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SELECTIVITY PROPERTIES OF SEPHADEX GELS IN COLUMN CHROMATOGRAPHY OF BIOLOGICALLY IMPORTANT PHOSPHATE ESTERS*

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SUMMARY

The selectivity properties of Sephadex gels toward various metabolic phosphates (nucleotides and coenzymes) have been examined by column chromatography using distilled water as eluent. These compounds are grouped within a range of K_d values from 0.22 to 0.39 on Sephadex G-10. Sephadex G-15 differs from G-10 in possessing a greater affinity for phosphates. The K_d values are only slightly less clustered on this gel, and there is but a negligible increase in the range of partition coefficients. Chromatography on G-25, on the other hand, gives a relatively good spread of K_d values. Several compounds have partition coefficients greater than 1.00. Whereas phosphate affinity decreases with increasing gel density, riboflavin, a nonphosphorylated reference compound, has the highest affinity for G-10 of all substances tested.

INTRODUCTION

The elution volume of a compound applied to a dextran gel column is the resultant of several factors, among which are the rate of solute diffusion within the bead and the steric exclusion of solute from the bead[1]. These factors are generally adequate in explaining the elution behavior of macromolecules, but may be of lesser importance when considering elution of certain small molecules, which are influenced strongly by adsorption[2-8] and charge effects[2,5-7,9-11].

With regard to the elution behavior of small molecules, the following observations seem to be significant. First, aromatic, heterocyclic, and to a lesser extent, aliphatic substances are adsorbed by dextran gels[2]. Aromatic amino acids, for example, show some adsorption when eluted with distilled water; it has been noted by PORATH[12] that dinitrophenyl derivatives of amino acids have greater adsorptivity than their

* The following abbreviations will be used: P = phosphate; d = deoxy; AMP, CMP, GMP IMP, TMP and UMP = adenosine-, cytidine-, guanosine-, inosine-, thymidine-, and uridine-5'-monophosphates, respectively; IDP and TDP = the corresponding diphosphates; FMN = flavin mononucleotide.

parent compounds. Additionally, the aliphatic amino acids, isoleucine, leucine and norleucine are adsorbed, but only in the presence of high salt concentrations[5].

Second, positively charged compounds, *e.g.*, basic amino acids, are adsorbed to the gel when applied in relatively small quantities and when distilled water is used as eluent under these conditions; however, acidic substances, such as acidic amino acids and phenolic (or heterocyclic) compounds with carboxylic acid substituents on the ring, are excluded from the gel under the same conditions[2,7]. Similarly, it has been reported that the decrease in retention time observed in the series AMP, ADP and ATP is the result of the increase in net negative charge[9].

The separation of nucleotides by dextran gel chromatography has received limited attention. HOHN AND POLLMANN[13], using a Sephadex* G-25 column and ammonium carbonate as eluent, found common nucleotides to emerge in the order UMP, CMP, TMP, GMP, 2′-AMP and 3′-AMP. However, ZADRAZIL *et al.*[14] established that phosphate buffer or ammonium carbonate, when used as eluent on a Sephadex G-25 column, cannot resolve UMP from CMP or AMP from GMP, but a mixture of the first two compounds can be partially separated from a mixture of the latter two. With the same dextran gel, a mixture of the 3′- and 5′-isomers of TMP can be resolved from 3′,5′-TDP in a triethylammonium bicarbonate system[15].

In comparison to G-25, less resolution of nucleotides has been found on Sephadex G-10. In this regard, MEZZASOMA AND FARINA[16] showed that dCMP, dAMP and dGMP could not be separated on G-10 with an acetate buffer eluent, and the 2′- and 3′-monophosphates of guanosine, uridine, cytidine and adenosine appeared in the void volume in a phosphate buffer system[17].

It is the purpose of this paper to report on the specificities of the Sephadex gels, G-10, G-15 and G-25, toward nucleotides and related metabolic phosphates with the use of relatively long columns and elution with distilled water. This work is intended to supplement a previous study on the separation of other metabolic phosphates by ion-exchange chromatography[18].

DEFINITION OF ELUTION PARAMETERS

The elution of solute will be characterized by the quantity K_d, which is defined as the partition coefficient for a substance between the internal aqueous volume of the gel available to acetone molecules and the aqueous phase outside the gel, and is given by[2]

$$K_d = \frac{V_e - V_o}{V_i}$$

where

V_e = volume of eluate from addition of sample to the maximum concentration of eluted substance,

V_o = volume of aqueous phase outside the gel grains and is found from the elution volume of Blue Dextran 2000 (ref. 19), and

V_i = the internal aqueous volume of the gel grains and is found from the elution volume of acetone less V_o (ref. 20).

* Mention of trade or company names does not imply endorsement by the Department over others not named.

The following interpretations will be given values of K_d:
$K_d = 0$; the solute is completely excluded from the gel grains[21].
$0 < K_d \leqslant 1$; the solute partially or completely penetrates the gel pores and may also interact by binding to the matrix.
$K_d > 1$; the solute is adsorbed to the matrix[21].

EXPERIMENTAL

Materials

The dextran gels used were Sephadex G-10, G-15 and G-25. Particle sizes ranged from 100–300 μ for G-25 to 40–120 μ for G-10 and G-15 as stated by the manufacturer (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.)[22]. Solutes used in chromatography were purchased from the Sigma Chemical Co., St. Louis, Mo., except that riboflavin was obtained from the Eastman Chemical Co., Rochester, N.Y., and were used without further purification.

Preparation of columns

The Sephadex gels were mixed with distilled water and allowed to swell for periods of time recommended by the manufacturer[22]. The fines were removed from the supernatant by repeated decantation. The glass column used in these studies was constructed from two 4 ft. sections of 1.1 cm tubing with a sintered glass disc and stopcock fused to the bottom. The column was filled with distilled water and the gel poured in slowly with the bottom outlet opened. A polyethylene disc was placed on top of the bed.

Column characteristics

Column dimensions were 1.1 × 239 cm (G-25 and G-15) and 1.1 × 231 cm for G-10. Respective void volumes (V_o) were 93 ml for G-25 and G-15 and 78 ml for G-10. Total volumes were determined by measuring the volume of water occupying the empty columns and were found to be 214 ml for G-25 and G-15 and 207 ml for G-10. Internal aqueous volumes (V_i) were 111 ml for G-25, 84 ml for G-15 and 69 ml for G-10.

Application and elution of samples

Solutes were dissolved in 4 ml distilled water (pH 6) and were applied in either their sodium, lithium or ammonium forms singly to the columns, and in the milligram quantities stated in Table I. Distilled water was used as eluent. Eluate was collected at a rate of 12 to 18 ml per hour and at room temperature (21°) in 3 ml fractions.

Analytical procedure

All substances were monitored by absorbance measurement at 260 mμ in a Gilford 220 spectrophotometer.

RESULTS AND DISCUSSION

Acetone standard and comparison of K_d values obtained on different gels

The elution properties of a given compound on dextran gels can be characterized by the parameter K_d. The use of the partition coefficient, however, is not without

limitation, because its calculation depends upon the accuracy of determination of the inner volume, V_i. The latter quantity, in many cases, has been derived from the manufacturer's water regain values by a method which has little theoretical justification, *i.e.*, that $V_i = aW_r$; where a is the dry weight of the gel and W_r is the water regain[23]. Presumably, the best method for calculating V_i involves determination of the distribution volume for tritiated water, though this procedure requires the use of an isotope monitor[24]. The simplest method necessitates measuring the elution volume of either NaCl[25], HCl[10] or acetone[20]. These solutes, however, may specifically interact with the gel through adsorption or charge effects, giving an abnormally large value for V_i. Therefore, the partition coefficients reported in this paper must be interpreted knowing that V_i was found from the elution volume of acetone.

Because of similar particle sizes, direct comparisons can be made between K_d values obtained using G-10 and G-15. However, strict comparisons between G-10 (or G-15) and G-25 data may lead to error, because of the larger particle size of the G-25 grains.

Sephadex G-10 chromatography

The elution volumes and partition coefficients for a select group of biologically important phosphate esters, applied singly to a G-10 column, are given in Table I. Molecular size and perhaps to a lesser extent net negative charge are factors which lead to exclusion of coenzyme-A. The long side-chain in this compound produces, in effect, a large tumbling radius, while it can be suggested that the three phosphate groups are probably associated with tightly bound water molecules[15] which effectively increase the molecular weight of the species in solution. The significance of steric

TABLE I

CHROMATOGRAPHIC DATA FOR NUCLEOTIDES AND RELATED COMPOUNDS ON SEPHADEX GELS

Compound	*Molecular weight*[a]	*Sephadex G-10*			*Sephadex G-15*			*Sephadex G-25*		
		Quantity (mg)	V_e *(ml)*	K_d	*Quantity (mg)*	V_e *(ml)*	K_d	*Quantity (mg)*	V_e *(ml)*	K_d
Blue Dextran 2000	2×10^6[b]	Sat. soln.	78	0	Sat. soln.	93	0	Sat. soln.	93	0
Coenzyme-A	768	3.3	78	0	1.9	120	0.32	3.2	123	0.26
IDP	429	6.1	84	0.09	6.4	141	0.57	8.9	156	0.57
FMN	514	7.0	93	0.22	3.8	141	0.57	2.8	156	0.57
Pyridoxal-P	250	4.6	93	0.22	10.3	132	0.46	9.0[c]	162	0.62
Vitamin B_{12}	1357	3.6	105	0.39	2.3	156	0.75	3.9	192	0.89
Pyridoxamine-P	250	3.1	96	0.26	3.1	138	0.53	5.0	195	0.92
Acetone	58	1% aq. soln. (v/v)	147	1.00	1% aq. soln. (v/v)	177	1.00	1% aq. soln. (v/v)	204	1.00
dUMP	308	5.7	93	0.22	2.7	129	0.43	2.3	222	1.16
IMP	348	7.9	96	0.26	3.5	159	0.79	2.4	243	1.35
dGMP	347	5.9	102	0.35	4.3	153	0.71	3.7	294	1.81
dCMP	307	5.8	96	0.26	5.2	141	0.58	2.7	294	1.81
Riboflavin	376	Sat. soln.	273	2.82	Sat. soln.	312	2.61	Sat. soln.	306	1.92
dAMP	331	7.1	105	0.39	3.9	156	0.75	2.5	330	2.14

[a] Weights of the free phosphoric acids.
[b] Pharmacia Fine Chemical Co., Technical Data Sheet No. 8, Piscataway, N.J.
[c] Pyridoxal-P applied to a G-25 column in the acid form.

hindrance for the retention of coenzyme-A is best seen by K_d values greater than zero obtained on Sephadex G-15 and G-25, which are gels providing greater pore accessibility. In addition, the three phosphate groups can be repelled by the fixed negative charges (believed to be carboxylate groups and presumably including oleate ions) in the gel matrix[5]; these are found in concentrations of 4.1 ± 2.8 and 45 ± 23 μequiv. per gram of dry gel in G-10 and G-25, respectively[10].

Six phosphates tested on Sephadex G-10, despite having varied molecular structures, elute with K_d values of 0.2, and thus gain equal, though limited, access to the gel pores. Vitamin B_{12} and the purine nucleotides, dGMP and dAMP (but not IMP) have slightly larger partition coefficients than the other phosphates, and this may be attributed to nonpolar interactions with the gel. It has been reported by BRAUN[26] that purine derivatives, in general, seem to be more strongly retained by Sephadex G-10 columns than are pyrimidine derivatives, although it should be stated that his eluent consisted of an electrolyte solution.

Riboflavin was included in this study as a matter of comparison, since it contains, as do the nucleotides, both a heterocyclic ring and a sugar moiety; however, it does not possess a phosphate group, and this contributes to its interesting elution properties. In this respect, riboflavin differs in K_d value from FMN by more than one order of magnitude.

Although Sephadex G-10 is unsatisfactory in effecting separations of selected metabolic phosphates in a distilled water elution system, it may have merit in grouping these substances with a range of K_d values from 0.22 to 0.39. Compounds of structure similar to riboflavin, for example, neutral or nonphosphorylated heterocyclic substances, perhaps may be separated from metabolic phosphates in this manner.

Sephadex G-15 chromatography

The selectivity properties of Sephadex G-15 toward the compounds previously tested on G-10 were investigated. Table I shows that partition coefficients found are at least approximately twice as great as the values obtained employing the G-10 column, except that riboflavin is adsorbed to a lesser degree. There is a slightly greater divergence of individual K_d values on G-15; this is exemplified by coenzyme-A and IDP. Additionally, those phosphates which showed the most affinity toward the tighter-meshed gel, vitamin B_{12} and the purine nucleotides (IMP, dAMP and dGMP), likewise have relatively larger partition coefficients than the other phosphates.

Sephadex G-15, therefore, provides an improvement over G-10 in phosphate affinity; however, K_d values on it are only slightly less clustered, and there is but a negligible increase in the range of phosphate partition coefficients, *i.e.*, 0.47 K_d units for G-15 as opposed to 0.39 units for G-10.

Sephadex G-25 chromatography

The same compounds previously tested on G-10 and G-15 were applied to a Sephadex G-25 column. Table I shows the relatively good spread of partition coefficients obtainable with this gel. In contrast to the smaller pore gels, K_d values for phosphates on G-25, in five cases, are greater than 1.00, thus indicating the role of adsorption in the elution process. No correlations, however, can be made between elution volume and molecular weight.

IMP, with one less phosphate than IDP, has considerably greater affinity for

G-25. Similarly, riboflavin interacts more strongly with the gel than does FMN as was previously noted for the G-10 and G-15 runs.

Fig. 1 is a composite which shows the elutions of phosphates on G-25. The quantities of each compound applied to the column are given in Table I. Coenzyme-A can be seen to tail, and some peak heading is apparent in the profile of pyridoxal phosphate, but this may be the result of column overloading of the latter compound.

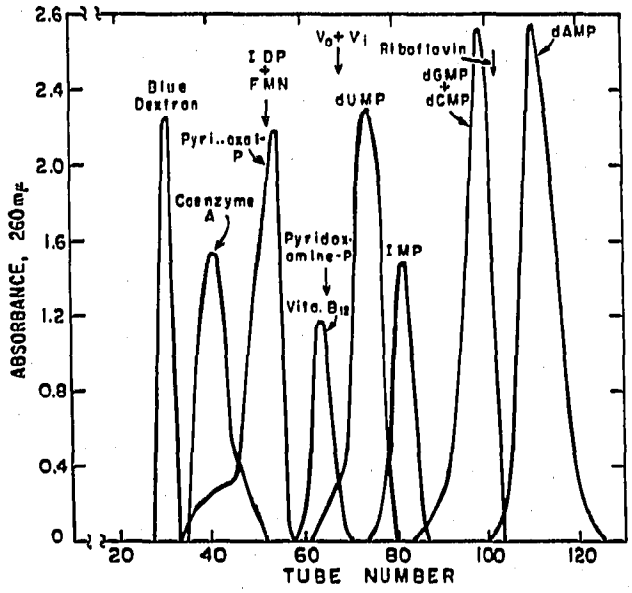

Fig. 1. Composite of phosphate elutions on Sephadex G-25. Column dimensions were 1.1 × 239 cm. Milligram quantities of each solute were applied individually to the column in 4 ml distilled water and eluted with distilled water. The eluate was collected in 3 ml fractions at a rate of 12 to 18 ml per hour. The elution volume of acetone is given by $V_o + V_t$. Operating conditions: room temperature (21°) and atmospheric pressure.

As evidenced by their partition coefficients, IDP and FMN cannot be separated on either G-25 or G-15; perhaps a partial resolution may be obtained with G-10, considering the small difference in K_d values on that gel. Similarly, vitamin B_{12} cannot be separated from pyridoxamine phosphate on Sephadex G-25, but G-15 has differential selectivity for them. Also, a greater difference in K_d values between dUMP and IMP is seen on G-15 as opposed to G-25. Finally, dGMP and dCMP have the same affinity when run on G-25, but differ slightly in affinity on G-15.

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