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STUDIES ON THE INTERACTION BETWEEN SMALL MOLECULES BY GEL FILTRATION IN SEPHADEX LH-20

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SUMMARY

A method for the study of the interaction between small molecules by gel filtration is described, based on the difference in the retention of the reactants and product in Sephadex LH-20 gel. The interaction between the anionic detergent sodium dodecyl sulphate and methylene blue and tryptophan in acidic solution is described.

INTRODUCTION

A considerable amount of information is available on the interaction of small molecules with Sephadex gels of high density, *e.g.*, Sephadex G-10, G-25 and LH-20[1–6]. The interaction is determined by hydrogen bonding, π-electron or hydrophobic interaction, depending upon the gel and solutes involved[2–6].

Recently, we observed that N-acetyl esters of aromatic amino acids are retained (by hydrophobic interaction) in Sephadex LH-20 (hydroxypropyl derivative of Sephadex G-25) gel[6]. It was also inferred that the presence of charges in the aromatic amino acids interfered with their retention in the gel. N-acetylphenylalanine, which carries a negative charge at neutral pH, is not retained in the gel although at acidic pH, where no effective charge is present, the molecule is considerably retained.

Based on the above observations, an attempt has been made to study the interaction between small molecules by gel filtration. When a group containing a relatively large non-polar moiety with charge(s) attached to it interacts with a similar group but with opposite charge(s), a complex having a reduced or no effective charge would result. This complex would be retained more than the reactants in the gel. In reactions where non-polar moieties having similar types of charge interact, the resulting complex, having a more effective charge, would be eluted before any of the reactants.

In this paper, we report a study of the first type. A Sephadex LH-20 gel column is equilibrated with a solution of reactant, R_I, at a desired concentration. A small amount of a solution of the other reactant, R_{II}, in which the total concentration of R_I is equal to the concentration at which the gel column has been equilibrated, is

then added to the column. If R_{II} interacts with R_I to form a complex, the sample in which R_{II} is equilibrated with R_I will be depleted with respect to R_I. As the mobility of the complex would be less than the mobility of R_I, the front depleted in R_I will move along the column faster than the complex. The resultant elution profile will show a trough below the base-line of R_I solution representing the depletion. This would be followed by the appearance of a peak of the complex enriched in R_I. From the areas of the troughs or crests of the complex resulting from different R_I concentrations with a constant R_{II} concentration, the binding parameters can be determined. This principle was used in order to study the interaction between the anionic detergent sodium dodecyl sulphate (SDS) and methylene blue (MB) and tryptophan.

MATERIALS AND METHODS

Sephadex LH-20 (Sigma. St. Louis, Mo., U.S.A.), L-tryptophan (Merck, Darmstadt, G.F.R.) and methylene blue (Stain Grade; BDH, Poole, Great Britain) were used. Both tryptophan and the dye showed a single elution peak when eluted in the gel. Sodium dodecyl sulphate was recrystallized twice from 50% ethanol. Columns of dimensions 11 × 1.8 and 12 × 2.1 cm with bed volumes (V_t) of 28 and 42 ml were used for the methylene blue and tryptophan studies, respectively. Distilled water was adjusted to pH 2.2 with hydrochloric acid (AnalaR).

The gel was soaked in the acidic solution (pH 2.2) for *ca.* 60 h before it was packed into the column. For equilibration by different concentrations of MB or tryptophan, the gel in the column was washed until the absorbance of portions of eluate from the gel was identical with the absorbance of the washing solvents.

A 1-ml volume of stock SDS was added to 1 ml of MB or tryptophan so that the concentration of the dye or amino acid was equal to its equilibration concentration in the gel column. Then 1 ml of the mixture was loaded into the column and 5-ml aliquots of eluate were collected, except around the trough, where the volume of each aliquot taken was 2 ml. The flow-rate was 25–30 ml/h. The concentration range for MB was $5 \cdot 10^{-6}$–$2.5 \cdot 10^{-5}$ M (SDS. $2.5 \cdot 10^{-3}$ M). The range of tryptophan concentration was $5.5 \cdot 10^{-5}$–$2.6 \cdot 10^{-4}$ M (SDS, $1 \cdot 10^{-2}$ M).

RESULTS

Elution profiles are shown in Fig. 1. The elution volumes of the dye and tryptophan were 85 and 78 ml, respectively. The lowest values of the troughs appeared at the positions of the elution peaks of the above two compounds within 5 ml. With MB, the visible depletion of its concentration, indicated by the movement of a zone having a low dye concentration, could be seen. This MB-depleted zone was followed by a dye-enriched (complex between the dye and detergent) front. The enriched front showed a gradual decrease in concentration (dissociation of the complex) as it moved through the length of the gel. By adjusting the flow-rate and volumes of aliquots collected, a separation of about 5–7 ml was obtained between the value when the trough reached equilibrium and the appearance of the complex. This procedure was followed for the tryptophan–SDS system also.

The number of moles of MB or tryptophan bound by SDS in the gel can be obtained from the procedure of Fairclough and Fruton[7]. The difference between the

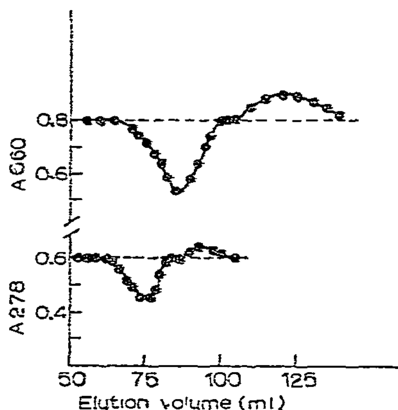

Fig. 1. Elution profile for measurements of the binding of MB (top) and tryptophan (bottom) with SDS at pH 2.2 and 25°. MB $1.1 \cdot 10^{-5}$ M, SDS $2.5 \cdot 10^{-6}$ M. Tryptophan $1.09 \cdot 10^{-4}$ M, SDS $1 \cdot 10^{-5}$ M.

base-line absorbance of each fraction constituting the trough was determined and used in the equation

$$\mu\text{moles bound} = \frac{\sum_i (\Delta A_i \times \text{ml}_i)}{\varepsilon \times 10^{-3}}$$

where ΔA_i is the difference between the base-line absorbance value and that of fraction i and ε is the molar absorptivity of either MB or tryptophan. The values of ε are $7.2 \cdot 10^4$ at 660 nm and $5.7 \cdot 10^3$ at 278 nm for MB and tryptophan, respectively.

The binding parameters, *viz.*, the number of binding site(s), n, and the association constant, k, were obtained from the double reciprocal plot of Klotz:

$$\frac{1}{\bar{\nu}} = \frac{1}{n} + \frac{1}{nkC}$$

where $\bar{\nu}$ is the number of moles of MB or tryptophan bound and C is the concentration of either MB or tryptophan with which the gel is equilibrated.

DISCUSSION

The first necessary criterion for the study of binding equilibria by gel filtration is that the minimum in the concentration in the depleted front (trough) should appear at the elution volume of the compound with which the column has been equilibrated. In the present study, the minima in the troughs appear within 5 ml of the elution volumes of both MB and tryptophan. The second criterion is that the appearance of the enriched front of the equilibrating solvent (complex) should not interfere with the measurements in the trough region. In this study, by adjusting the flow-rate and aliquots collected, a separation of 5–7 ml can be obtained between the value at which equilibrium of the depleted front is attained and the appearance of the complex-enriched front.

The double reciprocal plot in Fig. 2 shows a reasonably good linear relationship between $1/\bar{\nu}$ and $1/C$. The plot results in 1:1 stoichiometry and an association

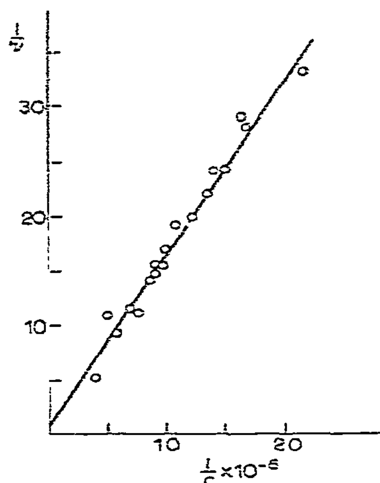

Fig. 2. Double reciprocal (Klotz) plot for the interaction between SDS and MB at pH 2.2 and 25°.

constant of $6.6 \cdot 10^4$ mole/l for the reaction between MB and SDS. The 1:1 stoichiometry indicates a simple metathetical reaction. The evidence of 1:1 complex formation for the above interaction is already available in the literature[8]. The association constant of the above reaction ($C_{16}H_{18}N_3S^-$ and $^-O_4SC_{12}H_{25}$) compares well with the association constant of $4.2 \cdot 10^4$ obtained from conductivity measurements for the reaction between the decyltrimethylammonium ion and azobenzene-4-sulphonate ($C_{13}H_{20}N^-$ and $^-O_3SC_{12}H_9$)[9].

Fig. 3 shows the double reciprocal plot for the tryptophan–SDS system. Of the various straight lines that can be drawn through the points, the one (shown in Fig. 3) resulting in an association constant of $2.8 \cdot 10^3$ and binding site (n) 1 represents most satisfactorily the binding isotherm (inserted in Fig. 3). These parameters describe the binding isotherm reasonably well in the region where the double reciprocal plot shows considerable scattering. The interaction between tryptophan and SDS can, therefore, be considered to be a simple metathetical reaction.

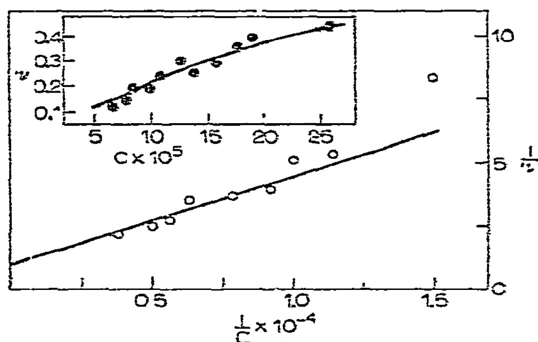

Fig. 3. Double reciprocal (Klotz) plot for the interaction between SDS and tryptophan at pH 2.2 and 25°. Insert: the line represents the binding isotherm between tryptophan and SDS with $n = 1$ and $k = 2.8 \cdot 10^3$ and the circles are experimental results.

The interaction between SDS and tyrosine and phenylalanine did not show any well defined trough by which studies such as those described above could be made. The complexes formed, probably having low association constants, dissociate rapidly while moving through the gel bed. This indicates that unless the association constant between the reactants are above certain value, the interaction between the molecules cannot be studied by the method described here.

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