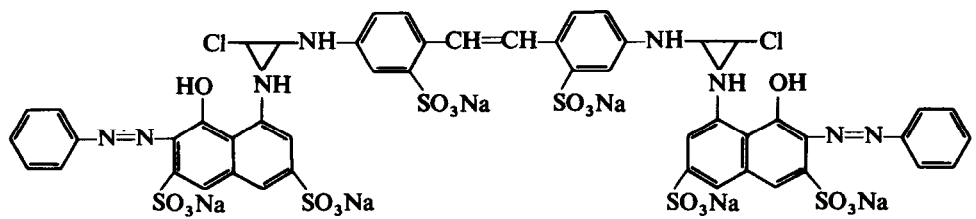

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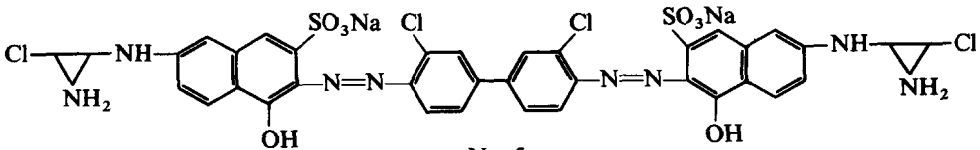

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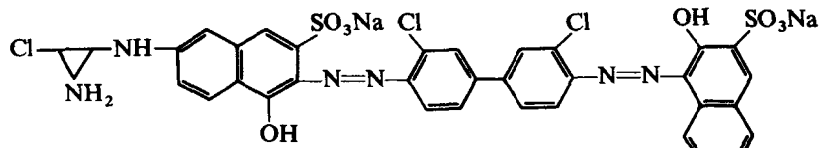

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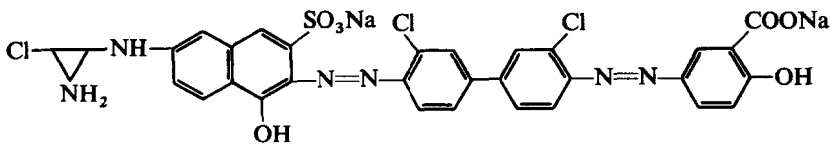

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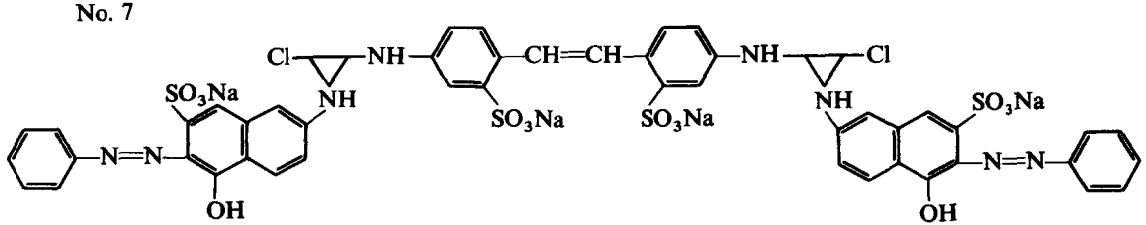

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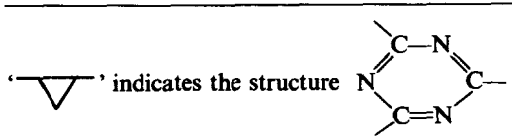
'  ' indicates the structure

TABLE 2
The Percentage Content of Albumin in Human Serum

Total protein content in human serum (mg/0·4 ml)	60·48
The percentage content of albumin in human serum (%)	54·00
Albumin content in human serum (mg/0·4 ml)	32·70

TABLE 3

C (10^{-2} μmol/ml)	*Wt* (mg)	*Pa* (%)	*Wa* (mg)	*Pr* (%)
1·21	13·99	87·7	12·27	37·5
14·42	32·00	86·3	27·62	84·5
23·54	35·60	80·1	28·51	87·2
44·92	39·42	74·9	29·53	90·3

C, concentration of dye-ligand; Wt, total binding capacity of human serum; Pa, percentage content of albumin in the binding proteins; Wa, binding capacity of albumin; Pr, recovery of albumin.
These symbols are used in all subsequent tables.

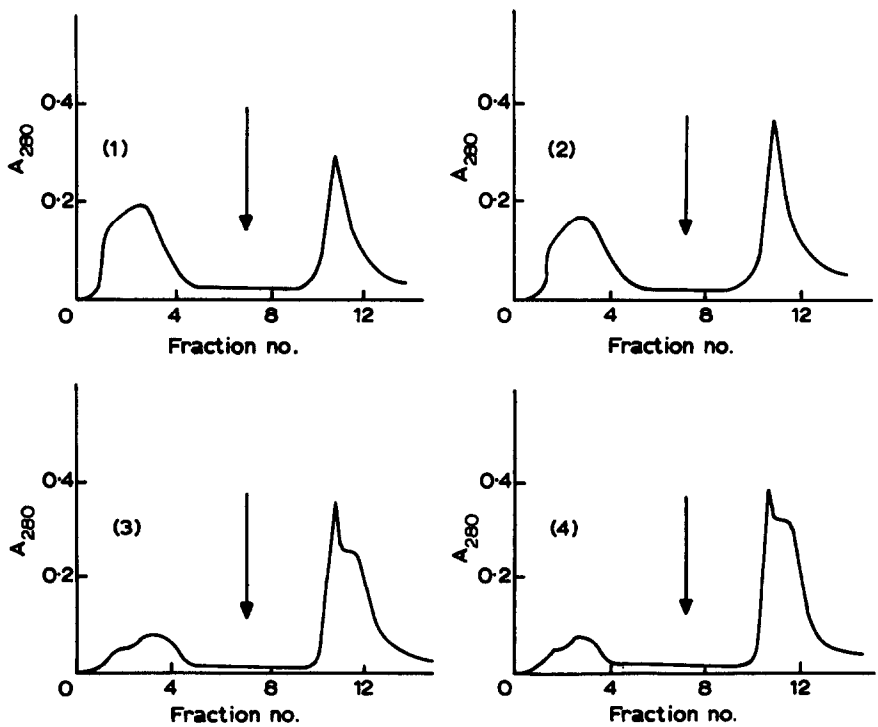


Fig. 3. The concentrations of dye-ligand were: (1) 0·0121 μmol/ml; (2) 0·1442 μmol/ml; (3) 0·2354 μmol/ml; (4) 0·4492 μmol/ml.

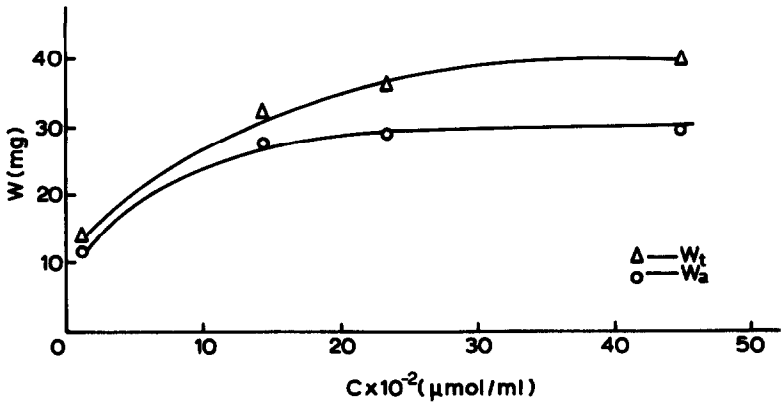


Fig. 4. Binding capacities of human serum and albumin at different concentrations of dye-ligand. The experimental conditions are shown in Fig. 2. Symbols refer to: △, human serum; ○, human serum albumin.

binding capacity of human serum increases rapidly. When the concentration of dye-ligand was above 0·15 μmol/ml, the binding capacity of human serum albumin increased slowly.

3.1.2 Effect of pH of the sodium phosphate buffer

The correct choice of pH is essential. Proteins tend to bind to immobilized dyes more tightly at lower pH values.

Figure 5 shows the change of elution profiles by affinity chromatography of human serum on dye No. 1 cross-linked agarose. Table 4 shows the change of binding capacities and purities of human albumin. With decreasing pH of the sodium phosphate buffer, the binding capacity of human serum albumin increases and its purity decreases. The best pH range for purifying human serum albumin was from 6·5 to 8·5.

Figure 6 shows the relation between the binding capacities and pH values; when the pH value was over 7·5, the percentage content of albumin in the binding proteins decreased slowly.

Figures 7 and 8 show that the bifunctional symmetrical reactive dyes Nos. 1 and 4 had better binding capacities for albumin than 2 and 3 dyes, and therefore the structural properties of the immobilized dyes play an important role in the interaction of the immobilized dyes with human serum albumin.

3.2 Results of UV spectrophotometric studies

Figures 9 and 10 show that the different dyes have different binding capacity, the binding capacity of dyes 1, 4, 5 and 8 (which are all bifunctional reactive

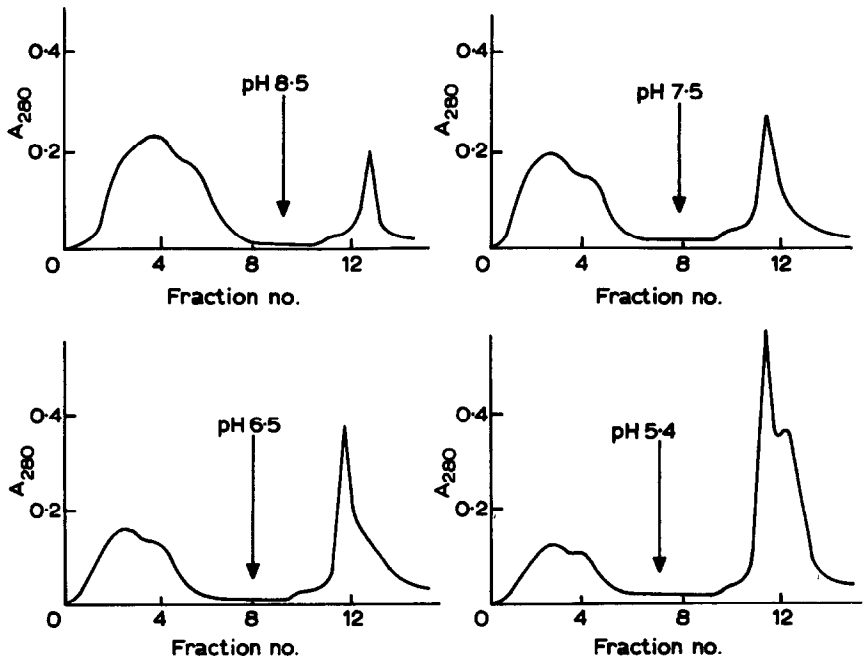


Fig. 5. Affinity chromatography of human serum using sodium phosphate buffers having different pH values; the ligand was dye No. 1 (0·1442 μmol/ml).

dyes having symmetric structure) were better than those of the other four dyes.

Table 5 shows that dyes 1, 4 and 5 had higher binding rates and Table 6 shows that although dyes 1, 4, 5 and 8 had greater binding capacity, their concentrations were lower.

Figures 11 and 12 show that, with decreasing pH of the sodium phosphate buffers, the binding capacity of human serum albumin increased.

These results from the UV spectrophotometric method were thus the same as the results using affinity chromatography. Compared to affinity chromatography, the UV spectrophotometric method was a simple and

TABLE 4

pH	*Wt (mg)*	*Pa (%)*	*Wa (mg)*	*Pr (%)*
4·9	44·35	63·2	28·03	88·7
5·4	36·55	74·6	27·27	86·5
6·5	31·06	86·0	26·70	84·5
7·5	29·41	89·6	26·35	83·4
8·5	28·50	91·8	26·16	82·8

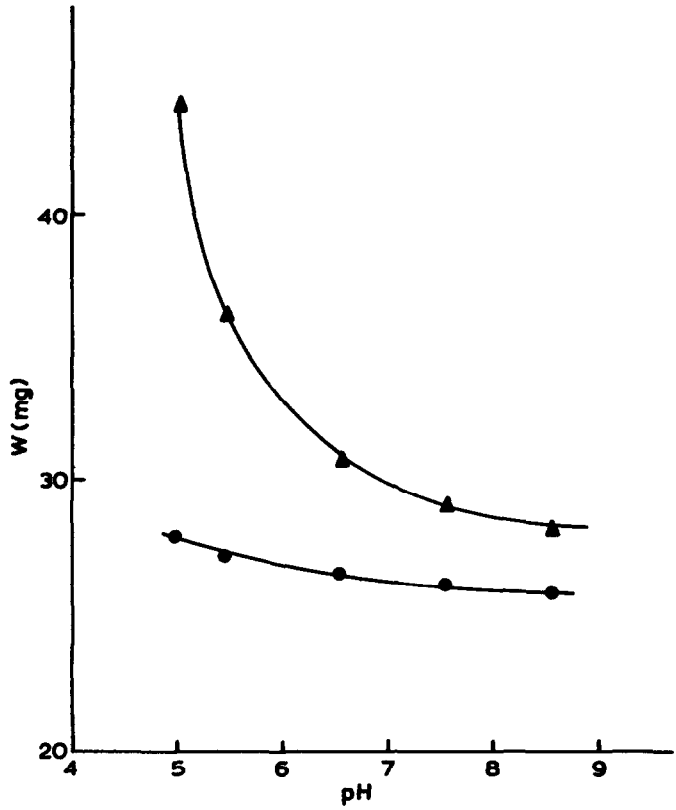


Fig. 6. Binding capacities of human serum proteins and albumin at buffers of different pH. Symbols refer to: ▲, human serum; ●, albumin.

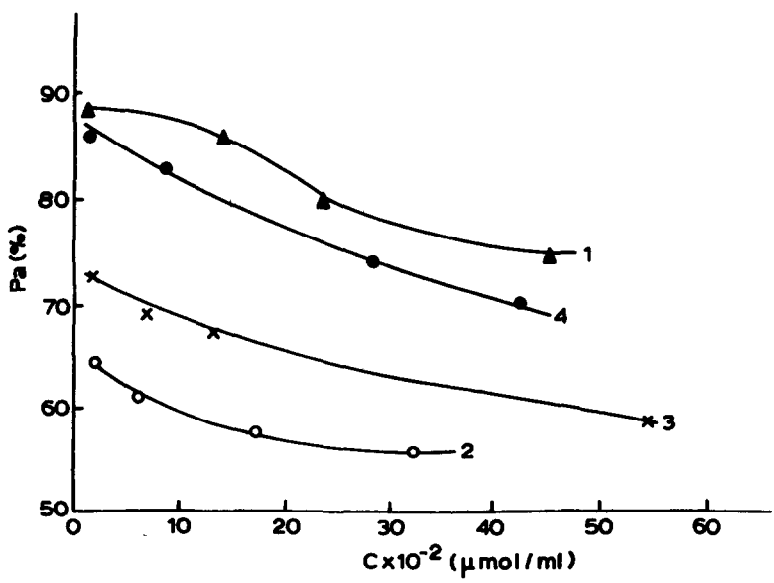


Fig. 7. The percentage content of albumin of four different dye-ligands (Nos 1–4).

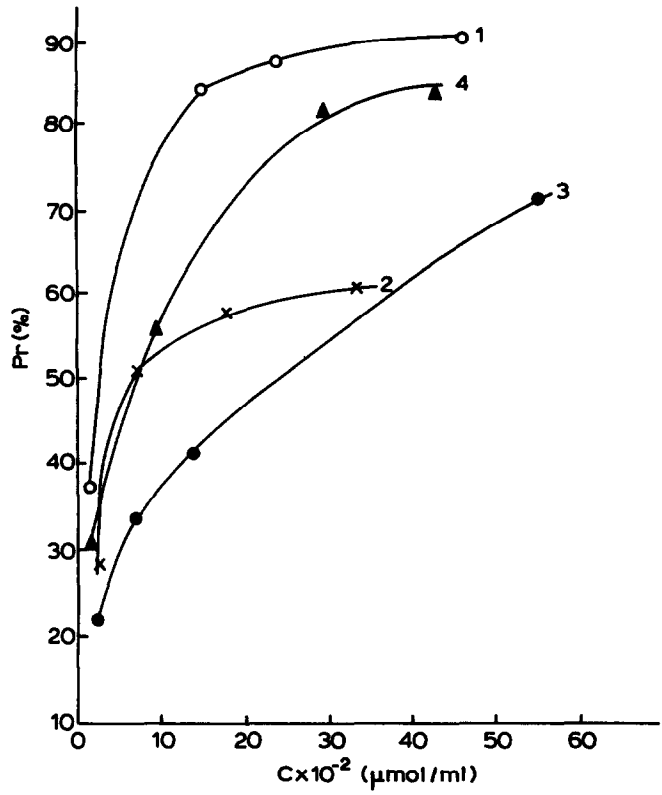


Fig. 8. The recovery of albumin for four different dye-ligands (Nos 1–4).

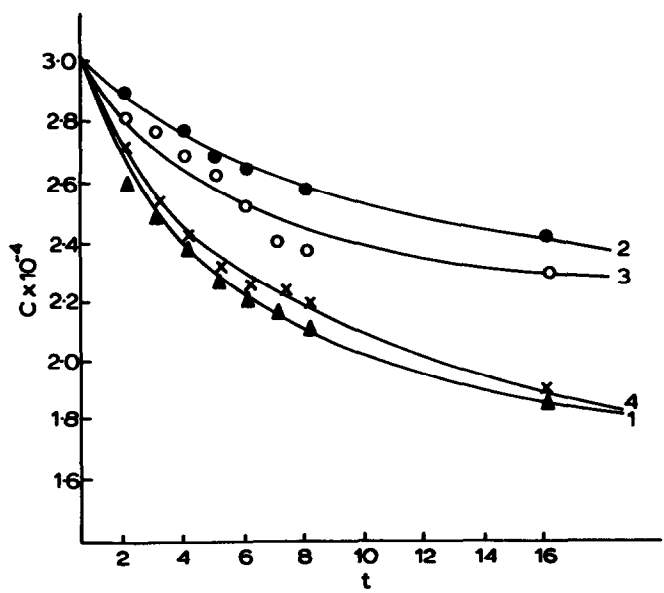


Fig. 9. Binding capacity of albumin of dye-ligands with 1–4 with change in time (C, concentration of albumin solution; t, time interval, every interval was 30 s).

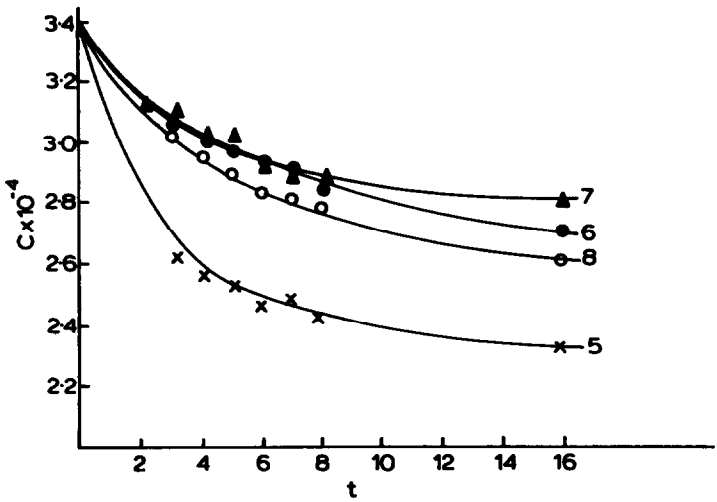


Fig. 10. Binding capacity of albumin of dye-ligands 5–8 with change in time.

rapid method for selecting a good ligand from a series of reactive dyes; it is also an effective technique in studying the relationships between the adsorbing capacity of the adsorbents for human serum albumin and the concentration of the adsorbents or the pH value of 10 mM sodium phosphate buffers.

3.3 Results of selecting matrices

Before larger scale chromatography can be carried out, a good matrix must be selected. An ideal matrix should be inert, stable (both chemically and physically) and available preferably in beaded form with large pores so that

TABLE 5
Binding Rate of Albumin with Dye-Ligands 1–8

No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
dc/dt	0·107	0·054	0·0613	0·0907	0·113	0·053	0·043	0·058

TABLE 6
The Concentration of Dye-Ligands

No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
$C \times 10^{-2}$ μmol/ml	7·62	7·93	15·82	7·86	7·40	9·20	14·82	7·92

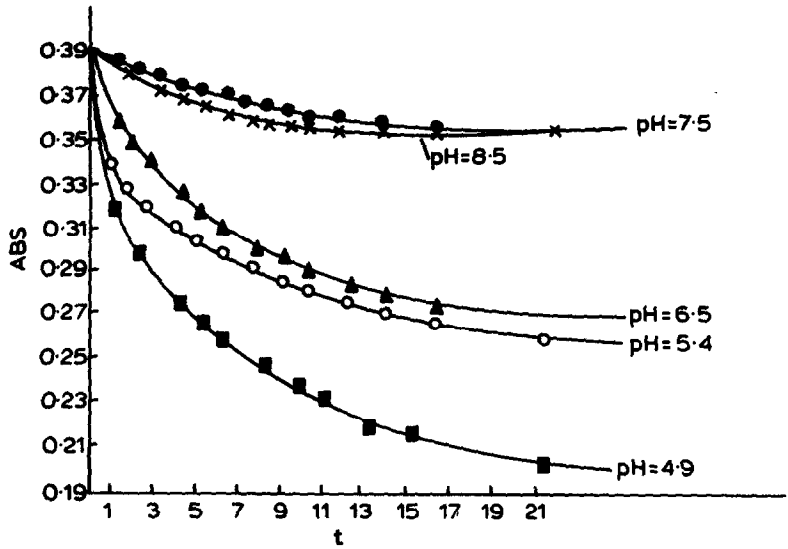


Fig. 11. The ABS value of albumin solution versus time, using sodium phosphate buffers of different pH values (the ligand was dye 6).

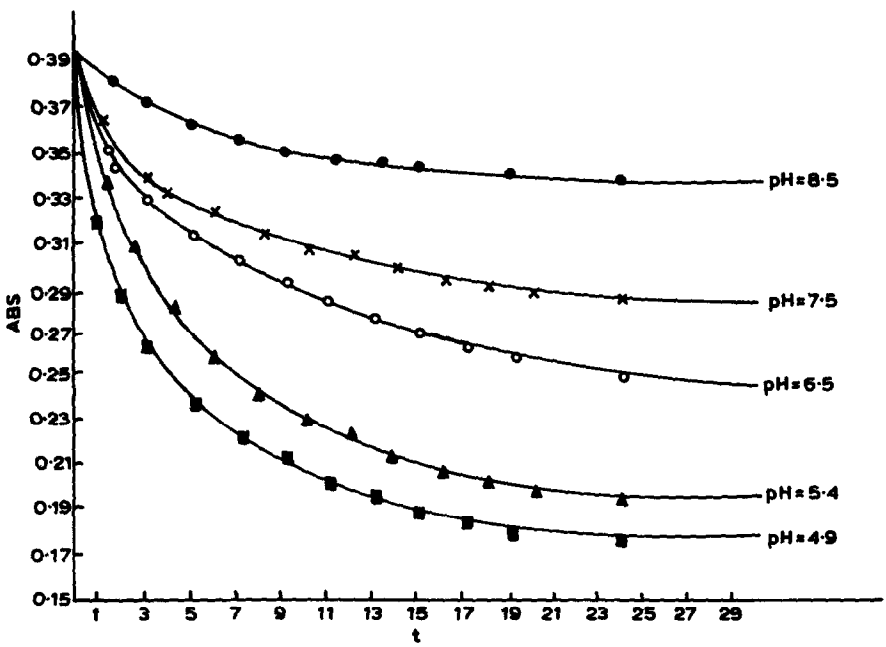


Fig. 12. The ABS value of albumin solution versus time using sodium phosphate buffers of different pH values (the ligand was dye 8).

TABLE 7
The Percentage Content of Albumin in Human Serum

Total protein content in 0·4 ml human serum (mg)	59·62
The percentage content of albumin in human serum (mg)	53·00
Albumin content in 0·4 ml human serum (mg)	31·60

TABLE 8

pH	*Wt (mg)*	*Pa (%)*	*Wa (mg)*	*Pr (%)*
4·9	14·30	88·6	12·67	40·1
5·4	13·60	90·4	12·29	38·9
6·5	12·57	94·0	11·82	37·4
7·5	12·29	94·8	11·66	36·9
8·5	11·94	94·7	11·31	35·8

The matrix was Xianfen bends.

TABLE 9

pH	*Wt (mg)*	*Pa (%)*	*Wa (mg)*	*Pr (%)*
4·9	18·23	84·1	15·33	48·5
5·4	17·44	85·9	14·98	47·4
6·5	16·21	87·5	14·91	44·9
7·5	14·89	89·3	13·30	42·1
8·5	13·37	89·3	11·94	37·8

The matrix was cross-linked dextran [G50].

TABLE 10

pH	*Wt (mg)*	*Pa (%)*	*Wa (mg)*	*Pr (%)*
4·9	20·48	83·3	17·06	54·0
5·4	20·07	85·2	17·10	54·1
6·5	19·49	86·9	16·94	53·6
7·5	17·52	89·1	15·61	49·4
8·5	16·24	89·7	14·57	46·1

The matrix was polyvinyl alcohol fibre.

TABLE 11

pH	*Wt* (*mg*)	*Pa* (%)	*Wa* (*mg*)	*Pr* (%)
4·9	14·22	84·9	12·07	38·2
5·4	13·50	87·5	11·82	37·4
6·5	12·77	89·8	11·47	36·3
7·5	12·22	91·8	11·22	35·5
8·5	11·64	92·3	10·74	34·0

The matrix was silanized silica.

TABLE 12

pH	*Wt* (*mg*)	*Pa* (%)	*Wa* (*mg*)	*Pr* (%)
4·9	41·83	68·3	28·57	90·4
5·4	36·38	76·7	27·90	88·3
6·5	31·21	87·8	27·40	86·7
7·5	29·58	91·1	26·95	85·3
8·5	29·25	91·5	26·70	84·7

The matrix was agarose beads.

the biomolecules may equilibrate with the interior part of the adsorbent without size exclusion effects; it should also be hydrophilic and possess sufficient modifiable groups to permit adequate substitution with dye.

In this paper, six different matrices were tried. Tables 8–12 list the results of isolating human serum using different dye-matrices with sodium phosphate buffers of different pH values.

Tables 8–12 and Fig. 13 show that of the six dye-matrices studied, dye-agarose beads had the best binding capacity and recovery. For large scale affinity chromatography, agarose beads were selected as the best matrix for the purification of human serum.

3.4 Results of large scale dye-ligand chromatography

The advantages of reactive dyes as ligands for purifying human serum albumin are their ease of preparation, economy, lack of complex organic ligand synthesis, reusability and the high capacity of dye matrices. These factors make larger-scale chromatography relatively cheap.

Tables 13 and 14 list the results of isolating human serum using different size columns and 10 mM sodium phosphate buffers of different pH. For the two larger columns, a sample of human serum (4 ml) was applied; this amount was 10 times greater than that for the small column (1 cm × 16 cm),

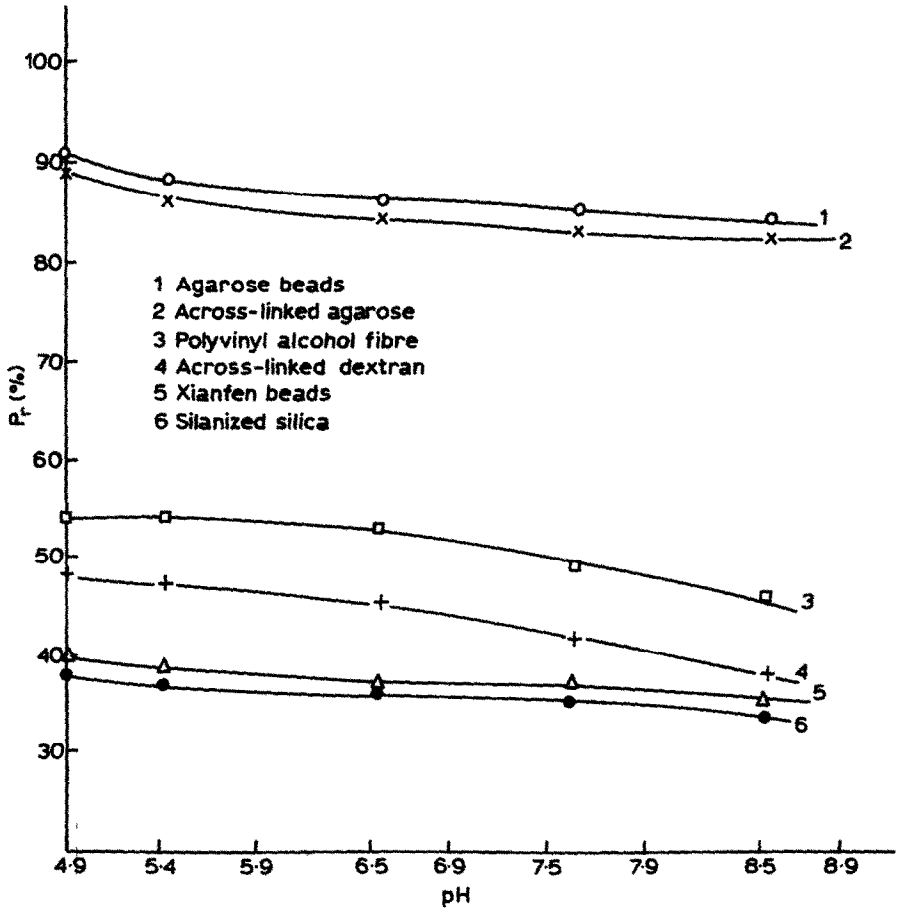


Fig. 13. Recovery of albumin using different matrices and the sodium phosphate buffer of different pH values.

TABLE 13

pH	*Wt (mg)*	*Pa (%)*	*Wa (mg)*	*Pr (%)*
4·9	36·30	72·7	26·39	83·5
5·4	31·44	81·3	25·56	80·9
6·5	27·24	90·7	24·71	78·2
7·5	25·76	93·6	24·11	76·3
8·5	25·66	93·1	23·89	75·6

3 cm × 20 cm column.

TABLE 14

pH	*Wt (mg)*	*Pa (%)*	*Wa (mg)*	*Pr (%)*
4·9	41·74	68·0	28·38	89·9
5·4	36·65	75·8	27·78	87·9
6·5	30·92	87·3	26·99	85·4
7·5	29·42	91·1	26·80	84·8
8·5	28·89	92·1	26·61	84·2

3 cm × 25 cm column.

used previously. For comparative purposes, the results in Tables 13 and 14 have been divided by 10.

It is apparent from Tables 13 and 14 and Fig. 14 that the 3 cm × 25 cm column is the most effective in the purification of human serum albumin, and from this a cost evaluation involving all start materials and other resources, on the basis of re-use of the column 20 times, was made. The cost (on

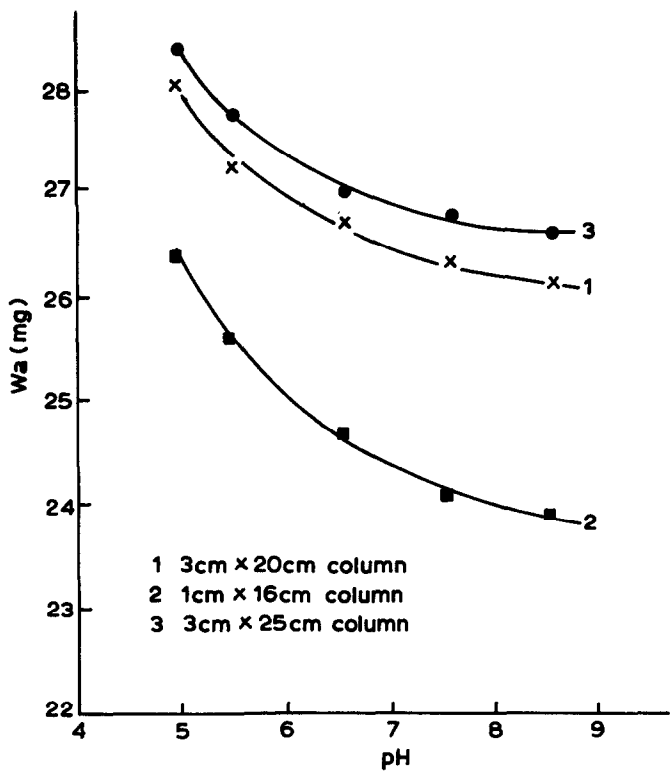


Fig. 14. Results of three different size columns.

exchange rates operative at the time of writing) was estimated at $2·68 (US) per 1 g human serum albumin.

4 CONCLUSIONS

Dye-ligand affinity chromatography is a very effective method for purifying human serum albumin. Dye-ligands with different structures have different affinities for human serum albumin and of the eight dye-ligands used in this paper, dyes 1, 4, 5 and 8 had the better affinity for human serum albumin. With decreasing pH of the sodium phosphate buffers, the binding capacity of human serum albumin increases and its purity decreases. With increasing concentration of the dye-ligand, the binding capacity of human serum albumin increases and its purity decreases. Of the six matrices used in this paper, agarose beads was the matrix in purifying human serum albumin.

A UV spectrophotometric for method using a circulating attachment is a new and effective technique for studying the relations between the adsorbtion capacity of the adsorbents for human serum albumin and the concentration of the adsorbents or the pH value of 10 mM sodium phosphate buffers.

Through larger scale chromatography, dye-ligand affinity chromatography is shown to be a very useful method, with potential industrial applications.

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REFERENCES

1. Clonis, Y. D., *Dye-ligand Chromatogr.*
2. Travis, J. & Pannell, R., *Clin. Chim. Acta*, **41** (1973) 49–52.
3. Angal, S. & Dean, P. D. G., *FEBS Lett.*, **96** (1978) 346.
4. Letherbarrow, J. & Dean, P. D. G., *Biochem. J.*, **189** (1980) 27.
5. Metcalf, E. C., Crow, B. & Dean, P. D. G., *Biochem. J.*, **199** (1981) 465.
6. Gianazza, E. & Arnaud, P., *Biochem. J.*, **201** (1982) 129.
7. Gianazza, E. & Arnaud, P., *Biochem. J.*, **203** (1982) 637.
8. Saint-Blancard, J., Kirzin, J. M., Riberon, P., Petit, F., Foucart, J., Girot, D. & Boschetti, E., *Affinity Chromatography and Related Techniques*, ed. T. C. J. Gribnan, J. Visser & R. J. F. Nivard. Elsevier Scientific, Amsterdam, 1982, pp. 305–312.
9. Dean, P. D. G. & Watson, D. H., *J. Chromatogr.*, **165** (1979) 311.
10. Ornstein, L., *Ann. NY Acad. Sci.*, **121** (1964) 321–49.