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Polyacrylamide adsorption chromatography of purines and related compounds

Synthetic resins of dextran ("Sephadex", Pharmacia) and polyacrylamide ("Bio-Gel", Bio-Rad) have been primarily employed in the separation of proteins by gel filtration. Both of these materials have strong sorption properties as well, especially for aromatic compounds. Sephadex G-10 and Bio-Gel P-2, the most highly cross-linked members of each series, offer a maximal surface area for adsorption of those compounds which diffuse freely throughout their interstices. Larger molecules (molecular weight greater than 700 in Sephadex G-10 and greater than 2,600 in Bio-Gel P-2) are excluded and are not adsorbed. When these gels are employed to study serum or urine on chromatographic columns, large molecules (primarily proteins) are eluted promptly in the void volume followed by nonadsorbed salts and lastly by adsorbed compounds in inverse order to their strength of adsorption. These preparations, then, combine gel filtration and adsorption chromatography in one step and thus offer a promising tool for the separation and study of small aromatic compounds in biological materials.

Recent studies in this laboratory[1] have shown that uric acid is strongly adsorbed to Bio-Gel and that this adsorption can be employed to isolate and quantify serum uric acid*. Other aromatic substances, including endogenous purine compounds, aromatic amino acids, and inhibitors of uric acid synthesis are also adsorbed to a varying degree. The effect of changes in buffer pH and molarity on such adsorption is evaluated in the present studies. Increased understanding of these effects should help elucidate the molecular mechanism of adsorption and may lead to improved methods for isolating these compounds.

Materials and methods

These studies were performed on two different columns, A and B, measuring 0.9 × 27 cm and 0.6 × 57 cm, respectively. Both columns were poured with Bio-Gel P-2 100–200 mesh, pumped at constant speeds of 10 cc or 15 cc per hour, and run in a cold room with mean temperature of 4°. The 1-cc test samples described below were layered manually under the eluting buffer with long-tipped serologic pipettes. On columns eluted with 2 *M* NaCl in 0.05 *M* phosphate, samples were applied directly to the top of the column and rinsed in with the buffer. Following buffer changes, at least five column volumes were eluted to ensure equilibrium before samples were applied. Neither column was repoured during the course of these studies and no loss of resolution occurred. All buffers contained 10 mg % sodium azide to retard bacterial growth. Column effluent was monitored at 280 nm with an LKB Uvicord II. Chemicals used were reagent grade. Allopurinol (4-hydroxypyrazolo[3,4*d*]pyrimidine) and oxipurinol (4,6-dihydroxypyrazolo[3,4*d*]pyrimidine), were kindly provided by Dr. STANLEY BLOOMFIELD of Burroughs Wellcome.

Three test solutions were employed in these studies, *viz.* (A) 1 mg % tyrosine, 1 mg % tryptophan, and 2 mg % uric acid; (B) 1 mg % hypoxanthine and 1 mg %

* *Editor's note.* Purine and pyrimidine bases have been studied before by several authors on polyacrylamide gels.

xanthine; and (C) 4 mg % allopurinol and 4 mg % oxipurinol. Each of these solutions was prepared in 0.05 *M* sodium phosphate buffer pH 7.0 and contained 4 g % (w/v) sucrose to permit layering under the eluting buffer, 20 mg % (w/v) human Cohn Fraction II as a marker of the exclusion volume (V_0) and 0.4 % (v/v) acetone as a marker of the inner volume (V_t). These standard solutions were applied sequentially under each of the conditions tested and the distribution coefficient of each compound was determined from the elution pattern.

Distribution coefficients for all studies are given as K_d values ($K_d = (V_e - V_0)/V_t$. V_0 is defined as the elution volume of the protein marker. V_t is arbitrarily defined as V_e (acetone) $- V_0$. It is recognized that acetone itself may interact slightly with the resin. The elution volume of acetone did not change, however, under any of the conditions studied. Any error introduced is therefore a constant one and does not conflict with the objectives of the study. Use of the acetone marker permits ready comparison between available reports of Sephadex studies[2] and the present work on Bio-Gel columns.

K_d values determined under similar conditions were the same for the two columns employed in this work and for a 0.9 × 57 cm column employed in preliminary studies. Duplicate determinations on the same column agreed within 0.1 K_d units. Values reported in this paper are the results of single determinations.

The effect of pH change was studied on column A equilibrated with buffers of pH 9.0, 8.0, 7.0, 6.0 and 5.0. All buffers were 0.05 *M* and were prepared using appropriate mixes of 0.05 *M* Na_2HPO_4 and 0.05 *M* NaH_2PO_4.

The effect of changes in molarity on adsorption to Bio-Gel was studied with sodium phosphate buffers, pH 7.0, prepared at molarities of 0.01, 0.05, 0.10, and 0.15. Additional molarities were tested in pH 7.0, 0.05 *M* phosphate buffers containing 0.10 *M*, 0.50 *M*, and 2.00 *M* NaCl. Column A was used for 0.01, 0.05, and 0.10 *M* studies. All other molarity studies were on column B.

Results

In studies of the effect of pH on adsorption to Bio-Gel, the 1-cc test samples were at pH 7.0. The column was previously equilibrated with, and then eluted by buffer of varying pH. These conditions were chosen in order to determine optimal conditions for separation and quantitation of these compounds from biological samples of neutral pH. The discrepancy between sample and column pH, however, does introduce a minor error in the specific evaluation of pH effects. This factor is caused by dilution of the eluting buffer by the sample itself and is greatest at the pH extremes. Distribution coefficients at pH levels other than 7.0 represent, to a small degree, intermediates between the eluting buffer pH and that of the sample.

Uric acid, xanthine, and oxipurinol experienced a marked decrease in adsorption as they became ionized in more alkaline buffers (Table I). The other compounds studied have higher pK values and it is not possible to say whether a similar change in adsorption would occur with their ionization. It is clear, however, that within this range increasing pH causes no significant change in adsorption of any of these compounds unless ionization occurs.

Also listed in Table I are K_d values on Sephadex G-10 calculated from the elution data of SWEETMAN AND NYHAN[2]. Acetone is again used as a marker of V_t and the buffer is 0.05 *M* phosphate pH 7.0. Adsorption to Sephadex is quantitatively

TABLE I

EFFECT OF VARYING pH ON DISTRIBUTION COEFFICIENTS OF TEST COMPOUNDS ON BIO-GEL P-2 COLUMNS

pK	*Compound*	*K_d values at varying buffer pH*					
		5.0	*6.0*	*7.0*	*7.0**	*8.0*	*9.0*
9.1	Tyrosine	2.2	2.2	2.1	—	2.1	2.1
9.4	Tryptophan	3.2	3.2	3.1	4.4	3.2	3.2
7.7	Oxipurinol	6.0	6.0	5.4	6.5	5.0	4.1
9.3	Allopurinol	3.8	3.8	3.6	4.5	3.7	3.3
8.9	Hypoxanthine	3.2	3.2	3.1	2.8	3.0	3.1
7.4	Xanthine	5.2	5.1	4.8	4.3	4.0	3.7
5.4	Uric acid	6.0	4.8	4.5	4.9	4.6	4.6

* Sephadex G-10 data recalculated from SWEETMAN AND NYHAN[2].

similar to that observed on Bio-Gel but there are significant qualitative differences. Thus hypoxanthine and xanthine are bound more tightly to polyacrylamide than to dextran while the converse is true of their analogues, allopurinol and oxipurinol. The amino acid tryptophan is also retained much longer on Sephadex than Bio-Gel.

The effect of varying molarity on test compound adsorption was studied over a range of 0.01 to 2.05 *M*. The same test solutions (in 0.05 *M* phosphate with 4% sucrose) were employed and, as in the pH studies, a small variation is introduced by dilution of the eluting buffer by the sample itself. This factor is not large, however, and may be disregarded in the purely qualitative interpretation of these data.

Adsorption of all test compounds was found to correlate inversely with the molarity of the eluting buffer (Table II). The one partial exception to this rule is sodium urate which had a lower distribution coefficient in 0.01 *M* than in 0.05 *M* buffer. A similar phenomenon at these molarities has been observed with adsorbed anions on Sephadex[3] where it has been attributed to ion exclusion.

Distribution coefficients for most test compounds agree well for the two 0.15 *M* buffers tested. The adsorption of sodium urate, however, is significantly diminished in 0.05 *M* phosphate buffer with 0.10 *M* NaCl (the K_d value is 3.8 as opposed to 4.4). Additional series of experiments were run with 0.10 *M* $(NH_4)_2SO_4$ and with 0.10 *M* LiCl, each being in 0.05 *M* phosphate buffer. K_d values for urate in these additional

TABLE II

EFFECT OF VARYING MOLARITY ON DISTRIBUTION COEFFICIENTS OF TEST COMPOUNDS ON BIO-GEL P-2 COLUMNS

Compound		*K_d values at varying buffer molarity*						
	M NaCl	—	—	—	—	*0.10*	*0.50*	*2.00*
	M PO_4	*0.01*	*0.05*	*0.10*	*0.15*	*0.05*	*0.05*	*0.05*
Tyrosine		2.3	2.1	2.0	1.9	2.0	—	1.8
Tryptophan		3.5	3.1	3.0	3.1	3.1	—	2.8
Oxipurinol		5.8	5.4	5.3	—	5.3	—	—
Allopurinol		3.8	3.6	3.5	—	3.5	—	—
Hypoxanthine		3.4	3.1	3.0	3.0	3.0	2.7	2.4
Xanthine		5.3	4.8	4.8	4.7	4.7	4.2	3.6
Uric acid		3.9	4.5	4.3	4.4	3.8	3.2	2.8

0.15 *M* buffers were 4.2 and 3.9, respectively. Distribution coefficients of the six other test compounds did not vary. Of the compounds tested then, the only ionized material appears to be affected by specific buffer components while unionized compounds are not affected.

Additional studies were performed with the same test solutions and pH 7.0 phosphate buffer on column B to evaluate the effects of elution rates and temperature on adsorption to Bio-Gel. Elution volumes did not vary over a range of 6–36 cc/h. At the higher rates of elution, however, there was significant broadening of the peaks with a resultant decrease in resolution. A prominent inverse correlation between temperature and adsorption was found for all compounds over a range of 4° to 21.5°. There was a mean fall of 30 % in adsorption of all compounds at the higher temperature. It is clear that low temperature operation significantly enhances the efficiency of such an adsorption column.

Discussion

In these experiments, no single set of conditions was encountered which would effectively separate all of the compounds tested. Application of Bio-Gel adsorption chromatography to the study of biological samples is further complicated by adsorbed compounds not included in these studies. It is presently feasible, however, to adapt this method for the isolation and study of the more highly adsorbed compounds: uric acid, xanthine, and oxipurinol. In fact, using this principle, a method for serum uric acid has been developed in this laboratory[1] and both xanthine and oxipurinol have been identified in the sera of patients treated with allopurinol.

In the course of this work, some of the characteristics of adsorption to Bio-Gel were more clearly defined and found to contrast with observations made by others on Sephadex columns. These contrasts may cast some further light on the molecular mechanisms of adsorption to both resins.

A clear inverse correlation was apparent between the buffer molarity and adsorption of all compounds tested on the Bio-Gel columns. Similar studies on Sephadex[4,5], however, have revealed increasing adsorption of aromatic compounds with increased buffer ionic strength. Those observations have been extended in this laboratory with similar direct correlation between buffer molarity and adsorption of uric acid to Sephadex G-10 and to cellophane tubing[6]. JANSON[4] has attributed this unusual effect to removal of water of hydration by higher molarity buffers. This then frees additional Sephadex binding sites for adsorption of other compounds by the processes of charge transfer (π electron interaction). The inverse correlation demonstrated in the present studies suggests a different mechanism for adsorption of aromatic compounds to Bio-Gel. The observations are entirely compatible with the concept that hydrogen bonding plays the major role in adsorption to such polyacrylimide resins[7,8].

An additional contrast between adsorption phenomena on Sephadex and Bio-Gel is seen on comparison of the K_d values obtained on representative compounds under identical conditions of pH and buffer composition. This is most apparent in examination of the distribution coefficients for hypoxanthine and xanthine as opposed to their analogues, allopurinol and oxipurinol. These molecules differ from the corresponding purine by transposition of the nitrogen and carbon atoms at positions 7 and 8 (Fig. 1). This change confers a marked increase in Sephadex adsorption, but

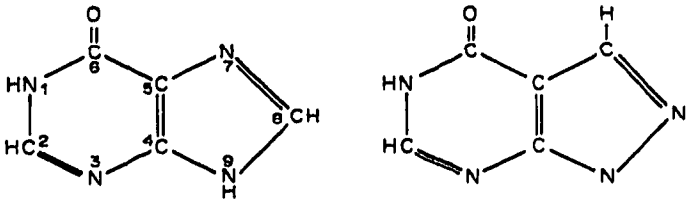

Fig. 1. Structure of hypoxanthine and allopurinol. Xanthine and oxipurinol are further oxidized at position 2. Uric acid is oxidized at positions 2, 6, and 8.

only a modest increase in interaction with Bio-Gel. Previous workers[9] have pointed out that N-7 introduces cross-conjugation into the principal resonance system of the purine molecule. When this nitrogen is replaced by a carbon atom, the cross-conjugation is eliminated and the resonance energy of the molecule is increased. Increased adsorption of the analogs to Sephadex may then be determined by the increased resonance energy as it is reflected in the π electrons of the purine molecule.

BROOK AND HOUSLEY[10] have recently shown, using buffers of increasing pH, that a marked fall in adsorption to Sephadex correlated closely with pK values of the compounds tested. In the present studies as well, there seems to be a clear fall in adsorption of uric acid, xanthine, and oxipurinol as these compounds become ionized. It appears entirely possible to determine pK values by study of such adsorption kinetics. Of more practical significance, however, is the possible prediction of optimal pH levels for separating adsorbed compounds based on knowledge of their pK.

The molecular basis of adsorption with either Bio-Gel or Sephadex is clearly complex and requires more extensive and sophisticated studies for precise characterization. Studies such as those reported here provide some insight into the fundamental mechanisms and are also useful in selection of appropriate conditions for specific problems in the separation of biological material.

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