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SEPARATION OF ALKYL DERIVATIVES OF URACIL BY SOLVOPHOBIC ADSORPTION CHROMATOGRAPHY ON SPHERON

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SUMMARY

Derivatives of such related substances as cytosine, uracil, thymine, 6-methyluracil, 5-ethyluracil, 5-propyluracil, 5-isopropyluracil, 5-cyclopropyluracil, 5-allyluracil, 5,6-trimethyleneuracil, 6-cyclopropyluracil, 5-cyclobutyluracil and 5-*tert.*-butyluracil have been separated on a column of Spheron P-300. Retention on the column was found to depend on the size of the non-polar part of the molecule. The chromatographic behaviour is analyzed according to the theory of solvophobic chromatography.

INTRODUCTION

Nucleic acid components can be separated by gas–liquid chromatography[1–3], thin-layer or paper chromatography[4,5] or ion-exchange chromatography[6–12], for which high separation efficiency has been achieved with pellicular supports[10–12]. The separation of purine and pyrimidine bases and of nucleosides on polyacrylamide gel has recently been reported[13], and it was assumed that the separation was mainly based on hydrophobic and ionic interactions between the separated substances and the polyacrylamide matrix of Bio-Gel P-2. Reversed-phase chromatography of nucleosides and their bases has also been recently reported[14].

In this paper, we describe the separation, on Spheron®, of a series of pyrimidine derivatives having aliphatic or alicyclic substituents of different sizes in position 5 or 6. Spheron is a macro-porous copolymer of 2-hydroxyethyl methacrylate with ethanediol dimethylacrylate; it has a high exclusion limit, a large specific surface and sufficient rigidity for use in HPLC[15,16]. The material has been used so far for the gel permeation chromatography of polydextrans[17], proteins[18,19] and viruses[20], for the affinity chromatography of enzymes[21] and the sorption chromatography of some natural macromolecules[19]. The internal surface of the material is highly polar, owing to the large number of hydroxyl groups, the gel swells in water, in solutions of low

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ionic strength it interacts only slightly with proteins[15], which are not denatured on its surface and retain their activity[21]. However, the high content of the cross-linking (less polar) ethanediol methacrylate, and the copolymer backbone (which has the character of an aliphatic hydrocarbon), can cause solvophobic interactions in highly polar media and the retention of compounds having lipophilic groups. This phenomenon was studied during the separation of our test compounds, *i.e.*, water-soluble derivatives of uracil with non-polar hydrocarbon residues of various sizes. The structure of the hydrophobic regions of Spheron apparently differs from that of other materials commonly used in reversed-phase liquid chromatography, but the results can be analyzed on the basis of energy interactions during solvophobic chromatography (Horváth *et al.*[22]).

EXPERIMENTAL

Chemicals

All the reagents used were of analytical grade and obtained from Lachema (Brno, Czechoslovakia). Ethanediol (analytical grade) was from Reanal (Budapest, Hungary) and Dextran T2000 from Pharmacia (Uppsala, Sweden).

Chromatographic support

The Spheron P-300 was a product of Lachema Brno, (Czechoslovakia) and the 20–40-μm wet-sieve fraction was used. The sorbent was extracted and the content of carboxyl-groups was decreased from an apparent 30 μequiv./g in the reaction with diazomethane to below the sensitivity of the analytical method[15]. The material had an exclusion limit at a mol. wt. of 500,000 for polydextran, and its specific internal surface area was 85 m^2/g; the maximum size of particles was 40 μm and the number-average diameter was 27 μm. The size-distribution curves of the particles and of the pores (measured by mercury porosimetry), the equilibrium adsorption values (BET method), the solvent-regain column permeability and some other parameters have been reported[15].

Apparatus for liquid chromatography

The chromatographic system consisted of a double pump with sapphire plungers (Proportional mini-pump 68005) connected to a 75-μl sample-injection loop, a precision-bore glass column (400 × 6.36 mm I.D.) with adjustable ends and a thermostatically controlled water jacket, and a spectrophotometer (type DVD, 245 nm) equipped with a 10-μl flow-through cell; all these components were produced by the Development Workshops of the Czechoslovak Academy of Sciences. Alternatively, a refractive index detector (Knauer 2050) or a conductivity detector (30-μl LKB 5321B) in combination with a Radelkis conductometer OK 102/1 could be used.

Chromatographic procedure

The columns were packed by the slurry technique, with a suspension in 20% ammonium sulphate solution at a flow-rate of 600 ml/h; the bed dimensions were always 240 × 6.36 mm. The inter-particle porosity[23] was determined with use of a 5% aqueous solution of Dextran T2000, then the porosity with 0.5 *M* potassium

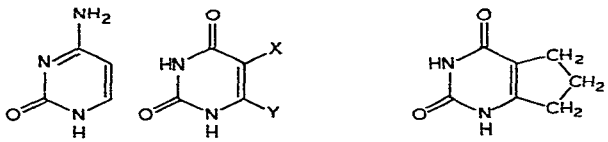
chloride at different flow-rates and with refractive index as well as conductivity detection.

All the derivatives to be chromatographed were dissolved in water and admitted through a 75-μl sample loop into the equilibrated chromatographic system. The amounts of the individual substances applied to the column are shown in Table I; other data for each series of separations are indicated in the appropriate table or figure. Usually, the individual compounds were applied in mixtures of four well-separated compounds, each mixture containing 5-cyclobutyluracil. A typical chromatogram is shown in Fig. 1.

TABLE I

STRUCTURES OF URACIL DERIVATIVES, AND ELUTION VOLUMES AT VARIOUS TEMPERATURES

Except where otherwise stated, conditions were as for Fig. 1, but the linear carrier velocity was 0.532 cm/sec. The compounds have the following structures:



I II – IX; XI–XIII X

Compound No.	*Compound*	*Substituent*		*Amount applied to column (μmole)*	*Elution vol. (ml) at*				
		X	*Y*		*20°*	*40°*	*60°*	*60°**	*80°*
I	Cytosine	—	—	1.5	8.00	7.90	7.50	6.80	
II	Uracil	H	H	1.5	8.20	8.00	7.90	7.40	6.14
III	Thymine	CH_3	H	2.5	10.80	9.70	9.10	8.70	7.40
IV	6-Methyluracil	H	CH_3	2.5	10.10	9.25	8.70	8.13	
V	5-Ethyluracil	C_2H_5	H	1.5	14.5	13.65	11.3	11.15	9.22
VI	5-Propyluracil	C_3H_7	H	1.5	22.6	20.4	16.6	16.4	12.43
VII	5-isopropyluracil	$(CH_3)_2CH$	H	1.5	22.0	19.8	15.9	15.8	
VIII	5-Cyclopropyluracil	C_3H_5	H	2.5	18.1	15.25	12.9	12.8	
IX	5-Allyluracil	CH_2:$CH \cdot CH_2$	H	2.5	19.1	15.95	13.0	12.8	
X	5,6-Trimethyleneuracil	See formula		1.5	14.6	13.50	11.7	11.8	
XI	6-Cyclopropyluracil	H	C_3H_5	2.5	16.9	14.3	11.8	12.3	
XII	5-Cyclobutyluracil	C_4H_7	H	1.5	37.0	30.0	23.0	23.0	16.3
XIII	5-*tert.*-Butyluracil	$(CH_3)_3C$	H	2.5	46.7	38.8	29.7	28.7	20.5

* Phosphate buffer solution (0.05 *M*); linear carrier velocity 0.178 cm/sec.

Properties of the chromatographed substances

Cytosine (I), uracil (II), thymine (III) and 6-methyluracil (IV) were obtained from Lachema. Other derivatives, synthesised by known procedures, were: 5-ethyluracil (V) and 5-propyluracil (VI)[24]; 5-isopropyluracil (VII) and 5-*tert.*-butyluracil (XIII)[25], 5-cyclopropyluracil (VIII) and 6-cyclopropyluracil (XI)[26], 5-allyluracil (IX)[27], 5,6-trimethyleneuracil (X)[28] and 5-cyclobutyluracil (XII)[29].

The solubilities of the compounds in the eluents were determined by stirring for 60 h with the eluent at 25° and centrifugation, the absorbance of the supernatant

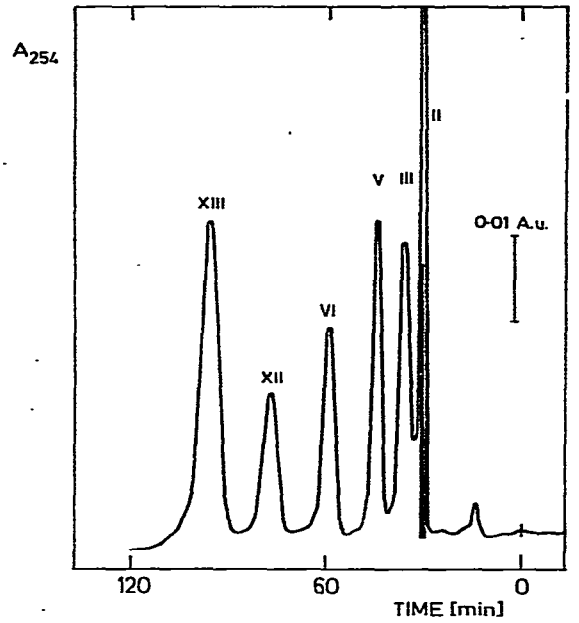


Fig. 1. Separation of uracil (II), thymine (III), 5-ethyluracil (V), 5-propyluracil (VI), 5-cyclobutyluracil (XII) and 5-*tert.*-butyluracil (XIII) on a column (240 mm × 6.36 mm) of Spheron P-300. Linear carrier velocity, 0.035 cm/sec; 80°; eluent, 0.078 *M* boric acid, –0.011 *M* sodium tetraborate (pH 8.66); sample, 75 μl.

liquid being measured with an Opton II PMQ spectrophotometer at 254 nm. The polarities of the eluents were determined from the charge-transfer bands in the visible spectrum, using the betaine form of 1-(2-methacryloyloxyethyl)-4-[2-(3-ethoxy-4-hydroxyphenyl)vinyl]pyridine[30] in a double-beam Specord UV–VIS spectrophotometer.

RESULTS AND DISCUSSION

Chromatography of alkylpyrimidine derivatives

Solvophobic chromatography of compounds I–XIII was carried out on a Spheron P-300 column. The separated substances are polar, owing to the pyrimidine ring, and they are water-soluble. Hydrocarbon substituents in positions 5 and 6 alter the hydrophobicity, so that the compounds are retained on the column by hydrophobic interactions with the packing and can thus be separated; this is indicated by the elution volumes shown in Table I at 20°, 40°, 60° and 80°.

The corresponding dependence of ln K_D (the distribution coefficient) on $1/T$ is shown in Fig. 2.

The lowest elution volume was found for cytosine (I), and the next lowest for uracil (II). Substitution of the hydrogen atom in position 5 or 6 of uracil prolongs the elution time according to the size of the hydrocarbon residue. For uracil derivatives with a three-carbon substituent in position 5 (compounds VI–IX), the elution volume at all temperatures increased in the order: cyclopropyl, allyl, isopropyl, propyl.

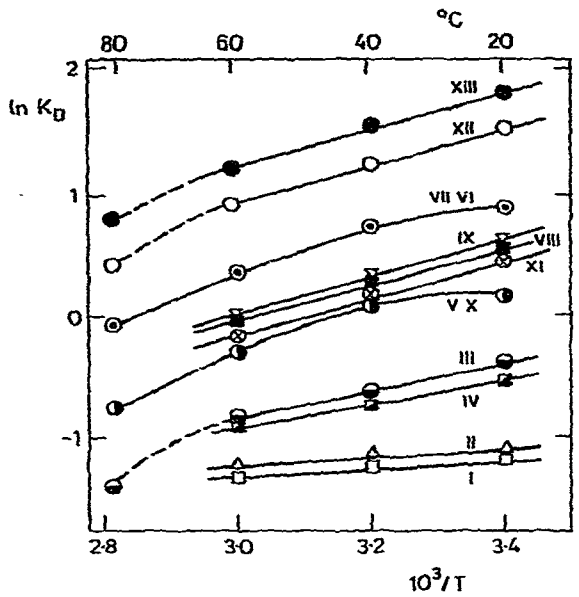


Fig. 2. Dependence of solute retention (expressed as ln K_D) on $1/T$. Experimental conditions as in Table I.

Uracil derivatives substituted in position 6 had smaller elution volumes than the analogous derivatives substituted in position 5; however, the difference was not great. The differences between the elution volumes of compounds VI–IX lead to the same conclusion as that formulated by Tanford for the hydrophobic interactions of amino acids[31], *viz.*, that the contribution to hydrophobic interaction is greater for hydrocarbon residues at the end of the aliphatic chain of the amino acid than for residues in the middle of the chain. Because of this fact, the elution volumes of 5-propyl and 5-butyl derivatives with linear or branched substituents are greater than those of the corresponding cyclic substituents. This is evidenced in higher values of K_D.

Horváth *et al.*[22] adapted the general theory of solvophobic interactions to column processes and proposed a theoretical basis for studying the controlling factors in solvophobic liquid chromatography. The fundamental equations expressing the capacity factor in several terms depending on the type of energy interactions may be written for K_D as follows:

$$\ln K_D = A' + B\frac{1-\lambda}{2\lambda}\cdot\frac{\mu^2_s}{\nu_s}\cdot\frac{1}{1-(\alpha_s/\nu_s)} + C\Delta A \qquad (1)$$

where

$$A' = \frac{\Delta F_{vdwassoc}}{RT} + \frac{\Delta F_{vdws}}{RT} + \frac{4.836\,N^{1/3}\,(K^e-1)\,V^{2/3}\,\gamma}{RT} + \ln\frac{RT}{P_0\,V}; \qquad (2)$$

$$B = ND/RT; \qquad (3)$$

$$C = N\gamma/RT; \qquad (4)$$

λ is a proportionality factor defined as $v_{s,l} = \lambda \cdot v_s$ (v_s being the molecular volume of the solute and $v_{s,l}$ that of the solute–ligand complex); α_s is the polarizability of the solute; $\Delta F_{vdwassoc}$ and ΔF_{vdws} are the differences in the energy association for van der Waals interactions in the gaseous state and the difference in energy of the van der Waals interactions between solute and solvent, respectively; μ_s is the static dipole moment of the solute; N is the Avogadro number; K^e is the energy required for the formation of a cavity; V is the molar volume of the solvent; γ is the surface tension of the solvent; ΔA is the contact surface area; P_0 is atmospheric pressure; R is the gas constant; T is absolute temperature; and D is a function of the static dielectric constant ε:

$$D = \frac{2(\varepsilon - 1)}{2\varepsilon + 1} \tag{5}$$

The uracil derivatives studied are closely related, differing in the hydrocarbon substituents, which are not particularly large in comparison with the size of the whole molecule. Thus, the volumes occupied by the individual derivatives are similar. The first term in eqn. 1 expresses the contribution of the van der Waals interactions, the energy required for cavity formation and an entropy factor; this first term for closely related solutes with commensurable molecular dimensions may be considered as constant (Horváth *et al.*[22]). The second term includes the dipole moment, the molecular volume, the polarizability of the solute and volume changes on binding; it, too, may be considered as constant in this context. The distribution factor is then a function of the size of the interacting solute surface and of the surface tension of the elution medium. Fig. 3 shows the dependence (at 20, 60 and 80°) of ln K_D on the surface area

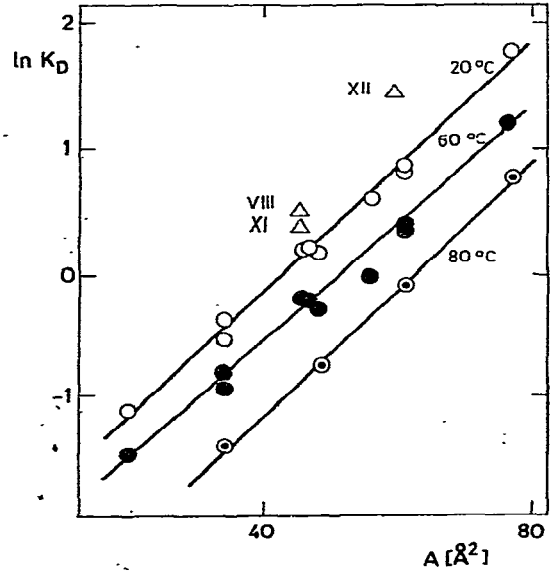


Fig. 3. Dependence of solute retention (expressed as ln K_D) on non-polar surface area (A). Experimental conditions as in Table I. The value of A was calculated for the individual compounds on the basis of the contributions of the non-polar hydrocarbon substituents and the non-polar parts of the uracil ring (see ref. 32), with no correction for "crowding".

of the non-polar groups for uracil derivatives with aliphatic substituents. The non-polar surface area for each compound was calculated from the group surface increments (see ref. 32) due to contributions from the hydrocarbon residues of the substituents and to the uracil ring. As the hydrocarbon substituents were short, no correction was made for "crowding". The dependence of ln K_D on the non-polar surface area in Fig. 3 is linear, and the lines for 20, 60 and 80° are almost identical in slope. This rectilinear relationship is not obtained for uracil derivatives with alicyclic substituents, *e.g.*, 5-cyclopropyluracil (VIII), 6-cyclopropyluracil (XI) and 5-cyclobutyluracil (XII). These derivatives have lower K_D values than have derivatives with aliphatic substituents with the same number of carbon atoms. However, their elution volumes and corresponding values of K_D are higher than would be expected from the dependence of ln K_D on the size of the non-polar surface. This is apparently due to the fact that the values of the non-polar hydrocarbon surface areas for cyclic substituents were calculated as the sum of the increments due to —CH and $-CH_2$ groups derived for aliphatic derivatives; consequently, the true non-polar surface area of these substituents is larger, and the contact area for alicyclic substituents with the same hydrocarbon surface area is greater, than that of the corresponding aliphatic derivative. It is of interest to note that the slopes of the lines in Fig. 3 are almost identical.

On the assumption that the first two terms of eqn. 1 are constant, the slope of the graph of ln K_D against ΔA should decrease with increasing temperature; for 20°, it should be about 1.4 times its value at 80°. The dependence of surface tension on temperature may be expressed by a modification[33] of the Eötvös equation.

$$\gamma = 96.13 - 2.749 \cdot 10^{-2} T - 1.871 \cdot 10^{-4} T^2 \quad (6)$$

If the surface tension of water is introduced into the third term of eqn. 1 from eqn. 6, we obtain the following equation, which clearly shows the dependence of the slope on temperature:

$$\ln K_D = K' + \frac{N}{R}(96.13 \cdot 1/T - 2.749.10^{-2} - 1.871 \cdot 10^{-4} T) \quad (7)$$

where K' is the sum of the first and second constant terms in eqn. 1. Comparison of the results in Fig. 3 and eqn. 7 shows the considerable difference between theoretical and experimental values. The slope of the dependence of ln K_D on ΔA in Fig. 3 changes little with temperature between 20 and 80°, the lines in Fig. 3 being almost parallel. Similarly, the value of the slope of this dependence does not correspond to the value calculated from eqn. 1. The values of the slopes in Fig. 3, and that estimated from the data of Horváth *et al.*[22], are only about 33% of the value calculated from eqn. 1. This can be accounted for by the fact that the non-polar contact surface area represents only part of the total non-polar surface[22]; a definite conclusion would require more data for several homologous series and for different sorbents.

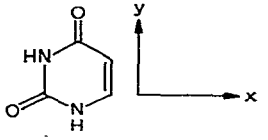
The observed dependence of ln K_D on the size of the interacting non-polar part of the surface of the compounds chromatographed was linear for all the aliphatic derivatives, *i.e.*, with substituents of up to four carbon atoms. According to the composition of the sorbent, one can assume that the size of the non-polar interacting part of the column material is relatively small. Hence one can expect deviations from the

linear dependence of ln K_D on ΔA for compounds with larger residues, as the interacting hydrophobic surface cannot be greater than these residues. The assumption that the interacting regions of the sorbent are relatively small in comparison with the large molecules is important from the point of view of interaction with proteins, and their denaturation; this question is at present being studied.

Table I shows that all the 6-substituted derivatives are eluted earlier and have lower K_D values than the analogous derivatives substituted in position 5, but the differences are not great. It can be assumed that all the parameters of eqn. 1 associated with molecular volume, cavity formation, contact area during solvophobic binding, surface tension and entropy change are equal for both the 5- and 6-substituted derivatives. Differences in the elution volumes and K_D values can thus be attributed to the second term of eqn. 1. The 5- and 6-substituted derivatives differ in the values of their static dipole moments; uracil (and especially cytosine) differ fundamentally not only in the dipole-moment value but also in its orientation. Table II shows the values of the dipole moment, and its orientation, calculated by the CNDO/2 method for cytosine (I), uracil (II), thymine (III) and 6-methyluracil (IV)[34].

TABLE II

VALUES OF DIPOLE MOMENTS AND THEIR x AND y COMPONENTS

Compound	*Dipole moment (debye units)* *			*Orientation of dipole moments*
	Total	*x*	*y*	
Cystosine	8.34	−8.05	−2.18	
Uracil	5.06	−3.17	3.95	
Thymine	4.84	−3.16	3.66	
6-Methyluracil	5.70	−3.66	4.36	

* Computed by the CNDO/2 method[34].

A brief study was made of the effect of pH on K_D values and the efficiency of separation; the results agreed with the above assumptions. Most of the experiments were carried out in a borate buffer of pH 8.66, and the results were compared with those obtained at pH 6.67 in a phosphate buffer at 60° (see Table I). The K_D values and the efficiency of separation were relatively unaffected by the change in pH. On decreasing the pH, the elution times of cytosine and uracil were decreased (the compounds have low K_D values). With those derivatives possessing a small hydrophobic surface, a change in one of the other factors affecting their retention will be more pronounced than with derivatives possessing a greater non-polar surface area. A decrease in pH of 2 units represents for most of the derivatives a large change in their ionization and hence a change in the size and orientation of the dipole moment; there is also a small change in molecular size.

During chromatography of purine and pyrimidine bases and nucleosides on polyacrylamide gel, Khym[13] noted considerable effects due to pH, pre-treatment of the column and the type of buffer used; we observed none of these effects. For pH 8.66, we used a borate buffer, which is essential for efficient separation of purine and pyrimidine bases, and of nucleosides, on polyacrylamide gel; the pH 6.67 buffer was phosphate, which has similar conductivity.

Effect of temperature

The slope of the graph of ln K_D on the interacting hydrophobic area as affected by temperature has already been discussed in part. In the range 20 to 80°, the elution volumes and K_D values decrease with increasing temperature (see Table I and Fig. 2); this means that the over-all enthalpy is negative. The dependence of ln K_D on $1/T$ is slightly curvilinear, but from 20 to 60°, it is well approximated by a straight line. Greater curvilinearity was found only for the 5-ethyl, 5-propyl, 5-isopropyl and 5,6-trimethylene derivatives of uracil (V, VI, VII and X, respectively).

Fig. 4 shows the dependence of the enthalpy change (ΔH) and the entropy change (ΔS) of the binding of the uracil derivatives to the sorbent on the size of their hydrophobic area. The enthalpy change is always negative; initially, its absolute value increases with the size of the hydrophobic surface (for the 5-propyl derivatives and those with larger area, it does not change much further). The entropy change, ΔS, is also negative and its absolute value for the binding to the sorbent increases with the size of the non-polar area, being maximal for the propyl and allyl derivatives; it then begins to decrease as the hydrophobic surface area increases.

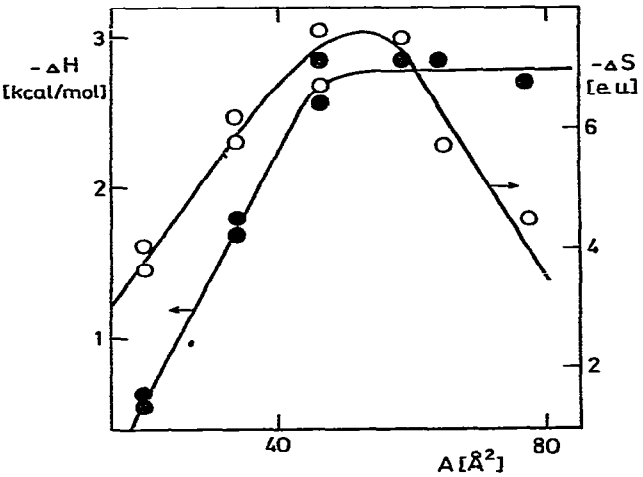


Fig. 4. Dependence of change in binding enthalpy (ΔH) and entropy (ΔS) for the sorption of compounds on the non-polar surface area (A). Experimental conditions as in Table I.

The positive slope of the graph of ln K_D against $1/T$ agrees with general experience in solvophobic chromatography and with the conclusions of Horváth *et al.*[22]. The conclusions from chromatographic experiments, however, are the opposite of those obtained in studies of low-molecular substances, *e.g.*, natural amino acids, or short-chain aliphatic alcohols in organic solvents and water. For amino acids with an aliphatic chain, there is a negative enthalpy change for transfer from a non-polar medium to water, and for aromatic amino acids there is practically no change. The enthalpy change is overcome by the large decrease in entropy, which is attributable to the more organized structure of water near the solute molecule[35]. For aliphatic alcohols with more than four carbon atoms, the effect of the hydroxyl group is negligible, more than two-thirds of the free energy being derived from the enthalpy

term, (in contrast to the predominant role of entropy for shorter alcohols[36]). The total free energy of transfer of alcohol to water is positive, and the enthalpy for C_1 to C_8 alcohols is negative, the lowest value being for the C_3 and C_4 compounds (for alcohols larger than C_8, it is positive). Our results show that the enthalpy does not change greatly with the size of the substituent, similar values of enthalpy being derived from the data of Horváth *et al.*[22]. Differences in the chromatographic behaviour of structurally related derivatives differing in the hydrophobic surface area are caused by differences in the entropy term. A more thorough interpretation of the differences between solute-pure solvent systems and chromatographic systems would require extension of the data to more hydrophobic substances and to thermodynamic changes accompanying the transfer of stationary phase to the solvent.

Effect of the elution agent

So far, we have discussed the effects of two factors (the solute and the temperature) on the behaviour of uracil derivatives during solvophobic chromatography. Another important factor, which is difficult to define, is the character of the eluent.

The elution power of the eluent during adsorption chromatography is usually described by the solubility parameter[37], which is defined as the square root of the cohesive energy density for the solvent and can be resolved into several components according to the type of energy interactions[37]. The agreement between experimental chromatographic data and values predicted from solubility parameters has not been highly satisfactory. A similar approach to the analysis of the theory of solvophobic chromatography was used by Horváth *et al.*[22]. For the eluents methanol–water and acetonitrile–water, these workers computed the dependence of the entropy of mixing, the van der Waals term, the electrostatic term and the surface tension on the solvent composition derived from eqn. 1. The values of interaction energy associated with interactions of the type charge dipole–induced dipole, *i.e.*, those expressed by the electrostatic and the van der Waals term in eqn. 1 (ref. 22), changed little with solvent composition. In the present work, we obtained somewhat different results. We correlated the change of ln K_D for such derivatives as 5-ethyluracil (V), 5-propyluracil (VI), 5-cyclobutyluracil (XII) and 5-*tert.*-butyluracil (XIII) with the change in the value of the semi-empirical polarity parameter for different eluents, from one of high salt content (10% of ammonium sulphate) up to 42% ethanediol. The semi-empirical solvent polarity parameter, which was introduced by Kosower[38], is based on the energy E_T (in kcal/mole) of the charge-transfer bands in the UV spectra of chromophores with a large dipole moment:

$$E_T = h \cdot \bar{\nu}_{max.} = \frac{2.859}{\lambda_{max.}} \cdot 10^5 \qquad (8)$$

where h is the Planck's constant, $\bar{\nu}_{max}$ is the frequency at the maximum of the charge-transfer band and $\lambda_{max.}$ is the wavelength of the maximum (in Å).

The extent of solvation of the ground and of the excited states of the chromophores used for solvent-polarity measurements has not been yet studied theoretically in detail, in contrast to the charge-transfer bands in the spectra of some inorganic anions[39]. From the analogy with the charge-transfer band properties, one can assume a considerable role of interactions of the type charge dipole–induced dipole between

the solute and the solvation medium on the value of E_T. This is confirmed by the fact that the semi-empirical polarity parameter was successfully correlated with a number of solvate-sensitive processes[30], *e.g.*, the rates of reactions taking place via important polar or charge intermediates. However, if one compares the course of the dependence of E_T on the composition of the solvent for the system methanol–water (and for other systems[27]) one sees it to be very different from the dependences calculated from eqn. 1 for the electrostatic and van der Waals terms[22]. The course of this dependence of ln K_D on E_T (Fig. 5) is reminiscent of the calculated dependence of the total value on the solvent composition, which includes all the contributions; this problem will require further detailed investigation. We attempted to compare the contributions of the above-mentioned interactions to the total values of the solubility parameters for various solvents[37] with their E_T values, but the results were not satisfactory.

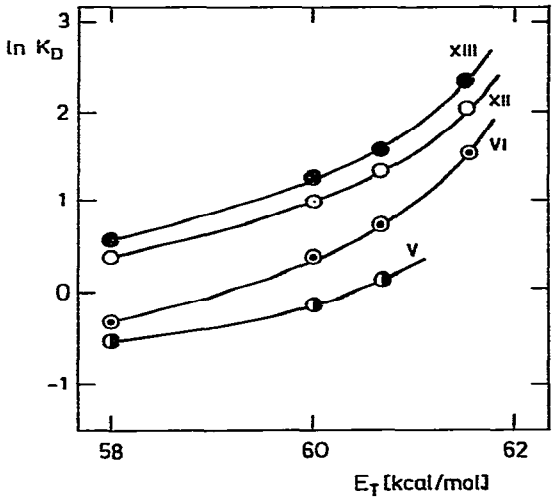


Fig. 5. Dependence of solute retention (expressed as ln K_D) on solvent polarity (E_T) for 5-ethyluracil (V), 5-propyluracil (VI), 5-cyclobutyluracil (XII) and 5-*tert.*-butyluracil (XIII). Eluent *a*, 10% ammonium sulphate solution in 0.078 *M* boric acid–0.011 *M* sodium tetraborate; eluent *b*, 0.078 *M* boric acid–0.011 *M* sodium tetraborate; eluents *c* and *d*, as eluent *b*, but with 20 and 42% of ethylene glycol added, respectively. Linear carrier velocity, 0.532 cm/sec; 60°; column as in Fig. 1. E_T values determined: eluent *a* 61.53, *b* 60.83, *c* 60.04, *d* 58.04 kcal/mol.

To express solvophobic interactions with the sorbent we used another empirical approach. Tanford[31] derived the following equation for the free energy change (ΔF) associated with the transfer of 1 mole of amino acid from ethanol (a non-polar medium) to water:

$$\Delta F = RT \ln \frac{M_{EtOH}}{M_{H_2O}} \tag{9}$$

where M_{EtOH} and M_{H_2O} are the solubilities of the amino acid in ethanol and water, respectively. If the same procedure is used for expressing the energy change associated

with the transfer of solute from the eluent to the less polar gel phase and ΔF is taken from chromatographic experiments, we obtain the equation:

$$\Delta F = -RT \ln \frac{M_{gel}}{M_{eluent}} = -RT \ln K_D \qquad (10)$$

Fig. 6 shows the dependence of K_D for 5-propyluracil (VI), 5-cyclobutyluracil (XII) and 5-*tert.*-butyluracil (XIII) on the solubility of the individual derivatives in the eluent (M_{eluent}); four eluents were studied. Fig. 6 shows that the value of M_{gel}, which expresses the capacity of the gel phase for solute, is constant but different for each of the derivatives studied (8.9 mM for VI, 1.9 mM for XII and 2.3 mM for XIII). Differences between the individual derivatives are probably attributable to different molecular dimensions.

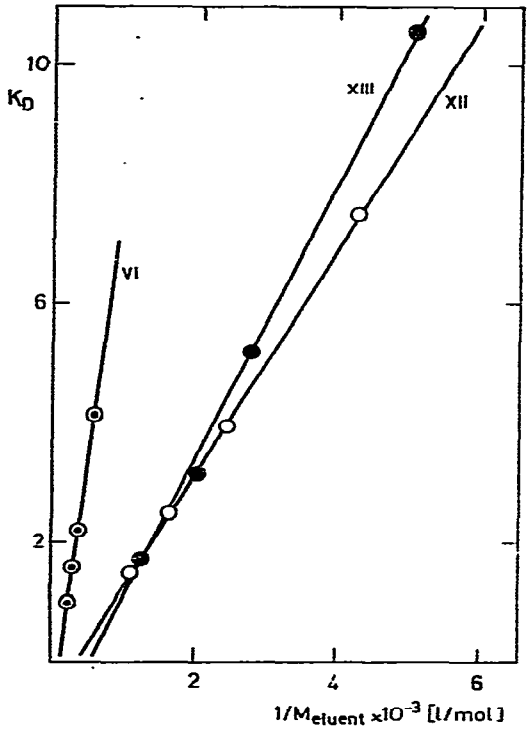


Fig. 6. Dependence of solute retention (expressed as K_D) for 5-propyluracil (VI), 5-cyclobutyluracil (XII) and 5-*tert.*-butyluracil (XIII) on solubility (M_{eluent}) in different elution agents. Conditions as in Fig. 5.

Separation efficiency

The dependence of the efficiency of separation on temperature for some derivatives is shown in Fig. 7; the HETP decreases somewhat with increasing temperature. Table III shows the values of HETP for all the derivatives studied, and its dependence on the carrier velocity at 60°. The dependence of the reduced HETP on

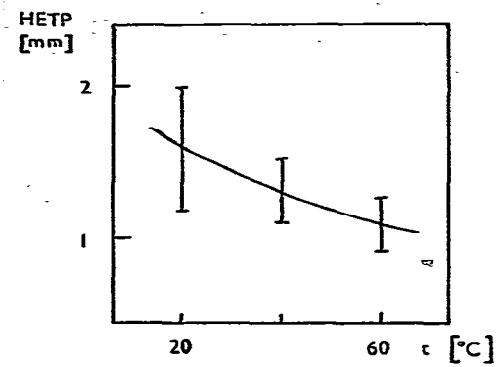


Fig. 7. Dependence of HETP on temperature. Conditions as in Table I. Linear carrier velocity 0.523 cm/sec.

TABLE III

DEPENDENCE OF HETP VALUES (mm) ON LINEAR CARRIER VELOCITY AT 60°
Conditions as in Table I.

Compound	*Linear carrier velocity (cm/sec)*						
	0.035	*0.0718*	*0.178*	*0.178**	*0.271*	*0.416*	*0.523*
Cystosine	0.22	0.29	0.38	0.39	0.49	0.74	1.14
Uracil	0.21	0.33	0.49	0.51	0.67	0.95	1.54
Thymine	0.17	0.26	0.44	0.37	0.55	0.84	1.17
6-Methyluracil	0.26	0.36	0.42	0.42	0.55	0.95	1.09
5-Ethyluracil	0.19	0.26	0.38	0.33	0.49	0.77	1.02
5-Propyluracil	0.24	0.28	0.43	0.39	0.55	0.80	0.96
5-Isopropyluracil	0.20	0.29	0.37	0.37	0.49	0.73	0.97
5-Cyclopropyluracil	0.22	0.28	0.45	0.35	0.53	0.75	0.98
5-Allyluracil	0.22	0.30	0.37	0.35	0.51	0.73	0.97
5,6-Trimethyleneuracil	0.26	0.30	0.40	0.37	0.60	0.85	1.08
6-Cyclopropyluracil	0.22	0.34	0.47	0.36	0.60	0.94	1.18
5-Cyclobutyluracil	0.21	0.32	0.40	0.37	0.49	0.67	0.91
5-*tert.*-Butyluracil	0.26	0.30	0.43	0.39	0.57	0.73	0.91

* Phosphate buffer solution (0.05 *M*).

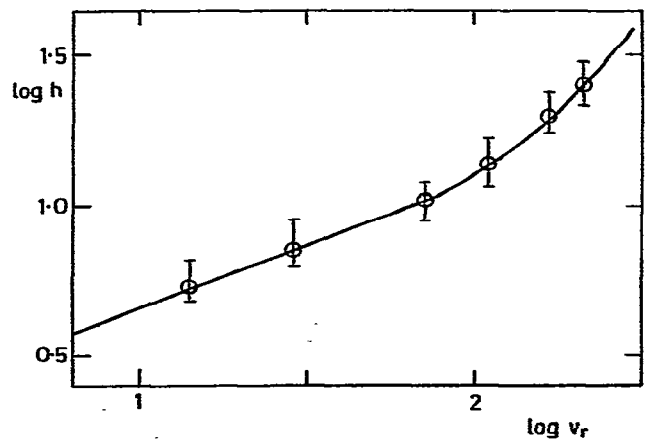


Fig. 8. Dependence of reduced plate height (h) on reduced carrier velocity (v_r). Column as in Fig. 1; 60°; eluent, 0.078 *M* boric acid–0.011 *M* sodium tetraborate (pH 8.66).

the reduced linear-flow velocity, v_r, is shown in Fig. 8. As the plate height of the column is proportional to the largest particle size within a given sieve fraction rather than to the average particle size[40], the value of 40 μm was introduced into eqns. 11 and 12:

$$h = \frac{HETP}{d_p} \tag{11}$$

$$v_r = \frac{v \cdot d_p}{D_m} \tag{12}$$

where d_p is the particle diameter, D_m the diffusion coefficient (10^{-5} cm^2/sec). The dependence of h on v_r is flat, and the efficiency of the filling is relatively high in view of the particle size.

The HETP values are also affected by extra-column contributions, one of the largest of which derives from applying the sample with a sample loop. For this reason, the separation under conditions shown in the experimental section, *i.e.*, with the sample loop, was compared with separation of some of the compounds, *viz.*, uracil (II), thymine (III), 5-ethyluracil (V), 5-propyluracil (VI), 5-cyclobutyluracil (XII) and 5-*tert.*-butyluracil (XIII) achieved after applying 10 μl of a more concentrated solution of the compounds directly in the centre of the top of a column of larger diameter; the separation is shown in Fig. 9. By eliminating the effect of sample application (and partly also the wall effect), the efficiency of the column was increased by a factor of 3.3 to 1.3 compared with the results shown in Table II.

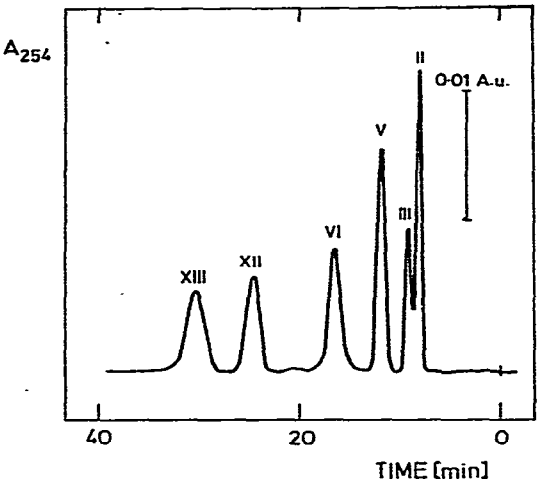


Fig. 9. Separation of uracil (II), thymine (III), 5-ethyluracil (V), 5-propyluracil (VI), 5-cyclobutyluracil (XII) and 5-*tert.*-butyluracil (XIII) on Spheron P-300 with direct sample injection (10 μl) on to the top of the column. Column, 270 mm × 8 mm; 20°; linear carrier velocity, 0.137 cm/sec; eluent as in Fig. 8.

CONCLUSIONS

Derivatives of uracil with hydrocarbon substituents in positions 5 and 6 were chromatographed on Spheron P-300; their retention on the column was dependent

on the area of their hydrophobic surfaces. The linear dependence of ln K_D on the hydrophobic area was established for three temperatures. In contrast with the theory of solvophobic chromatography formulated by Horváth *et al.*[22], we found that the slopes of the lines for different temperatures were identical. The dependence of the changes in enthalpy and in entropy during binding to the chromatographic support on the area of the non-polar surface was also determined. The effect of changes in pH, ionic strength, buffer type and eluent on the retention of the compounds on the column was studied; retention was little affected by these factors. The distribution coefficients of some of the compounds in different eluents were correlated with the polarities of these eluents and with the solubilities of the chromatographed compounds. The efficiency of the support in dependence on temperature and flow-rate has been determined.

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