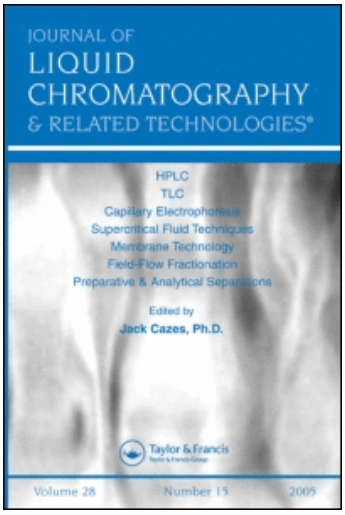

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ADSORPTION EFFECTS OBSERVED IN GEL PERMEATION CHROMATOGRAPHY OF THIOURACIL

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ABSTRACT

In gel permeation chromatography (GPC), several compounds deviate from the molecular volume/elution count relationship which is prepared using satured hydrocarbons. In this paper, this problem is investigated in detail using thiouracil in aqueous solution as a model chromatographic adsorbate. The concentration dependences of elution counts and peak heights prove the adsorption of thiouracil on Sephadex G-25 when water is the solvent. Thus to investigate further the mechanisms of adsorption responsable for the chromatographic behaviour, thiouracil-Sephadex interactions were investigated by studying equilibrium adsorption. Isotherms of type IV of BDDT classification were found which are typically associated with a weak adsorption such as physisorption, on a porous solid. The effect of water structure perturbants, ionic strength and pH on this adsorption was consistent with the hypothesis that with water as a solvent both aromatic adsorption and electrostatic interaction are the determinants of the affinity of this gel for a thiouracil compound. This may be particularly useful since results of equilibrium adsorption isotherms are frequently used to develop liquid chromatographic theories.

INTRODUCTION

GPC through Sephadex gels has been widely applied for the determination of molecular weights of peptides and macromolecules other than proteins, and the determination of binding parameters of macromolecules with low-molecular-weight ligands.

However many organic compounds are eluted from Sephadex beds at higher elution counts than are to be expected from data for aliphatic hydrocarbons (1-2). The phenomenon is assumed to be due to the adsorption of solute molecules on to the gel. If adsorption of solute molecules occurs, the peak height or the area under the peak will not be proportional to the concentration, because a certain number of solute molecules will stay longer in the columns than the rest.

Since Sephadex is a cross-linked dextran gel of a porous nature, the surface of the adsorbent can be external or internal. The former is that which constitutes the physical limits of the solid, the latter, the internal surface, is that which corresponds to the pores of the material. The size of these pores is of considerable importance, given that in order that their surface can adsorb it is neccesary that the adsorbate molecules can pass through them.

There is considerable evidence (3-6) that a hydrophobic interaction (7-8) is involved in the affinity of the more highly cross-linked, but otherwise unsubtituted dextran gels for non-polar and weakly polar compounds, but there has been little discussion concerning mechanism of adsorption in liquid chromatography with Sephadex gel when other adsorbate-adsorbent interactions occur.

As part of our research on thiocarbamides of low molecular weight, we observed an anomalous behaviour in the thiouracil elution through a Sephadex bed while compounds more apolar such as propylthiouracil have a normal behaviour. To investigate further the mechanisms of adsorption responsible for this chromatographic

behaviour, we conducted equilibrium adsorption studies of thiouracil on Sephadex G-25 (fine).

EXPERIMENTAL

Uracil, 2-thiouracil, 6-n-propyl-2-thiouracil and human serum albumin (A 1887) were bought from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephadex G-25 fine grade was bought from Pharmacia Fine Chem. (Uppsala, Sweden). 6-n-Propyluracil was synthesized according to the method of Lindsay et al (9).

GPC

Gel permeation chromatography was performed in a Pharmacia K 16/40 column which was packed Sephadex G-25 fine gel according to the instructions from the manufacturer (10). The flow rate of 1.0 ml/min was controlled with a LKB peristaltic pump. The column was equilibrated with phosphate buffer 0.066 M, pH 7.4 at 37°C. All solutions were carefully degassed before use. One ml of sample was injected into the column. Samples were directly monitored at 254 nm with a LKB detector, and occasionally with a Beckman - Spectrophotometer model UV-3600 for 270 nm detection.

Adsorption of thiouracil by Sephadex

Adsorption experiments were conducted at 0°C, 21°C and 37°C. For the preliminary kinetic experiments, 20.0 ml of a 0.50 mM solution of thiouracil in phosphate buffer were added to flasks -- containing Sephadex. Aliquots of the supernatants (3.0 ml) were then removed after specific periods of time. Concentrations of thiouracil were determined by spectrophotometric titration, using a Beckman UV-3600 spectrophotometer.

For the final equilibrium adsorption studies, 20.0 ml of va-- rious concentrations of thiouracil in phosphate buffer were added to flasks containing Sephadex. After at least 1 h 30 min and occasional swirling, supernatant concentrations of thiouracil were de

termined by spectrophotometric titration. Amounts adsorbed by Sephadex were determined by the difference between the initial and final concentrations (Details of this experimental method may be found in ref. 11).

RESULTS AND DISCUSSION

Elution behaviour by GPC

The elution behaviour of several carbamides in a Sephadex bed was studied. Since Sephadex permeation chromatography is widely used at neutral pH in biomolecular analysis, a phosphate buffer eluent was used. The concentration dependence of peak heights was measured for various compounds. Figure 1 shows these results. In propyluracil and propylthiouracil the peak heights are completely proportional to the concentration. These straight lines pass through the origin. In thiouracil, the peak heights vs. concentration curves, however, do not pass through the origin. Since if adsorption of solute molecules takes place on the gel surface, the peak heights in GPC will not be proportional to the concentration of solutes because of retardation of a certain number of solute molecules and the thiouracil molecules are adsorbed on the Sephadex. Figure 1 demostrates that the peak of the thiouracil disappears at a certain concentration. The solute molecules are completely adsorbed on the gel surface at the concentration where the peak disappears.

As shown in Figure 2, the elution volume in thiouracil decreases with increasing concentration because the elution volume is considerably increased by adsorption at low concentration. On the other hand, the behaviour in propyluracil and in propylthiouracil is contrary to that of thiouracil and is quite normal for GPC (12). The concentration dependence of the elution volume also proves the occurrence of adsorption in thiouracil.

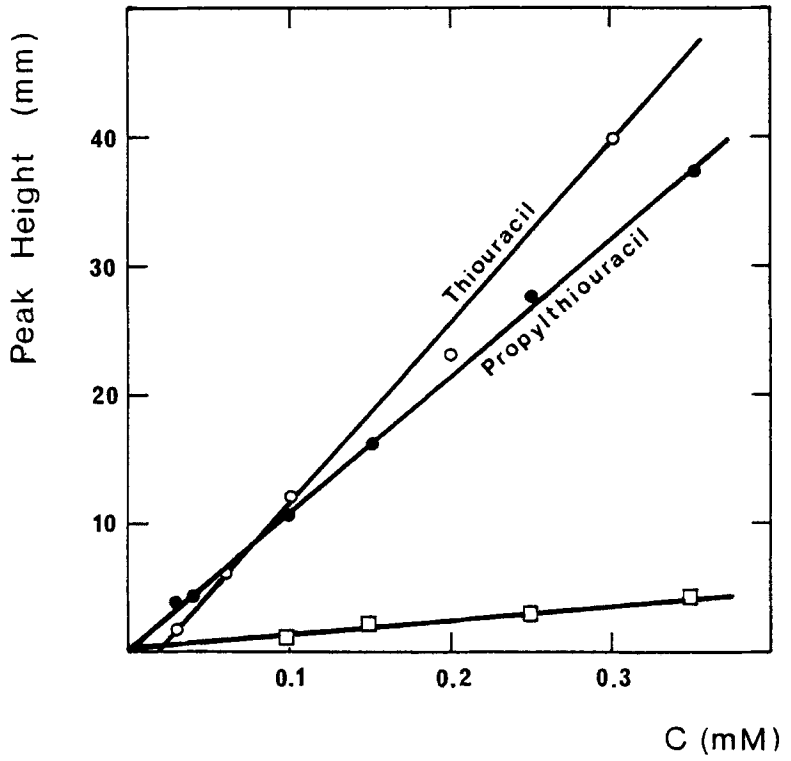

FIGURE 1.- Concentration dependence of peak heights in phosphate buffer solution.

Equilibrium adsorption isotherms

A series of experiments was first conducted to determine the time for attainment of adsorption equilibrium. A plot of mM g-1 adsorbed vs. time (Figure 3) indicated that adsorption equilibrium for thiouracil was attained in ca. 20 min.

The percentages of the adsorption of thiouracil by Sephadex G-25 (fine) are shown in table 1. In no case is the 23% adsorption per gram of gel passed. By means of the results described in this table, one different value of the capacity of single layer per gram of adsorbent, Xm, was obtained at every temperature

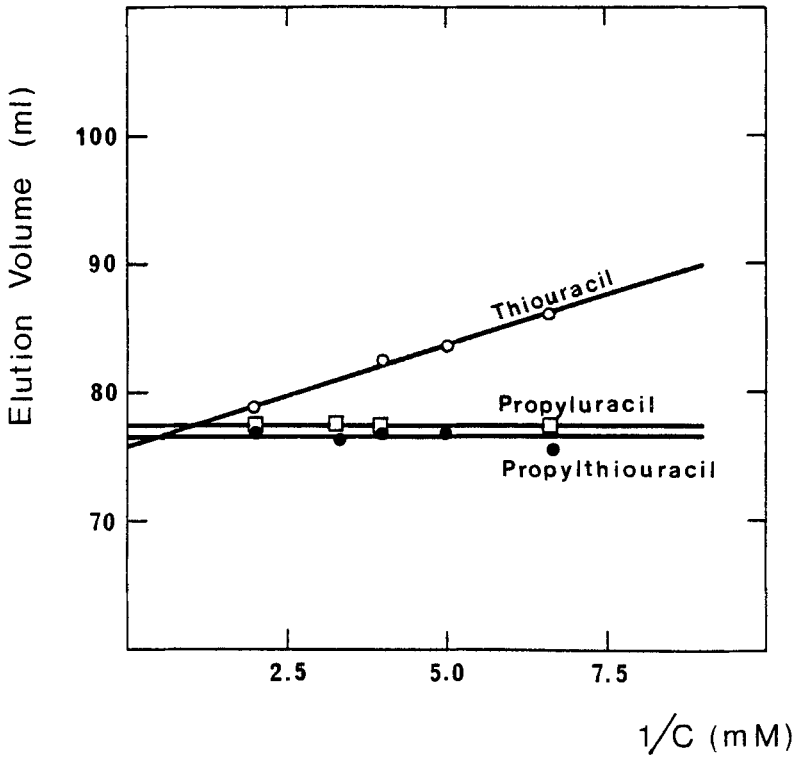

FIGURE 2.- Concentration dependence of elution volumes in phos-- phate buffer solution.

used. One Xm value of 2.00 was calculated by the adsorption of thiouracil at 37°C, being the Xm values calculated at 21°C and 0°C of 1.11 and 1.00 respectively, that is, the capacity of the single layer decreased with a decrease in the temperature.

The complete adsorption curves for thiouracil at 0°C, 21°C and 37°C (Figure 4) have the general appearance of type IV (low affinity phenomenon) of the Brunauer, Deming, Deming and Teller (BDDT) classification. Type IV curves, with hysteresis and saturating sections, are typically associated with weak adsorption -

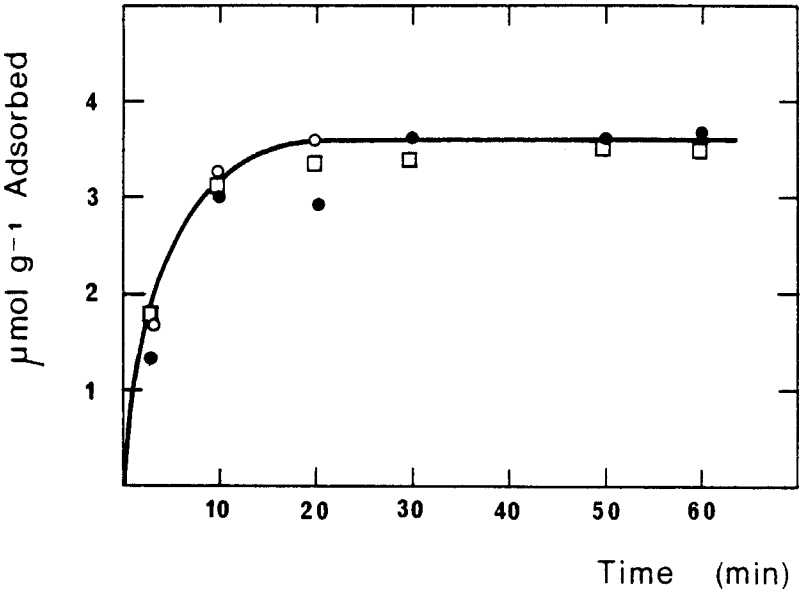

FIGURE 3.- Kinetics of adsorption of thiouracil on Sephadex G-25 (fine) at 0°C (□), 21°C (o) and 37°C (●). Thiouracil concentration was 0.50 mM.

TABLE 1

Thiouracil Values Adsorbed for Various Concentrations of Adsorbate at Three Temperatures

Thiouracil	Thiouracil adsorbed on Sephadex G-25 (fine)					
(μM)	μmol/g at 0°C	%	μmol/g at 21°C	%	μmol/g at 37°C	%
25.4	0.007	0.0	0.022	2.2	0.133	11.5
51.6	0.070	0.1	0.113	5.5	0.323	13.8
105.5	0.178	0.2	0.315	7.5	0.694	15.0
212.8	0.634	0.3	0.826	9.7	1.427	15.7
314.3	1.022	0.3	1.297	10.3	2.607	19.0
419.1	1.110	0.3	1.475	8.8	3.377	18.3
431.7	1.600	0.4	1.840	10.6	3.390	18.2
532.2	2.250	0.5	2.080	9.8	4.140	18.1
636.8	1.960	0.3	2.660	10.5	5.460	19.8
740.6	1.180	0.2	3.330	11.3	6.000	19.1
846.6	3.110	0.4	3.410	10.1	8.270	22.2

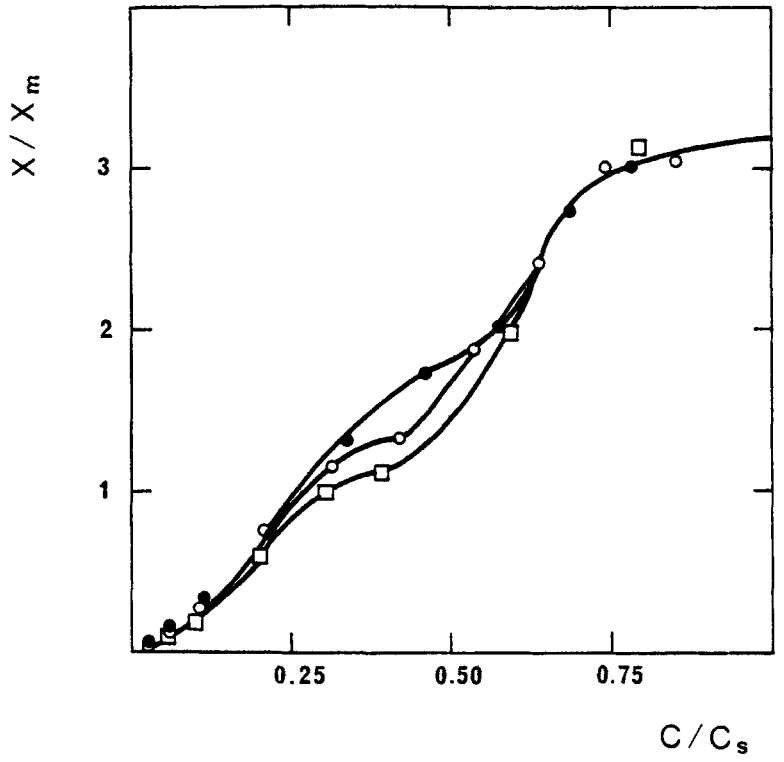

FIGURE 4.- BET isotherms at 0°C (□), 21°C (o) and 37°C (●) for the adsorption of thiouracil on Sephadex G-25 (fine).

such as physisorption, on a porous solid.Curves similar in appearance have been reported for adsorption of benzylthiouracil (11). In the first zone of the isotherm, at low thiouracil concentration, the pores behave in the some way as on the external surface. However, if the adsorption continues a moment is reached when these pores are filled to saturation, and it is impossible for the solid to take up more molecules of adsorbate.

The adsorption equilibrium constant is higher than 2 for the three temperatures studied. At 0°C the adsorption is stronger --

TABLE 2

Thiouracil Values Adsorbed on Sephadex G-25 (fine) in Aqueous Systems Containing One Water Structure Perturbant or Some Simple Electrolytes, at Severals pHs.

	μmol g-1 adsorbed			
	pH 4.4	pH 7.4	pH 9.0	pH 10.4
Water	2.90	2.52	2.59	2.54
2.0 M urea	2.74	2.23	2.30	2.25
0.2 M NaCl	3.07	2.75	2.78	2.81
0.2 M Na_2HPO_4	2.87	3.07	2.55	2.87
0.2 M Na_2SO_4	3.00	2.78	2.80	2.81
0.2 M KI	3.06	3.54	3.01	2.67

Thiouracil concentration was 0.40 mM and temperature 37°C.

than at greater temperatures, and an inflection point appears in the 0.42 mM concentration of thiouracil.

These experiments were also performed with other carbamides (6-n-propyl-2-thiouracil, uracil) and adsorption was not observed in any of them, which is in agreement with the elution behaviour of these compounds in a Sephadex G-25 (fine) bed.

Table 2 compares the effects on the adsorption values of thiouracil in aqueous solutions of one perturbant (urea) and of simple electrolytes, at severals pHs. The fact that in urea the thiouracil adsorbed is reduced by about 11% (Table 2), is consistent with the supposition of the existence of a water-dependent hydrophobic interaction.

On the other hand, as shown in table 2, the adsorption of thiouracil to Sephadex increases with decreasing pH. The greater adsorption values correspond to pH 4.4. This behaviour could be interpreted as due to electrostatic interaction. Electrostatic interactions on Sephadex are due to the fact that the cross-linked

dextran chain contains a few terminal carboxylic groups. With the more tightly cross-linked Sephadex type G-25, the effect of the fixed charged groups may be particularly noticeable when the solvent is deionized water, and the gel will act as a weak cation - exchanger with very low capacity. Thus small amounts of cations will be adsorbed. This is, in fact, what is observed (Table 1). Besides, drastic changes in pH definitely strongly influenced the behaviour of the solute interaction on the gel (Table 2). The possible explanation for the higher adsorption at pH 4.4 is that - either there occurs a rearrangement of the π-electrons in the - thiouracil molecule due to the new charged group, or that the molecule is now more positively charged. A combination of these two effects is,of course,also possible.

Moreover, by the addition of small amounts of an electrolyte to the distilled water at neutral or basic pH the thiouracil is adsorbed in greater ratio (Table 2). This can be caused either - by an increase in the number of adsorption sites available or by an increase in the strength of the interaction due to a decrease in the size of the water hydration layer which prevents the solute-gel interaction.

Thus both aromatic adsorption and electrostatic interactions appear to be answerable for the behaviour of the thiouracil with Sephadex G-25.

This compromise between the two counter-balancing effects are in concordance with the behaviour described by Janson (1) for aromatic solutes containing an ionizeable group and for aromatic ampholytes.

Since on Sephadex G-25 (fine) the amount of uracil adsorbed is lesser than the thiouracil adsorbed, the tiol group should play an important role in the thiouracil electrostatic interactions, with redistribution of the charges within the thiouracil molecule. This fact should permit the development of liquid chromatographic methods for specific separations of amines and thioami--

nes on gels containing cross-linked dextran chains. By a suitable choice of the medium it is possible to utilize the molecular sieve effect as well as the electrostatic interaction and aromatic adsorption effect of the Sephadex gel in the separation of certain nitrogenized bases, such as the purines and pyrimidines.

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