SEPHADEX G-10 ADSORPTION CHROMATOGRAPHY OF PURINES AND RELATED COMPOUNDS

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Sephadex G-10 adsorption chromatography was investigated in a search for means of analyzing and isolating the purines of human urine and other biological fluids. Available methods of anion and cation exchange chromatography do not adequately separate the different methyl isomers. Paper chromatographic systems are not capable of separating the large quantities needed for determination of the low specific activities expected after *in vivo* radioactive tracer studies.

Adsorption chromatography of aromatic and heterocyclic compounds, including many purines, was first investigated on Sephadex G-25 by GELOTTE[1]. These studies were extended by ZADRAŽIL *et al.*[2] and HOHN AND POLLMAN[3], but they were applied only to the common bases encountered in the nucleic acids, nucleosides and nucleotides.

For the study of the separation of purines, Sephadex G-10 was chosen because the higher mass to volume ratio of this gel than those of G-25 and G-50 would be expected to increase greatly the magnitude of adsorption, provided the smaller pore size did not exclude the molecules. Excellent separations of many purines were obtained under appropriate conditions of pH, ionic strength and flow rate. Analytical and preparative applications were investigated and a relation between elution volume and purine ring substituents was found.

METHODS AND MATERIALS

Preparation of Sephadex G-10 columns

Sephadex G-10 (water regain 1.0 ± 0.1 g/g; particle size 40–120 μ) obtained from Pharmacia was suspended in distilled water overnight then rinsed in succession with several volumes of the following solutions by suspension, settling, and decantation: 0.1 *N* NaOH, distilled water, 0.1 *N* HCl, distilled water, 0.1 *N* NaOH, and twice with 0.05 *M* sodium phosphate buffer, pH 7.0. After deaeration under aspirator vacuum for 30 min, a 1:1 slurry of the prepared gel in the buffer was poured into the column. In early studies, a 0.9 × 100 cm column was used which was made from glass tubing with a porous polyethylene disc at the bottom. In the later studies a 1 × 100 cm LKB column was used also with a porous polyethylene disc. After the gel had settled under buffer flow (3–4 p.s.i. pressure) for several days, excess gel was removed from the top and a porous polyethylene disc placed on top.

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Regeneration was not necessary after use of the column for most runs. Exceptions were urine in which case strongly adsorbed components would appear in the following run, and before separations involving uric acid where it was necessary to be certain uric acid degrading bacteria were removed from the column. Regeneration was done with several column volumes of 0.05 N NaOH followed by pH 7.0 buffer. One column was used for more than a year without loss of resolution; the top few centimeters of gel were replaced when they became discolored as was the polyethylene disc when it became clogged.

Column accessories

The ultraviolet absorbance of the effluent from the column was monitored with a Vanguard Automatic U.V. Analyzer and the fractions collected. In early studies constant flow rate was maintained with applied air pressure, but with the LKB column a Beckman Accuflow pump was used. The column was eluted with 0.05 M NaH_2PO_4 buffer adjusted to pH 7.0 with NaOH. Purines and related compounds were obtained from various commercial sources except for 1,7-dimethylxanthine, 2-dimethylamino-6-hydroxypurine, azathioprine, allopurinol and alloxanthine, which were generously provided by Miss GERTRUDE ELION of Burroughs Wellcome.

Column volume determination

Samples of known volume were applied on the column and rinsed in with a small volume of buffer. The elution volume (V_e) was determined from the time of application of the sample to the center of the peak on the chromatograph. No corrections for detector dead volume (1.4 ml) were made. The column volumes were determined with a solution of dyed blue dextran (Pharmacia) (0.04 %, w/v) and acetone (0.6 %, v/v) recorded at 260 mμ. The dextran (mol.wt. 2×10^6) provided the void volume (V_0) or the volume outside the gel beads. The acetone (mol.wt. 58) gave the void volume plus internal volume ($V_0 + V_i$). The internal volume $V_i = V_e$ (acetone) $-V_0$ gave the volume of liquid within the gel that was accessible to small molecules. Adsorbed compounds had elution volumes greater than acetone.

RESULTS

Selection of buffer and pH

The column was eluted with 0.05 M NaCl as described by GELOTTE, who had shown that anions were excluded from Sephadex G-25 at lower ionic strengths, presumably because of the small number of carboxyl groups in the matrix (about 10 microequivalents per gram dry gel*). The influence of the ionic strength of the eluate on the elution volume of uric acid, an anion above pH 6 (acidic pK = 5.4) is shown in Table I for the 1.0 × 100 cm column. Hypoxanthine, xanthine, guanine and adenine each had a specific elution volume. These elution volumes were identical in either 0.05 M NaCl or 0.05 M phosphate. This indicates that for neutral molecules, elution volume is independent of ionic strength.

To determine the effect of pH on elution volumes, the following buffers were used with the four purines of major interest on the 0.9 × 100 cm column (Table II).

The only large change in going from pH 7 to 5 occurred with adenine, which is

* From Pharmacia literature.

TABLE I

EFFECT OF ELUENT IONIC STRENGTH ON ELUTION VOLUME OF URIC ACID ON SEPHADEX G-10

Eluent (all pH 7)	*Ionic strength (calc.)*	V_e *(ml)*
0.05 *M* Phosphate	0.080	
Dextran		32.5
Acetone		61.0
Uric acid		171.0
0.05 *M* NaCl	0.050	
Uric acid		115.0
0.001 *M* Phosphate	0.002	
Uric acid		52.0
Distilled water	0.000	
Uric acid		50.5

the only purine with a basic p*K* close to 5. Apparently protonation decreases adsorption. At high pH, adsorption was almost totally lost for all four purines, probably due to competition by hydroxyl ions, which are known to be adsorbed to Sephadex[1]. That this low level of adsorption is not due to the fact that the purines are in an ionic form, is indicated by the considerable adsorption of uric acid (V_e = 171 ml) at pH 7 where it is anionic.

Phosphate buffer at 0.05 *M*, pH 7.0 was chosen for standard elution. Most purines are unionized at this pH and at higher or lower pH, the adsorption of some purines was decreased. The ionic strength was sufficient to prevent exclusion of anions. The buffer is transparent in the ultraviolet. Fig. 1 shows the elution chromatogram at 260 mμ for the major purines and pyrimidines on the 1.0 × 100 cm column of Sephadex G-10 eluted with 0.05 *M* phosphate, pH 7.

Fig. 2 is a composite chromatogram of the elution profiles of the methylated xanthines, showing that methyl groups reduce adsorption of the compounds to Sephadex G-10. Although 1,3-dimethyl- and 1,7-dimethylxanthine have the same elution volume, they can be readily distinguished by their ultraviolet spectra in basic solutions. 1,3-Dimethylxanthine has a λ_{max} at 278 mμ and 1,7-dimethylxanthine has a λ_{max} at 290 mμ.

TABLE II

THE EFFECT OF ELUENT pH ON ELUTION VOLUMES OF PURINES ON SEPHADEX G-10

pH	*Buffer*	V_e *(ml)*			
		Hypoxanthine	*Xanthine*	*Guanine*	*Adenine*
5.00	0.05 *M* NH_4 acetate	101	144	186	198
7.00	0.05 *M* Na phosphate	101	140	178	229
10.31	0.05 *M* Tris	27	42	53	78
	Basic p*K**	2.0	0.8	3.3	4.2
	Acidic p*K**	8.9	7.4	9.2	9.8

* Obtained from Cal Biochem Data Sheet.

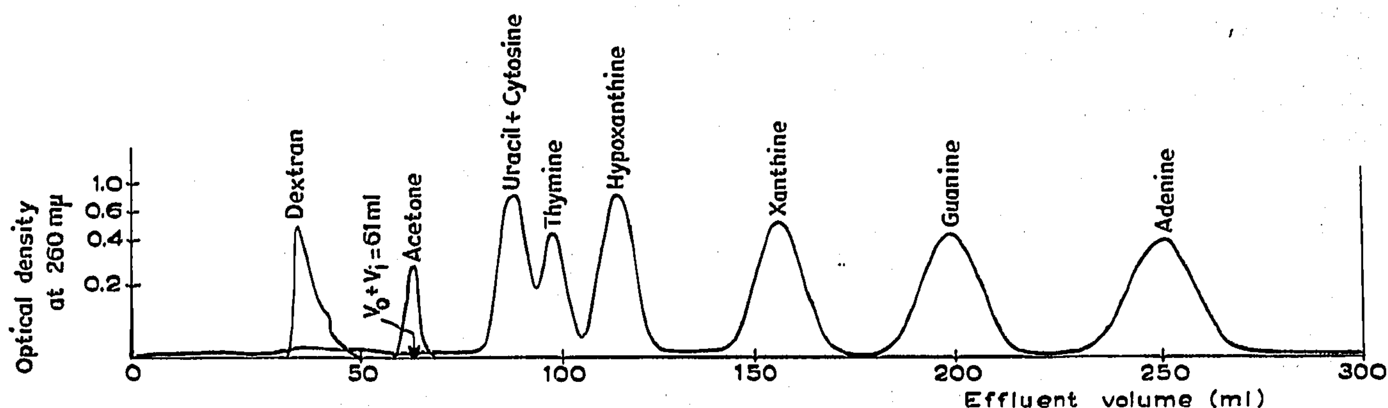


Fig. 1. Chromatogram of purines and pyrimidines on 100 × 1.0 cm Sephadex G-10.

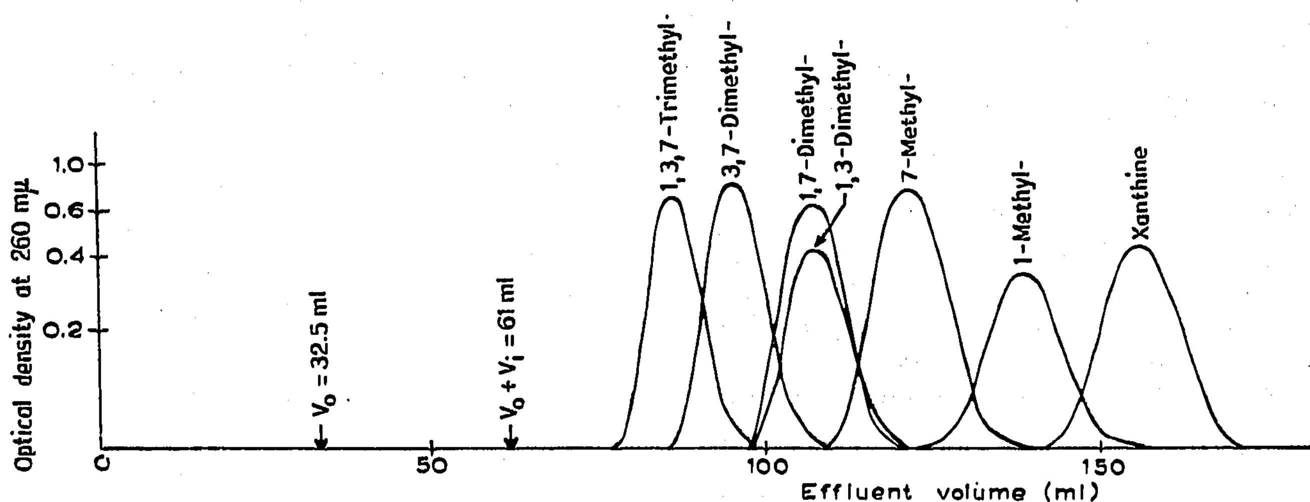


Fig. 2. Composite chromatogram of methylxanthines on 100 × 1.0 cm Sephadex G-10.

Selection of flow rate

On the 0.9 × 100 cm column there was a linear relationship between applied air pressure and flow rate over a range of 0–6 p.s.i. and 19.1 to 82.7 ml/cm²/h. A solution of hypoxanthine and adenine was chromatographed at these two flow rates and the height equivalent to a theoretical plate (HETP) calculated from the band spreading according to[4]:

$$H = 5.54 \left[\frac{\text{elution volume (ml)}}{\text{width at 1/2 height (ml)}}\right]^2$$

where H equals the number of theoretical plates in the column and HETP = (column height in cm)/H. These data are shown in Table III. The ratio (HETP high flow)/(HETP low flow), was 2.50 for hypoxanthine, and 1.75 for adenine. Therefore, the column was approximately twice as efficient at the lower rate, and peaks were sharper. With the installation of the constant flow pump, a flow rate of 24.4 ml/cm²/h was used, with a back pressure of 3.5 p.s.i. A complete chromatograph was obtained in 24 h at this flow rate.

TABLE III

EFFECT OF FLOW RATE ON THE EFFICIENCY OF 1.0 × 100 cm G-10 SEPHADEX COLUMN

Flow rate ($ml/cm^2/h$)	*Hypoxanthine*			*Adenine*		
	V_e	*H*	*HETP*	V_e	*H*	*HETP*
82.7	101	111	0.90	222	144	0.70
19.1	101	276	0.36	222	252	0.40

Recovery

The recovery of chromatographed purines was quantitative. Recoveries of uric acid are shown as an example in Table IV. Immediately before each run, the column was rinsed with 0.05 *N* NaOH followed by pH 7 buffer. The fractions were collected under toluene to protect uric acid from degrading bacterial contamination. The uric acid was assayed in the fractions by the enzymatic spectrophotometric method[6], and the radioactivity was determined by liquid scintillation counting in a dioxane–naphthalene mixture. The values were well within the limits of the assay methods.

TABLE IV

RECOVERY OF URIC ACID FROM 1.0 × 100 cm SEPHADEX G-10 COLUMN

Sample	*Applied* (*mg*)	*Recovery* (%)	*Applied* (*mμ curies*)	*Recovery* (%)
Urate 2-^{14}C, 1.0 ml in 0.1 *N* Li_2CO_3	1.68	98.9	11.04	96.3
Urate 2-^{14}C, 1.0 ml in 0.1 *N* Li_2CO_3	1.038	99.9	8.944	101.8
Mean		99.4		99.0
Urine, 4.0 ml	0.652	100.9		
Urine, 5.0 ml	0.732	93.3		
Urine, 8.0 ml	0.783	104.4		
Urine, 5.0 ml	0.793	102.7		
Mean		100.0		99.0
S.D.[a]		4.4		4.9

[a] S.D. estimated from range by S.D. = range/*C* where *C* equals a constant depending on the number of determinations given in table in ref. 5.

Reproducibility of chromatographic position of elution

Volumes were determined from time of sample application and every fifth fraction was collected in a graduated centrifuge tube to check for constancy of flow rate. Samples were applied to the 1.0 × 100 cm column in 1.0 ml of 0.1 *N* Li_2CO_3. Table V illustrates the elution volumes of four purines at various times over a 5 month period after the column was prepared.

Over a period of one year, nine 1.0 ml samples of uric acid in 0.1 *N* Li_2CO_3, 0.05 *M* phosphate, or urine were chromatographed. The mean elution volume was 171.0 ml with a standard deviation of 2.4 ml estimated from the range. Similarly, in chromatography of five urines of 4–5 ml (pH 6–10), the mean elution volume of uric acid was 171.1 ml with an estimated standard deviation of 4.7 ml.

These and other data indicate that the elution volume is largely independent of sample volume and pH. There was no change in elution volume for uric acid in amounts of from 0.02 to 10.0 mg, although there was tailing of the peak at the higher concentrations.

TABLE V

REPRODUCIBILITY OF ELUTION VOLUMES OF PURINES ON SEPHADEX G-10

Months	V_e *(ml)*			
	Hypoxanthine	*Xanthine*	*Guanine*	*Adenine*
0	113	156	199	252
1	113	156	198	251
5	113	156	199	253

Elution data on purines and related compounds

The elution parameters of a large number of purines, pyrimidines and related compounds are given in Table VI.

Definition of elution parameters

With Sephadex gel chromatography, the partition coefficient K_d, defined as $K_d = (V_e - V_0)/V_t$, has been used to characterize elution volumes since it is independent of column dimensions[1,7]. For nonadsorbed compounds, $K_d = 0$ for high molecular weight substances which are excluded and 1 for low molecular weight compounds which enter the gel freely. Substances of intermediate molecular weight have K_d values between 0 and 1. Adsorbed compounds have K_d values greater than 1 and with Sephadex G-10, as high as 14.

Sephadex G-10 has very small pore sizes and gives different V_t values depending on the size and structure of the molecule used in its determination. For the 1.0 × 100 cm column eluted with 0.05 M phosphate at pH 7.0, the values for $V_0 + V_t$ for several compounds are shown in Table VII. Dyed blue dextran gave a value for V_o of 32.5 ml. It appeared arbitrary to define one of these as $V_o + V_t$; therefore, a corrected volume $V_e°$ was defined as:

$$V_e° = (V_e - V_0)/V_0 = K_d\ (V_t/V_0)$$

and

$$\text{Log}\ V_e° = \log K_d + \log (V_t/V_0) = \log K_d + \text{constant}$$

DISCUSSION

Relation of elution volume to purine structure

Differences in the structure of the adsorbed purine molecules result in differences in their elution volumes and the amount of difference can be predicted using a treatment analogous to the ΔR_M functions successfully applied by BUSH[8] to the R_F values of steroids in paper chromatography.

$V_e°$ may be considered as proportional to an adsorption coefficient, and the

TABLE VI

ELUTION VOLUMES OF PURINES AND RELATED COMPOUNDS

Compound	V_e *(ml)*	$V_e°$	*Log* $V_e°$
Purines			
6-Succinoaminopurine	52	0.58	—0.236
6-Trimethylammoniumpurinide	65	1.00	0.000
1-Methyladenine	75	1.31	+0.116
2-Hydroxypurine	82	1.52	+0.183
6-Carboxypurine	82	1.52	+0.183
9-Methylhypoxanthine	83	1.56	+0.194
Caffeine	86	1.56	+0.184
Adenine-1-N-oxide	91	1.80	+0.256
1-Methylhypoxanthine	92	1.85	+0.266
7-Methylhypoxanthine	93	1.74	+0.240
Theobromine	95	1.79	+0.254
6-Chloro-9-methoxymethylpurine	103	2.17	+0.337
Purine	106	2.12	+0.326
1,7-Dimethylxanthine	107	2.28	+0.359
Theophylline	107	2.15	+0.410
Hypoxanthine	113	2.57	+0.366
6-Methylpurine	116	2.32	+0.366
7-Methylxanthine	120	2.54	+0.406
6,8-Dihydroxypurine	122	2.76	+0.442
Isoguanine	135	3.17	+0.501
1-Methylxanthine	139	3.09	+0.490
N^2-Dimethylguanine	144	3.43	+0.536
7-Methylguanine	153	3.70	+0.568
Xanthine	156	3.59	+0.555
6-Methoxypurine	159	3.60	+0.556
1-Methylguanine	166	3.88	+0.589
Uric acid	171	4.09	+0.612
8-Hydroxy-7-methylguanine	174	4.36	+0.640
N^2-Methylguanine	184	4.41	+0.645
2-Aminopurine	187	4.77	+0.679
Guanine	199	4.85	+0.686
6-Chloropurine	206	5.06	+0.704
8-Hydroxyguanine	225	5.92	+0.772
6-Methylaminopurine	250	6.69	+0.826
Adenine	251	6.38	+0.805
6-Mercaptopurine	275	7.09	+0.851
6-Bromopurine	278	7.57	+0.880
6-Dimethylaminopurine	281	7.65	+0.884
6-Cyanopurine	284	7.73	+0.889
6-Methylmercaptopurine	319	8.82	+0.946
Imuran (Azathioprine)	371	10.42	+1.018
6-Iodopurine	400	11.32	+1.054
2,6-Diaminopurine	439	12.52	+1.098
6-Thioguanine	555	16.07	+1.206
6-Furfurylaminopurine	788	23.24	+1.367
6-Phenylaminopurine	1,754	52.97	+1.724
Purine ribosides, deoxyribosides and ribonucleotides			
7-Methylinosine	53	0.64	—0.196
5′-GMP	56	0.66	—0.179
2′,3′-AMP	59	0.87	—0.093
7-Methylguanosine	59	0.735	—0.133
Inosine	77	1.265	+0.102
2′-Deoxyinosine	78	1.29	+0.112

(*continued on p. 669*)

TABLE VI (*continued*)

Compound	V_e (*ml*)	$V_e°$	*Log* $V_e°$
Xanthosine	104	2.07	+0.316
Guanosine	122	2.60	+0.415
2'-Deoxyguanosine	126	2.70	+0.432
Guanosine (ribose $\xrightarrow{IO_4}$ dialdehyde)	142	3.37	+0.528
Adenosine	153	3.50	+0.544
2'-Deoxyadenosine	153	3.50	+0.544
8-Azapurines			
8-Azahypoxanthine	188	4.78	+0.679
8-Azaguanine	258	6.94	+0.842
8-Azaxanthine	298	8.17	+0.912
8-Azaadenine	421	11.95	+1.078
8-Aza-2,6-diaminopurine	441	12.57	+1.099
Pyrazolo[3,4-d]pyrimidines			
4-Hydroxy	160	3.70	+0.569
4,6-Dihydroxy	217	5.38	+0.731
4-Amino	383	10.28	+1.012
Pyrimidines, nucleosides and nucleotides			
5'-CMP	44	0.36	—0.452
5'-UMP	45	0.39	—0.407
Pseudouridine	64	0.97	—0.014
Uridine	69	1.11	+0.048
Cytidine	70	1.14	+0.059
Pyrimidine	70	1.15	+0.060
Thymidine	75	1.32	+0.121
Cytosine	85	1.61	+0.207
Uracil	87	1.67	+0.222
5-Methylcytosine	87	1.68	+0.227
Orotic acid	91	1.80	+0.256
Thymine	96	1.94	+0.289
Miscellaneous aromatic and heterocyclic compounds			
Creatinine	61	0.88	—0.057
Urea	71	1.18	+0.074
Imidazole	73	1.25	+0.097
Allantoin	78	1.40	+0.148
Cyclohexanone	80	1.47	+0.168
Hippuric acid	84	1.58	+0.200
Pyridine	105	2.22	+0.328
Pyrazole	117	2.60	+0.416
4-Aminoimidazole-5-carboxamide	154	3.53	+0.548
L-Tryptophan	158	3.56	+0.552
Pyrrole	173	4.33	+0.637
Chlorogenic acid	444	12.66	+1.103
Benzimidazole	474	13.58	+1.132

TABLE VII

ELUTION VOLUMES OF SMALL MOLECULES ON SEPHADEX G-10

Compound	*Mol. wt.*	*1.0 ml of this concn. applied*	V_e (*ml*)	*Detection method*
Acetone	58	0.082 *M*	61	Absorbance 260 mμ
KCl	74	0.05 *M*	61	AgCl ppt.
Glycine	75	0.67 *M*	47	Absorbance 220 mμ
KH_2PO_4	136	0.50 *M*	47	$KClO_4$ ppt.

log of $V_e°$ proportional to the difference in the chemical potential of the molecules on the gel and the molecules in the buffer. This follows from the relation between the adsorption coefficient k, which is an equilibrium constant, and the difference in chemical potential (μ) of the molecules in the two phases[8].

$$\log k = \Delta\mu / 2.33\ RT$$

The lower the chemical potential in the gel compared to the buffer, the larger the adsorption coefficient and the larger the elution volume.

If one assumes that each chemical group in a molecule contributes a fixed fraction of the difference in chemical potential in the two phases, and that this property is independent of the other groups present in the molecule, then each group should also contribute a fixed amount to the log $V_e°$ of the compound. Thus the addition of a single group to a molecule should give a Δ log $V_e°$ independently of the other groups present. Δ log $V_e°$ values have therefore been calculated for various classes of compounds in which the Δ log $V_e°$ represents the difference between the log $V_e°$ of the parent purine and that of the substituted compound. In Table VIII a number of substituents are listed in groups according to their positions on the purine ring in order to illustrate the effect of substitution on Δ log $V_e°$. The Δ log $V_e°$ values for purines and their ribosides measure the contribution of a ribosyl group in the 9-position of the purine ring to the adsorption to Sephadex G-10, and give confirmation of the validity of the above assumptions.

TABLE VIII

Δ log $V_e°$ VALUES FOR SUBSTITUENTS ON THE PURINE RING

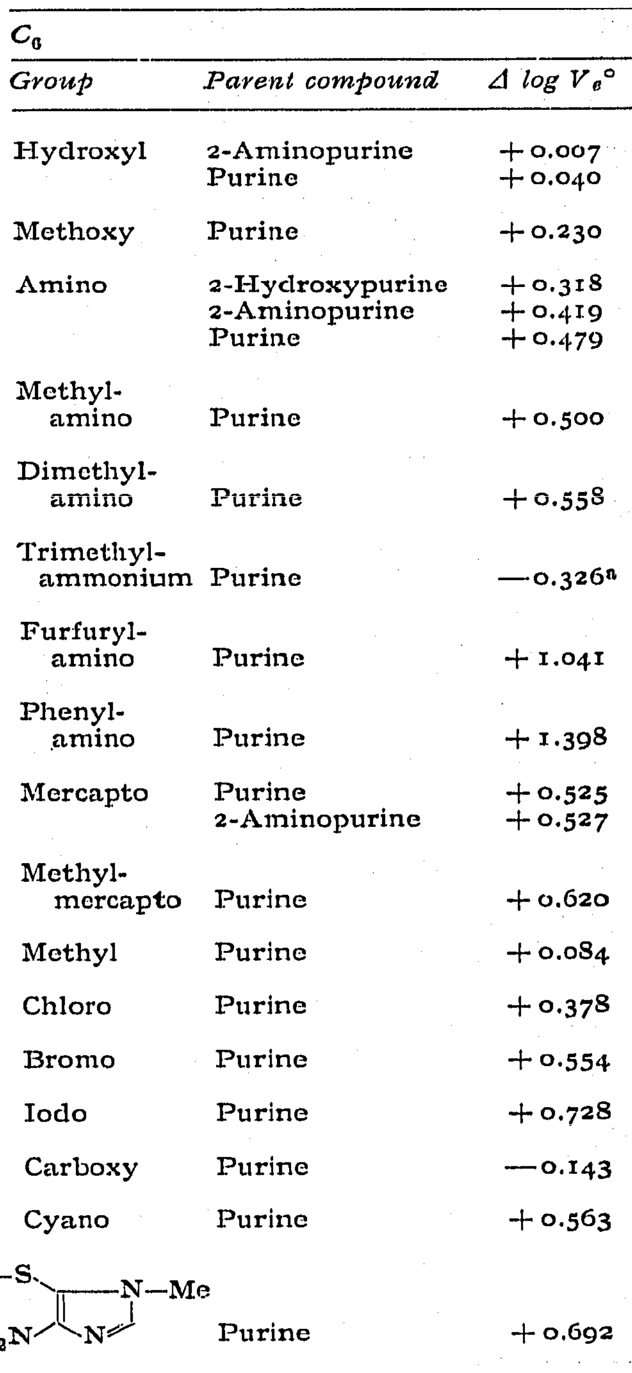
N_1		
Group	*Parent compound*	*Δ log $V_e°$*
Methyl	7-Methylxanthine	−0.049
	Xanthine	−0.065
	Theobromine	−0.070
	Guanine	−0.097
	Hypoxanthine	−0.100
	Adenine	−0.689[a]
N-Oxide	Adenine	−0.549[a]

C_2		
Group	*Parent compound*	*Δ log $V_e°$*
Hydroxy	Adenine	−0.304
	Purine	−0.143
	7-Methylhypoxanthine	+0.166
	6,8-Dihydroxypurine	+0.170
	Hypoxanthine	+0.189
	Inosine	+0.214
Amino	Adenine	+0.293
	Inosine	+0.313
	2′-Deoxyinosine	+0.320
	Hypoxanthine	+0.320
	7-Methylhypoxanthine	+0.328
	Purine	+0.353
	6-Mercaptopurine	+0.355
Methylamino	Hypoxanthine	+0.279
Dimethylamino	Hypoxanthine	+0.170

(continued on p. 671)

TABLE VIII (*continued*)

N_3		
Group	*Parent compound*	*Δ log $V_e°$*
Methyl	7-Methylxanthine	—0.152
	1-Methylxanthine	—0.158
	1,7-Dimethyl-xanthine	—0.175



C_6		
Group	*Parent compound*	*Δ log $V_e°$*
Hydroxyl	2-Aminopurine	+0.007
	Purine	+0.040
Methoxy	Purine	+0.230
Amino	2-Hydroxypurine	+0.318
	2-Aminopurine	+0.419
	Purine	+0.479
Methyl-amino	Purine	+0.500
Dimethyl-amino	Purine	+0.558
Trimethyl-ammonium	Purine	—0.326[a]
Furfuryl-amino	Purine	+1.041
Phenyl-amino	Purine	+1.398
Mercapto	Purine	+0.525
	2-Aminopurine	+0.527
Methyl-mercapto	Purine	+0.620
Methyl	Purine	+0.084
Chloro	Purine	+0.378
Bromo	Purine	+0.554
Iodo	Purine	+0.728
Carboxy	Purine	—0.143
Cyano	Purine	+0.563
—S—; N—Me; O_2N; N	Purine	+0.692

(*continued on p. 672*)

TABLE VIII (*continued*)

N_7

Group	*Parent compound*	*Δ log $V_e°$*
Methyl	Guanine	−0.118
	Hypoxanthine	−0.126
	8-Hydroxyguanine	−0.132
	1-Methylxanthine	−0.133
	Theophylline	−0.148
	Xanthine	−0.149
	Inosine	−0.298[a]
	Guanine	−0.548[a]

N_9

Group	*Parent compound*	*Δ log $V_e°$*
Ribosyl	Xanthine	−0.239
	Adenine	−0.261
	Hypoxanthine	−0.264
	Guanine	−0.271
	7-Methylhypo-xanthine	−0.436[a]
	7-Methylguanine	−0.536[a]
2′-Deoxy-ribosyl	Hypoxanthine	−0.254
	Guanine	−0.254
	Adenine	−0.261
5′-P-Ribosyl	Guanine	−0.865
2′,3′-P-ribosyl	Adenine	−0.898
Methoxy-methyl	6-Chloropurine	−0.367
Methyl	Hypoxanthine	−0.172

[a] Introduces ⊕ in ring.

C_8

Group	*Parent compound*	*Δ log $V_e°$*
Hydroxyl	Xanthine	+0.057
	7-Methylguanine	+0.072
	Hypoxanthine	+0.076
	Guanine	+0.086

TABLE IX

CALCULATION OF Δ log $V_e°$ VALUES FOR RIBOSE[a]

Purines	V_e	$V_e°$	*log V_e*	*Purine ribosides*	V_e	$V_e°$	*log $V_e°$*	*Δ log $V_e°$*
Hypoxanthine	113	2.32	+0.366	Inisone	77	1.26	+0.102	−0.264
Xanthine	156	3.59	+0.555	Xanthosine	104	2.07	+0.316	−0.239
Guanine	199	4.85	+0.686	Guanosine	122	2.60	+0.415	−0.271
Adenine	251	6.38	+0.805	Adenosine	153	3.50	+0.544	−0.261

[a] Conditions: 1.0 × 100 cm Sephadex G-10, pH 7.00, 0.05 *M* phosphate.

Table IX shows the constancy of the $\Delta \log V_e°$ values, which are negative for a ribosyl group, indicating decreased adsorption. Consideration of the $\Delta \log V_e°$ for the ribosides of hypoxanthine, xanthine, guanine and adenine yields very similar values. The mean $\Delta \log V_e°$ for a 9-ribosyl substitution is —0.259 with a standard deviation of 0.016 estimated from the range.

Thus the determination of $\Delta \log V_e°$ values for various chemical groups located at different positions on the purine ring makes it possible to predict elution volumes of compounds containing many groups. The usefulness is indicated by an example, 6-thioguanine, whose elution volume can be calculated as follows:

$\log V_e°$ (6-thioguanine) = $\log V_e°$ (purine) + $\Delta \log V_e$ (6-SH) + $\Delta \log V_e°$ (2-NH_2)
$V_e = (V_e° + 1) V_0$, where V_0 = 32.5 ml. From Tables VI and VIII,
$\log V_e°$ (purine = + 0.326
$\Delta \log V_e°$ (6-SH) = + 0.525
$\Delta \log V_e°$ (2-NH_2) = + 0.353

$\log V_e°$ (6-thioguanine) = + 1.204, $V_e°$ = 16.0, and V_e = 552 ml

This predicted value may be compared to the experimental V_e = 555 ml (Table VI).

Some interaction between groups does occur, particularly between the amino and hydroxyl group in positions 2 and 6 of the purine ring, so that $\log V_e°$ values are not always additive although they usually are.

Dependence of $\Delta \log V_e°$ values on ring location

The most extensive data for the variation of $\Delta \log V_e°$ values with ring position is for methyl groups, as shown in Table X. In addition, there are anomalous $\Delta \log V_e°$ values when the introduction of a methyl group causes the formation of a positive charge in the ring by quaternization of a nitrogen as in the examples shown in Table XI. The data indicate that the ring nitrogens are of major importance in adsorption to Sephadex G-10, with N_1 least important. A high electron density contributes to adsorption as introduction of a positive charge into the heterocyclic ring greatly reduces adsorption. That the decrease in adsorption by methyl substitution on the

TABLE X

$\Delta \log V_e°$ VALUES FOR METHYL GROUPS ON PURINES

Ring or attached amino group position substituted						
N_1	N_3	N_7	N_9	C_6	N^2 (*amino*)	N^6 (*amino*)
—0.065	—0.152	—0.149	—0.172	+0.084	—0.041 (1st)	+0.021 (1st)
—0.070	—0.158	—0.148				
—0.097	—0.175	—0.126			—0.109 (2nd)	+0.058 (2nd)
—0.049		—0.133				
—0.100		—0.132				
		—0.118				
—0.078	—0.162	—0.134 (means)				

TABLE XI

Δ LOG $V_e°$ VALUES FOR METHYL GROUPS ON POSITIVELY CHARGED NITROGEN

Parent compound	*Methylated compound*	Δ *log* $V_e°$
6-Dimethylaminopurine	6-Trimethylammoniumpurinide	−0.326
Inosine	7-Methylinosine	−0.298
Guanosine	7-Methylguanosine	−0.548
Adenine	1-Methyladenine	−0.689

ring nitrogens is not due to the presence of a bulky group on the ring is shown by the increase in adsorption with a methyl group on the 6 carbon, which could contribute to the electron density at the 1 or 3 nitrogen by induction or hyperconjugation.

It is concluded that Sephadex G-10 is an excellent adsorbent for the separation of many purines, particularly the methyl isomers. For general use, a neutral buffer with ionic strength sufficient to prevent exclusion of anions is appropriate, but changes of ionic strength or pH can be used to separate certain purines with differing pK values. The sharpness of the peaks depends on flow rate, but adequate separations are obtained in 24 h with a 100 cm column. The elution volumes are highly reproducible, and together with the Δ log $V_e°$ treatment, they can aid in identification of unknowns, or prediction of elution volumes of compounds not yet run.

Quantitative recovery makes possible quantitation of the compounds in the fractions by optical or chemical means. In this laboratory, direct chromatography of urine has been used to determine urinary excretions of alloxanthine (4,6-dihydroxypyrazolo[3,4-*d*]pyrimidine) after ingestion of the drug allopurinol[9]. Furthermore, a riboside of the drug was detected in the urine by analysis of the fractions at the predicted elution volume, although the peak was obscured by hippuric acid.

Sephadex G-10 adsorption chromatography promises to be useful for preparative work in purine syntheses where products can frequently be separated from starting material. For example, 1-methylhypoxanthine was readily separated from 1-methyladenine after nitrous acid deamination. Also adenine-1-N-oxide was completely separated from adenine after acidic hydrogen peroxide oxidation. Application is also found in nucleoside chemistry where periodate oxidized guanosine was separated from guanosine.

The mechanism of adsorption of heterocyclic compounds to Sephadex G-10 is under investigation. Analysis of the effects of ring substituents on purines indicates that the nitrogens are of major importance. Since the introduction of a positive charge in the ring greatly reduces adsorption, whereas a negative charge has little or no effect, it is reasonable to implicate high electron density at the nitrogens with their unshared pairs of electrons as the primary site of adsorption. If so, the groups in the Sephadex expected to interact with the unshared electron pairs of the nitrogen would be the hydroxyl groups. This is currently under investigation, as are possible correlations of log $V_e°$ values with physical properties of the purines.

SUMMARY

The adsorption chromatography of purines and related heterocyclic compounds on Sephadex G-10 was investigated under different conditions of pH, ionic strength

and flow rate and found to give excellent separations. The elution volumes were highly reproducible and recovery quantitative. A relation between a log function of the elution volumes and substituents on the purine ring was shown and the additivity of the resulting Δ log $V_e°$ values used to predict elution volumes. Some analytical and preparative applications have been undertaken.

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