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INTERACTION OF AMINO ACIDS, N-ACETYL AMINO ACID ESTERS, THYMINE AND ADENINE WITH SEPHADEX LH-20 GEL

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SUMMARY

N-Acetyl aromatic amino acid esters, tryptophan, adenine and thymine show strong retention in Sephadex LH-20 gel in an aqueous phase. The decreased retention in 6 *M* urea, the absence of retention in absolute methanol and the increased retention at higher temperatures in the aqueous phase indicate that hydrophobic interaction is responsible for the observed retention of the amino acids and their esters in the gel. Adenine was found to be retained by polar interaction in the gel. The increased retention of the solutes in the presence of different electrolytes suggests that the lyotropic effect is more important than the ionic strength effect. The relevance of the results obtained with amino acids and esters to the conformational aspects of proteins in aqueous solution is discussed.

INTRODUCTION

Separation in gel filtration is considered not to involve any interaction of molecules with the gel matrix; large molecules leave the gel bed before the smaller molecules and all molecules are eluted before the bed volume of the gel[1–5]. However it was observed that certain low-molecular-weight substances interact with Sephadex gel of high density (increased extent of cross-linking in the dextran polymer), when smaller molecules are eluted before the larger molecules and all molecules are eluted after the bed volume of the gel[1,6]. The observed retention has been ascribed to hydrogen bonding and dispersion interaction between the solutes and the gel[7,8].

Determann and Lampert[9] have recently demonstrated that with Sephadex LH-20, which is a hydroxypropyl derivative of Sephadex G-25, the retardation of saturated and unsaturated aliphatic acids, aromatic acids, benzene and phenol can be explained not by hydrogen bonding or dispersion interaction but by "hydrophobic bonding" between the solutes and the gel. The main object of their work was to identify the nature of the interactions present in the gel and to utilise them as a basis for new separation methods.

This paper reports a study of the interaction of aromatic amino acids and N-acetyl aromatic amino acid esters with the Sephadex LH-20 in water and in the presence of electrolytes. The relevance of these results to the conformational aspects

of protein molecules is discussed. In addition, the interaction of the two nucleic acid bases adenine and thymine with the gel has also been studied.

EXPERIMENTAL

Sephadex LH-20 beads (particle size 25–100 μm) were obtained from Sigma (St. Louis, Mo., U.S.A.) and β-phenylalanine (Phe) from BDH (Poole, Great Britain). L-Tyrosine (Tyr) and DL-tryptophan (Try) were reagent-grade materials from E. Merck (Darmstadt, G.F.R.). N-Acetyl-L-phenylalanine methyl ester (APE), N-acetyl-L-tyrosine ethyl ester (ATYE) and N-acetyl-L-tryptophan ethyl ester (ATRE) were grade I chemicals from Cyclo Chem. (Los Angeles, U.S.A.). Adenine (A) and thymine (T) were obtained from Calbiochem. (Los Angeles, U.S.A.) and were recrystallized twice from water. N-Acetyl-L-phenylalanine (APA), m.p. 145–146°, was obtained from the Biochemistry Discipline of this Institute.

The pH of distilled water was adjusted to 7.0 and it was used as the solvent. The salt effect was also studied at this pH. For pH 2.5 and 9.0, hydrochloric acid–potassium chloride (ionic strength, $\mu = 0.03$) and triethanolamine–hydrochloric acid ($\mu = 0.03$) buffers, respectively, were used. Chemicals were of reagent grade and used without further purification.

Thermostatic control was achieved by circulating water at the desired temperature through the outer jacket of the column. At all pH values, without salt, the column dimensions were 16.1 × 2.1 cm (bed volume, $V_t = 54$ ml). In the presence of salts and other reagents, the gel shrank and the bed volume changed (see Results) and V_t was determined in each instance by weighing the amount of water required to fill the column to the top of the bed of the gel. The elution of the compounds was followed spectrophotometrically with a Carl Zeiss spectrophotometer. The absorbance of Phe, APE and APA in both the aqueous phase and methanol were measured at 257.5 nm, Tyr and ATYE at 275 and 278 nm in aqueous solution and methanol respectively, and Try and ATRE at 280 and 283 nm, respectively. Both A and T were measured at 264 nm in water and methanol. The void volume, V_0, was determined with Blue Dextran 2000 and found to be 22 ml. Lampert and Determann[10] observed that V_0 is independent of temperature. The flow-rate was 60 ml/h. The elution volume, V_e, was reproducible within 5 ml for all compounds.

RESULTS

Effects of pH, temperature and salt on the LH-20 gel

The bed volume, V_t, of the gel was found to be independent of pH. The gel matrix shrank with increase in temperature; V_t decreased from 54 ml at 25° to 44 ml at 50° for 13 g of swollen gel at pH 7.0. An increase in the salt concentration also resulted in shrinking of the gel. For example, V_t is 42, 45 and 52 ml, respectively in 0.333 *M* sodium sulphate solution, 1 *M* sodium chloride solution and 1 *M* sodium perchlorate solution.

Elution pattern of different solutes in LH-20 gel

The elution volumes, V_e, of the solutes are shown in Fig. 1. Skewed elution patterns were observed with ATRE and to a lesser extent with Try. The elution pat-

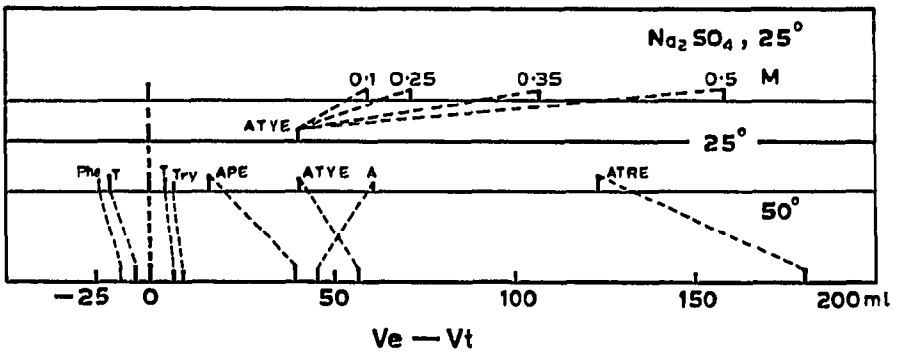

Fig. 1. Schematic diagram showing the relative peak positions of the elution profile at pH 7.0 in Sephadex LH-20 gel at different temperatures. The concentrations used were: Phe and APE, $8 \cdot 10^{-3}$ *M*. Tyr and ATYE, $5 \cdot 10^{-3}$ *M*; Try, ATRE, A and T, $1 \cdot 10^{-3}$ *M*. The figure also shows the effect of increasing Na_2SO_4 concentration on the peak position of the elution of ATYE.

terns with ATRE, particularly at high salt concentration, showed a maximum absorbance region spread over an elution volume of 10 ml, and the volume representing the mid-point of this region was taken as V_e for ATRE in these solutions. A 15-fold change in the concentration of the amino acid or the ester and a 25-fold change with A and T did not result in any change in V_e values at concentrations above $\cdot 10^{-3}$ *M*.

Of the amino acids, Phe and Tyr were eluted before V_t, whereas Try was eluted after V_t (Fig. 1). The first two amino acids at 50° were eluted later than at 25°, although both were eluted before V_t (Fig. 1); Try did not show any marked change in V_e compared with the value at 25°. In the presence of 0.5 *M* sodium sulphate solution, the values of $V_e - V_t$ for Phe and Tyr are −3.0 and 5.0 ml, respectively, compared with values of −14 and −11 ml, respectively, in water, indicating a tendency for these solutes to be retained in the gel in the presence of electrolytes. The $V_e - V_t$ values for Try in water and 0.1 *M* and 0.5 *M* sodium sulphate solution are 6, 9 and 22 ml, respectively, indicating its stronger retention in the presence of electrolytes compared with the other two amino acids.

The ester molecules are considerably retained in the gel in the order APE < ATYE < ATRE and an increase in temperature increases the retention of the molecules (Fig. 1). The effect of 6 *M* urea is to reduce the retention of the esters (Fig. 2), while 8 *M* aqueous methanol reduces V_e (not shown here) but to a lesser extent than 6 *M* urea. The effect of absolute methanol was marked; all solutes eluted before V_t (Fig. 2). The effect of an increase in salt concentration was to increase the retention of the esters (Fig. 1). In order to compare the increased retention of the solutes in the gel matrix, we followed the procedure used by Determann and Lampert[9], who defined a parameter $A_1 = (V_e - V_t)/g$ (g is the weight of the dry gel in grams); A_1 values for the compounds are plotted in Figs. 3 and 4. It can be seen that in the same salt solution, the retention increases in the order ATRE > ATYE > APE (Fig. 3). In different salt solutions, the retention of the solutes decreases in the order Na_2SO_4 > NaCl > $NaClO_4$ (Fig. 4).

A showed a high retention, which was greater than that of ATYE in water. T, however, was retained to a small extent (Fig. 1) and temperature did not have any significant effect on its V_e value. A was eluted earlier at 50° than at 25°, which was in

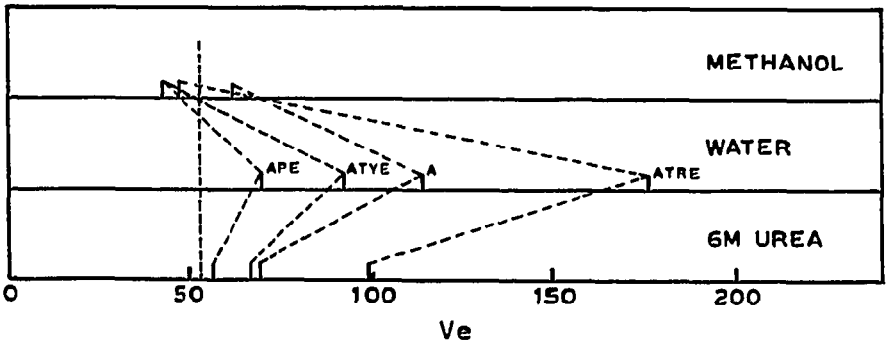

Fig. 2. Schematic representation of the effects of absolute methanol and 6*M* urea on the position of elution peaks of the solutes in Sephadex LH-20. The vertical dotted line is the position of the bed volume, V_t, of the gel.

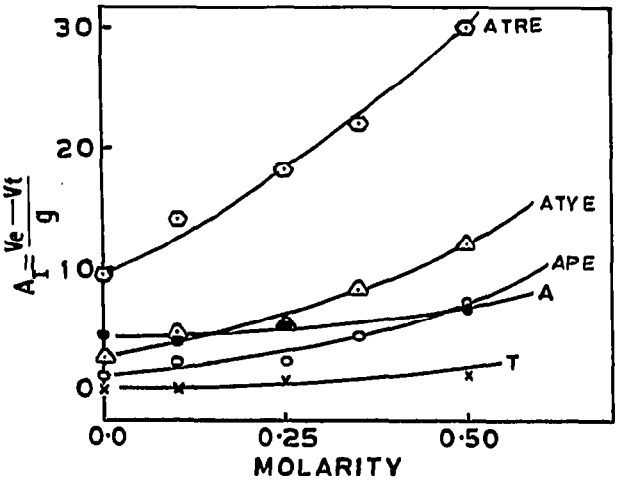

Fig. 3. Effect of Na_2SO_4 concentration on the retention of different solutes in Sephadex LH-20. The term A_I is defined in the text. pH, 7.0; temperature, 25°.

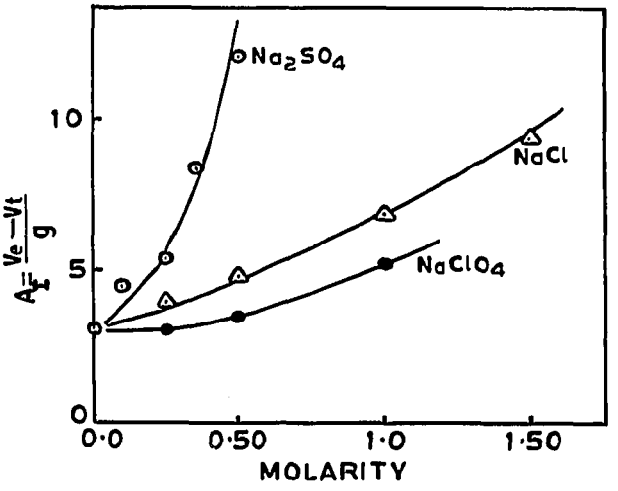

Fig. 4. Effects of concentration of different salts on the retention of ATYE in Sephadex LH-20. For A_I, see text. pH, 7.0; temperature, 25°.

contrast to the results with the other solutes in this investigation. In 6 *M* urea the retention of A decreased, but in absolute methanol A showed some retention which was absent with the esters. In the presence of electrolytes, the retention of both A and T increased. The retention of T was much lower than those of A and esters.

DISCUSSION

Shrinkage of the gel

Lampert and Determann[10] and Determann and Lampert[9] observed shrinkage of LH-20 gel with increase in temperature and ionic strength, as was observed in this work. They suggested that the increase in the interaction of different groups present in the dextran chain is responsible for the shrinkage of the gel at higher temperature, whereas the competition for water molecules between the solvated polymer chain and ions of the electrolytes favours the ions and results in shrinkage of the gel. The present results show that the shrinkage in sodium sulphate solution is greater than in sodium chloride solution, whereas sodium perchlorate solution does not result in any shrinkage (see Results). This indicates that the lyotropic effect (which depends on the nature of the salt rather than its concentration) is more important in shrinking Sephadex LH-20 gel.

Adsorption in the gel

Obrink *et al.*[11] showed that the elution of the molecules in Sephadex gels is not governed by the surface forces between the gel matrix and the eluting molecules. The interaction of solutes with the gel and their consequent retention may arise from Van der Waals interaction, hydrogen bonding, π-electron interactions (in the case of solutes having π-bonds) and hydrophobic interaction of the solutes with the Sephadex gel. The observed retention is always the sum of these different interactions, which sometimes compete or overlap with each other[9]. Determann and Lampert[9] concluded that hydrophobic interaction of some long-chain aliphatic acids, benzoic acid, phenol and benzene with Sephadex LH-20 in an aqueous phase is responsible for the observed retention of these solutes. The suggestions by Streuli[12] and Janson[13] that π-electron interactions of the aromatic systems with the Sephadex gel may be responsible for the retention have been refuted by Determann and Lampert[9], from their observation that anisole and nitrobenzene, which are supposed to increase and decrease the π-electron density, respectively, in the benzene ring, are both retained more than benzene in the gel.

In order to identify the nature of the interaction responsible for the retention of the solutes studied here, the compounds were eluted in aqueous methanol, urea and methanol and at higher temperatures. The decreased retentions in aqueous methanol and urea indicate that hydrogen bonding or hydrophobic bonding or both are responsible for the observed retention of the esters, as both of these interactions are weakened in these solutions[14,15]. The absence of any interaction of the esters with the gel in methanol indicates that hydrogen bonding between the esters and the gel is absent and hydrophobic interaction is most probably responsible for the retention in the aqueous phase. This is confirmed by the increase in the retention at higher temperatures, as hydrogen bonding and Van der Waals forces are weakened as the temperature increases. The endothermic nature of the retention shows that the process is entropy-

controlled and the interaction is hydrophobic in nature. It has been observed that the hydroxyl group in tyrosine does not impart any hydrophilic properties to the molecule, and in fact tyrosine is more hydrophobic than phenylalanine[16]. Similarly, it has been found that the tryptophanyl side-chain is the most hydrophobic of all protein side-chains[16]. The hydrophobic interaction of the esters with Sephadex LH-20, therefore, most probably involves these side-chains with the hydroxypropyl group of the gel.

Phe and Tyr were eluted before the bed volume, indicating that they do not interact with the gel, although Try was retained by the gel (Fig. 1). It has been reported that ions, particularly anions, are excluded from the Sephadex gel matrix, which indicated the absence of interaction of the compounds that carried anions with the gel bed[6]. Brook and Housley[17] studied the elution of phenol and substituted phenols from Sephadex G-10 in the presence of 0.1 *M* sodium hydroxide solution, where all of the solutes are in the anionic form, and observed that although phenol was eluted before V_t of the gel, certain monosubstituted phenols were considerably retained, indicating that exclusion and interaction can take place simultaneously. The results with the amino acids are similar. The retention of benzene and phenol, as observed by Determann and Lampert, and the esters studied here suggests that these groups are capable of hydrophobic interaction, either alone or as a part of neutral molecule. The reduced or lack of retention of amino acids that carry similar groups suggests that the hydrophobic groups are incapable of showing interactions of comparable magnitude when they are part of a charged molecule. In other words, the presence of charges in the vicinity of a hydrophobic group, as in amino acids, would reduce their hydrophobicity.

That the presence of charge in the molecule influences the interaction of molecules is further shown by the V_e values at different pHs (Table I). The esters with no charge had the same V_e values at the three pHs, within the limit of experimental error. The amino acids that are anionic at pH 9 and zwitterionic at pH 7 were eluted at the same position. At pH 2.5, where the amino acids are positively charged, retention of Tyr and an increased retention of Try were observed, which shows that anions probably interfere more than cations with the hydrophobic interaction. The most

TABLE I

EFFECT OF pH ON THE ELUTION VOLUME (V_e) OF THE COMPOUNDS AT 25°
V_t = 54.0 ml (see text).

Compound	V_e *(ml)*		
	pH 2.5	*pH 7.0*	*pH 9.0*
APE	68	70	69
ATYE	92	92	89
ATRE	185	180	150
Phe	45	41	37
Tyr	53	42	42
Try	79	60	62
APA	86	37	37
A	55	114	119
T	—	58	—

remarkable effect of anions was observed with acetylphenylalanine, which was eluted first of all the solutes at pH 9 and 7, and was strongly retained at pH 2.5, no effective charge being present in the molecule.

Effect of electrolytes

The retention of solutes in the gel, as discussed above, indicates interaction of the solutes with the gel. Alternatively, the elution position can be considered to reflect the tendency of the solutes to leave the bulk aqueous phase in favour of the environment of the gel[18]. The increased retention of the esters in the presence of electrolytes showed the increased preference of the solutes to leave the bulk aqueous phase, *i.e.*, salting-out of the compounds. The increased retention, however, also indicates stronger hydrophobic interaction of the solutes with the gel in the presence of electrolytes. The decreasing retention of the solutes in the series $Na_2SO_4 > NaCl > NaClO_4$ showed that the lyotropic effect is more important than the ionic strength effect, as was observed previously from the salting-out behaviour of similar esters from solubility/distribution experiments[19]. The amino acids in sodium sulphate solution (where only the salt effect on amino acids was studied) show a tendency to remain in the gel matrix (see Results) compared with their retention in water. This is interesting, because at moderate salt concentrations, charges on the amino acids would be stabilised compared with their stabilities in water, and Try should have eluted earlier than in water. The results show that favourable interaction of the charges with the salt would be overcome by unfavourable interactions of the hydrophobic groups with the salt ions.

Interaction of adenine and thymine with the gel

Adenine was the second most strongly retained solute studied; T, however, showed much less interaction with the gel. The strong interaction of A with the gel is not dominated by hydrophobic interactions, like the esters, as shown by its retention in methanol and its decreased retention at higher temperatures. This result, on the other hand, indicates the presence of polar interactions of A with the gel. Hydrogen bonding alone cannot explain the greater retention of A observed, as T has the same number of possible hydrogen bonding sites. The elution of A before V_t at pH 2.5 indicates that its amino group ($pK_1 = 4.2$) plays a significant role in the observed retention. This may be due to the high electron density at the N-1 position with its unshared pair of electrons as the primary site of interaction with the OH function of the hydroxypropyl group attached to the dextran ring[20].

A and, to a lesser extent, T are retained more in the presence of electrolytes compared with their retentions in water (Fig. 3). This indicates that salting-out of the solutes occurs in a similar manner to that observed by Robinson and Grant[21] from solubility studies of the compounds. The increased retention in electrolytes is surprising, as polar interactions by which A is adsorbed on the gel should decrease in the presence of electrolytes and thus should lead to less retardation. The observed increased retardation suggests that other interaction(s) predominate over the polar interaction in the presence of electrolytes.

Application of the results to protein in solution

Kauzmann[22] suggested that hydrophobic interaction derived from the non-polar side-chains present in the peptide backbone is mainly responsible for the com-

pact configuration of native protein molecules. Since then, much work has been carried out to obtain qualitative or semi-quantitative estimates of these hydrophobic interactions, especially in denaturing solvents such as urea and guanidine hydrochloride[23–27]. These studies were mostly carried out with model compounds such as aliphatic and aromatic hydrocarbons and amino acids. Doubt has been expressed, however, on the relevance of using the results obtained with these above model compounds, as it was considered that a hydrocarbon moiety attached to a polar backbone may behave in a manner different from that of the hydrocarbon molecules; also, the presence of charges in the neighbourhood of a hydrophobic group may influence its capability to interact, which would otherwise occur when similar groups are part of peptide chain[24,28]. Recently, a series of blocked amino acids ($CH_3 \cdot CO \cdot NH \cdot CH(R) \cdot COOC_2H_5$) were studied in order to determine the contribution of the side-chains, R, towards the free energy of transfer in different denaturing solvents[19,29–31]. These compounds were thought to be better models for a peptide unit in a protein molecule than either amino acids or hydrocarbon molecules. The contributions towards the free energy of transfer of R groups derived from these compounds in urea and guanidine hydrochloride solutions were found to be considerably less than those obtained from similar R groups attached to amino acids[29,30].

That the gels can be used to study the interactions present in a protein molecule has been shown by St. Pierre and Jencks[18] and more recently by von Hippel *et al.*[32], who studied the interaction of different salts and denaturants with a polyacrylamide gel that they considered as a model for amide groups present in a protein molecule. This investigation presents evidence that the capability of the groups to interact hydrophobically would be reduced to a great extent in the presence of charges and therefore extension of the use of the values obtained from amino acid studies to studies with proteins should be carried out with caution.

Kauzmann[22] suggested that the increased hydrophobic interaction of nonpolar groups in salt solutions is responsible for the stability of native protein molecules in those solutions. His suggestion was based on the decreased solubility of certain nonpolar solutes in electrolyte solutions. The present results most probably indicate that hydrophobic interactions would increase in electrolyte solutions, which would depend more on the nature of the ion than on ionic strength. Further, a favourable interaction between an electrolyte and ions present in a molecule would be dominated by an unfavourable interaction of hydrophobic groups with the electrolyte.

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