THE APPLICATION OF GEL FILTRATION TO THE MEASUREMENT OF THE BINDING OF PHENOL RED BY HUMAN SERUM PROTEINS*

MELVIN LEE AND JOSEPH R. DEBRO

Department of Preventive Medicine, University of California School of Medicine, San Francisco, Calif. (U.S.A.)

(Received June 18th, 1962)

INTRODUCTION

The development of gel filtration techniques, as described by PORATH AND FLODIN[1] and by GELOTTE[2], offers a convenient method for studying the binding of small molecules by proteins. WILCOX AND LISOWSKI[3] have used these techniques to investigate protein–metal complexes, and DEMOOR *et al.*[4] have carried out studies on the binding of corticoids by proteins, using gel filtration to separate the free and protein-bound corticoids.

The association of phenol red with proteins has been extensively examined by means of equilibrium dialysis and ultrafiltration. GROLLMAN[5] has demonstrated that the binding of phenol red by serum is due primarily to the albumin fraction. This albumin–phenol red association was shown to be pH dependent, with a maximum at about pH 4.5. No binding was demonstrable above pH 8 or 9. SMITH AND SMITH[6] found that the binding of phenol red by serum was proportional to the concentration of albumin. RODKEY[7] examined the binding of phenol red by sera of several species and studied the effect of hydrogen ion concentration on the association constants for phenol red and bovine serum albumin.

This communication is concerned with the application of gel filtration to measurements of the binding of phenol red by human serum and by human serum albumin, and suggests a method for routine serum albumin analysis. We have examined some of the factors which influence the association of phenol red with serum and serum albumin.

METHODS

Gel filtration experiments were carried out using the cross-linked polysaccharide Sephadex G-25** (medium particle size). The bed material was washed with distilled water. Fine particles were removed by repeated washing and decanting. The Sephadex, suspended in distilled water, was poured into columns 1.1 cm in diameter fitted with a coarse sintered glass disc. Unless otherwise stated, all columns were poured to a height of 12.5 cm.

* Supported, in part, by a donation from Mr. and Mrs. WINSTON S. COWGILL, in memory of CHARLES A. CHRISTENSON.

** Pharmacia, Uppsala, Sweden.

Human serum α-globulin (fraction IV) and crystalline human serum albumin were purchased from Pentex, Inc., Kanakee, Ill. Human serum γ-globulin was obtained from Squibb and Co. Serum proteins were dissolved in isotonic saline to a concentration of 4 g/100 ml. Phenol red (phenolsulfonephthalein, sodium salt) was obtained from Distillation Products, Rochester, N.Y. and was used without further purification. It was dissolved in distilled water to a concentration of $5 \cdot 10^{-3}$ M. Human serum was obtained by venipuncture and refrigerated, but not frozen, until needed.

Unless otherwise noted, all experiments were carried out in the following manner. One ml of serum was mixed with 2 ml of phenol red solution. The pH was adjusted to the desired point with dilute HCl or NaOH, with rapid agitation. After standing for 1 h at room temperature, an aliquot, usually 100 μl, was applied to the column and elution was started with distilled water. The effluent was collected in 10-drop fractions, using an automatic fraction collector equipped with a drop counting unit. To each fraction was added 1 drop of N NaOH and 3.0 ml of distilled water. The optical density of the solution was measured at 520 mμ, using a Bausch and Lomb Spectronic 20 Spectrophotometer.

Total protein and albumin were determined according to HAWK, OSER AND SUMMERSON[8], using the biuret reagent.

The bound phenol red was calculated as percent of total phenol red (100 × O.D. of bound phenol red/total O.D. of phenol red). All column separations were carried out in duplicate.

RESULTS

Fig. 1 shows the elution pattern of human serum albumin alone and of a mixture of human serum albumin and phenol red, pH 4.5. It may be seen that there are two phenol red peaks, one of which is superimposed on the protein peak and represents

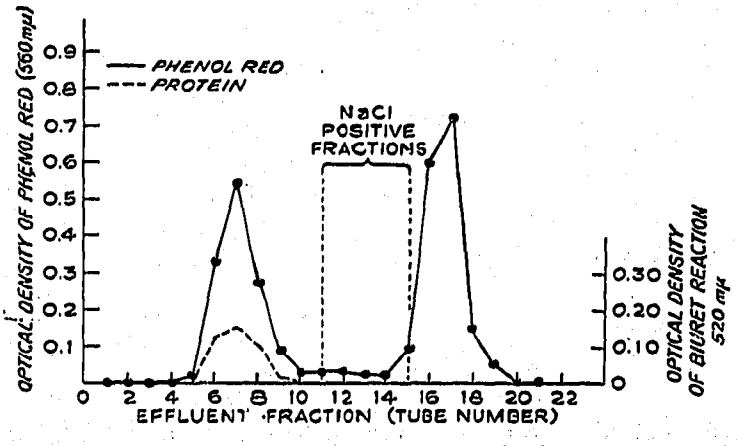

Fig. 1. Elution pattern of human serum albumin alone and of a mixture of human serum albumin and phenol red, at pH 4.5. Details of technique are given in text.

protein-bound phenol red. The second peak represents free, or unbound, phenol red. Some interaction between phenol red and the Sephadex is suggested by the fact that the free phenol red peak lags slightly behind the elution of added NaCl.

Sephadex appears to remove some protein-bound molecules, such as triiodothyronine, from protein, so that the apparent extent of binding is influenced by the

length of the column[9]. Fig. 2 shows the effect of varying the column length on the binding of phenol red by human serum. It is clear that phenol red binding is not influenced by column lengths between 7.5 and 12.5 cm and, therefore, the precise height of the bed material is not critical. A column length of 12.5 cm was chosen in order to obtain a satisfactory separation between the two phenol red peaks.

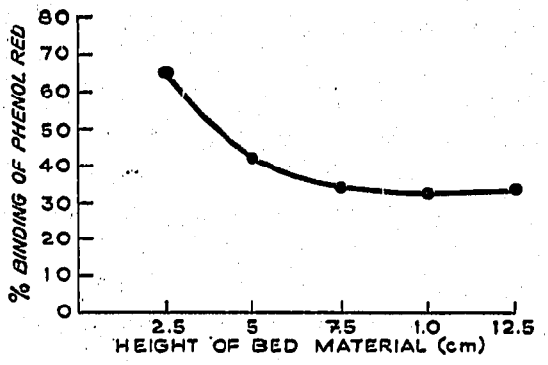

Fig. 2. Effect of Sephadex column length on the binding of phenol red by human serum.

Fig. 3 illustrates the influence of hydrogen ion concentration on the extent of binding of phenol red by human serum albumin. It is seen that there is a maximum binding at about pH 4.0–4.5 and a plateau between pH 6 and pH 7.5. The binding falls to zero at about pH 8.5.

The interaction of human serum γ-globulin (4 g/100 ml) with phenol red was studied over the range of pH 5–9. No binding could be demonstrated at any point. The interaction of human serum α-globulin (4 g/100 ml) with phenol red was measured at pH 4.5. Under these conditions approximately 0.5 % of the phenol red was bound by the protein, whereas about 25 % of the phenol red was protein-bound when a similar concentration of albumin was employed at pH 4.5.

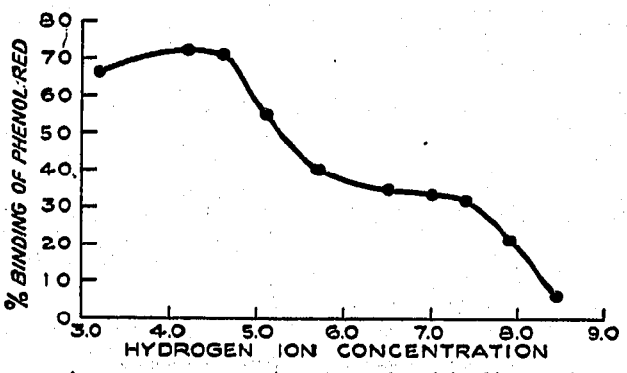

Fig. 3. Influence of hydrogen ion concentration on the binding of phenol red by human serum albumin.

The above results would indicate that, under the conditions described, virtually all of the phenol red bound by human serum is attached to albumin. The relationship between albumin concentration and phenol red binding is shown in Fig. 4. It may be seen that a direct proportionality exists between protein concentration and phenol red binding over the range of concentration of albumin of 4 mg/ml to 20 mg/ml. This is true both of crystalline human serum albumin and pooled human serum.

TABLE I

COMPARISON OF THE RESULTS OF SERUM ALBUMIN DETERMINATIONS BY SODIUM SULFATE FRACTIONATION AND BY PHENOL RED BINDING

Serum	*Albumin concentration (g/100 ml)*	
	Sodium sulfate	*Phenol red binding*
1	3.7	3.6
2	3.8	3.8
3	3.8	3.7
4	4.7	4.9
5	4.5	4.7
6	4.9	4.7
7	3.9	4.0
8	4.9	5.2
9	3.7	3.9
10	4.6	4.8

That this method is applicable to the measurement of albumin in human serum is shown by Table I. The albumin concentrations of several randomly selected human sera were determined by sodium sulfate fractionation and by phenol red binding. In most cases the two methods agree to 0.2 g/100 ml or better, a maximum difference of about 5 %.

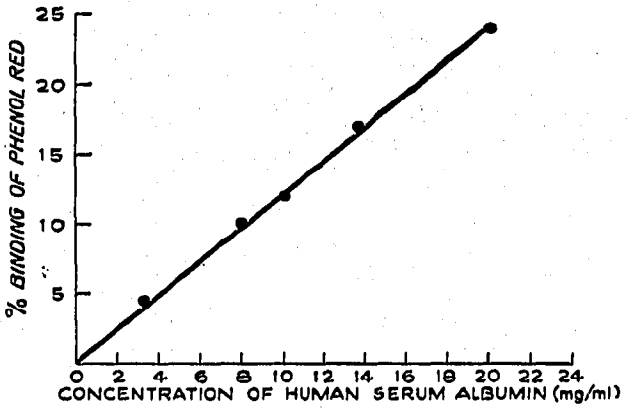

Fig. 4. Relationship between albumin concentration and the extent of binding of phenol red.

DISCUSSION

Application of gel filtration, using such materials as Sephadex, would seem to be well suited to the study of protein–small molecule binding. It is inherently simpler and less time consuming than is equilibrium dialysis, the most common technique used in the past. However, gel filtration cannot be applied to all systems, since the gel binds some small molecules sufficiently tightly so that it competes with protein for these compounds. However, where interaction with the bed is weak, the method can be used with good results.

It would appear that the albumin–phenol red system offers a good model for studying this application of gel filtration. Over reasonable column lengths the binding of phenol red by protein is constant and the weak interaction of phenol red with the bed material is sufficient to produce a good separation of bound and free forms of the dye.

Gel filtration studies have shown, as have ultrafiltration experiments, that phenol red is bound by human sera and that this binding is primarily due to the albumin in the serum. It would appear that gel filtration would offer a possible method for determining serum albumin levels and albumin/globulin ratios.

SUMMARY

The application of gel filtration to studies of the interaction of phenol red with serum proteins has been examined. Phenol red is bound primarily by serum albumin, although a comparatively minute amount is bound by serum α-globulins. Serum γ-globulins do not bind phenol red. The combination of phenol red with albumin is pH sensitive, with a maximum at pH 4.5. No binding was observed above pH 8.5. The extent of binding of phenol red by albumin within the concentration range tested, is proportional to the concentration of phenol red. The use of this technique for the determination of human serum albumin concentrations is demonstrated.

REFERENCES

1 J. PORATH AND P. FLODIN, *Nature*, 183 (1959) 1657.
2 B. GELOTTE, *J. Chromatog.*, 3 (1960) 330.
3 P. E. WILCOX AND J. LISOWSKI, *Federation Proc.*, 19 (1960) 333.
4 P. DEMOOR, K. HEIRWEGH, J. F. HEREMANS AND M. DECLERCK-RASKINS, *J. Clin. Invest.*, 41 (1962) 816.
5 A. GROLLMAN, *J. Biol. Chem.*, 64 (1925) 141.
6 W. W. SMITH AND H. W. SMITH, *J. Biol. Chem.*, 124 (1938) 107.
7 F. L. RODKEY, *Arch. Biochem. Biophys.*, 94 (1961) 38.
8 P. B. HAWK, B. L. OSER AND W. H. SUMMERSON, *Practical Physiological Chemistry*, 13th Ed., Blakiston Co., New York, 1954, p. 605.
9 M. LEE AND S. MIYAMOTO, unpublished observations.