ADSORPTION PHENOMENA ON SEPHADEX®

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Although it was the molecular sieving properties of the dextran gels which first received the greatest attention, other interactions were also soon observed. In 1960, PORATH[1] and GELOTTE[2] described a number of observations where substances showed a behaviour differing widely from what might be expected from their molecular size, *i.e.* their behaviour could not be interpreted as only a restricted diffusion into and through the gel phase caused by steric hindrance.

In general there are only two ways in which a solute can depart from its "true" elution volume, *viz.* either by appearing earlier or by appearing later. Substances which appear later are retarded either by adsorption or by electrostatic interaction, whereas an early elution is caused primarily by ion exclusion or sometimes by complex formation or aggregation.

The electrostatic interactions (including ion exclusion) are due to the fact that the cross-linked dextran chains contain a few terminal carboxylic groups. With the more tightly cross-linked Sephadex types, G-50, G-25, G-15 and G-10, the effect of the fixed charged groups may be particularly noticeable when the eluant is deionized water, and the gel will act as a weak cation exchanger with very low capacity. Thus small amounts of cations will be adsorbed, and anions may be completely excluded, especially when very small samples are applied to the gel bed. For a heavy sample load, as in the desalting of high polymer solutions, the effect will be observed as a front tailing of the salt zone. This means that a complete desalting is not possible on these gels. In most cases it is of minor importance since the relative amount of salt eluted together with the high polymer material is low. To effect complete desalting it is recommended that a solution of a volatile salt is used as eluent and afterwards the volatile salt is removed by lyophilization.

These electrostatic interactions were first discovered in the early dextran gels which contained much higher concentrations of fixed negatively charged groups (carboxylic groups) relative to the present gels. MIRANDA and collaborators[3] thus utilized the ion exchange properties of Sephadex G-25 and G-50 obtained with deionized water for the reversible retention of low molecular weight basic proteins such as toxins of scorpions, ribonuclease and lysozyme. GLAZER AND WELLNER[4] studied the binding capacity of Sephadex G-50 in distilled water for lysozyme, ribonuclease and serum albumin.

As this ion exchange effect is completely eliminated by the addition, to the medium or to the sample, of small amounts of an electrolyte, it is of minor importance and of little interest in most gel filtration experiments. Nevertheless one has to bear it in mind when interpreting elution data at low ionic strength.

Here, the term "adsorption" is meant to describe any kind of non-electrostatic

interaction between a solute and the dextran gel matrix which for the solute causes an anomalously high K_d.

The K_d value is defined as $V_e - V_0/V_i$. V_e is the elution volume of the substance, V_0 is the void volume, and V_i is the inner volume, *i.e.*, the sum of the internal aqueous volumes of the gel grains. V_i can be calculated from $V_i = g \cdot W_r$, where g is the dry weight of the gel present in the column and W_r is its water regain in grams per gram.

Thus a molecule which is completely excluded has a K_d of zero, while a low molecular weight solute which can diffuse freely into and through the grains should have a K_d value of about 1. In reality this is not the whole truth because part of the inner volume, V_i, is water of hydration which is firmly bound to the polysaccharide framework in the gel grains and is inaccessible to the solute molecules. In Sephadex G-25 a K_d value of about 0.8, and in Sephadex G-10 a K_d value of about 0.75, indicate therefore a non-restricted diffusion in the gel column. When exact values of V_i are desired, one probably should use tritiated water as recommended by MARSDEN[5] in a recent paper, where he critically examines this method.

Among the adsorption phenomena, the affinity of the dextran gel matrix for aromatic and pseudo-aromatic substances is particularly striking. However, this is not a unique property of the dextran gels. It is in accordance with the affinity characteristics found for adsorption to cellulose[6]. A planar structure and an extending system of conjugated bonds in the solutes favour adsorption, and in view of the chemical similarity between dextran gels and cellulose similar mechanisms are probable. The observations of LATHE AND RUTHVEN in 1956[7] on the behaviour of many substances on starch columns support this view.

In most cases, the adsorption isotherms on dextran gels, have been found to be linear, and the solutes are therefore eluted as symmetrical peaks. This is also valid for substances with very high K_d values.

As this affinity for π-electron rich compounds to the dextran gel matrix has been shown to be the most important interaction utilized for separation, the discussion of this effect will dominate this paper.

The larger the proportion of the solute molecule that consists of a gel-interactive group, and the higher the matrix density of the Sephadex gel used, the more pronounced is the adsorption effect compared with the molecular sieving effect. Consequently it is in the fractionation of low molecular weight solutes on the most tightly cross-linked Sephadex types that the most applications are expected to be found, since the chemical differences among the small solutes are much greater than among the macro-molecules usually studied in the gels of high "water regain".

Let us consider the different effects which may influence the behaviour of a solute which has some sort of "aromatic interaction" with the Sephadex gel matrix.

First, molecular sieving is always the underlying effect and always, most important, plays a decisive role as regards the selection process implied by the terms penetration and exclusion. In fact, no useful chromatography can be accomplished with substances which are too large to penetrate the gel.

Second, when discussing the mechanism of adsorption of solutes to the gel matrix one has to distinguish between the *pure adsorptivity* which is based on the structure of the substance and *superimposed effects* such as the ionic strength and pH of the eluent, and the effect of the few carboxylic groups present.

If the solute is uncharged, and possesses no acidic or basic properties, it is

practically insensitive to changes in pH and ionic strength. It is merely influenced by the presence of substances in the medium which compete for the adsorption sites in the Sephadex gel matrix. Such substances are for example pyridine, phenol, acetic acid and urea.

If the solute is charged or if it contains an ionizeable group and/or if it is an ampholyte, drastic changes in pH will definitely strongly influence the behaviour of the solute on the column. The possible explanation for this is that either there occurs a rearrangement of the π-electrons in the molecule due to the new charged group, or that the highly charged molecule is now surrounded by a larger ionic double layer which prevents the interacting group of the gel matrix from entering the adsorption sites. A combination of these two effects is of course also possible.

In distilled water, the K_d values for the acidic aromatic substances are a compromise between the two counter-balancing effects, aromatic adsorption and ion exclusion. By the addition of small amounts of an electrolyte to the distilled water the ion exclusion effect, which depends on the small amounts of fixed carboxylic groups mentioned, is eliminated and the aromatic charged solute is retarded to an extent which is determined by the degree of the ionic strength of the eluent, *i.e.* the adsorption increases with increasing salt concentration. This is caused either by an increase in the number of adsorption sites available or by an increase in the strength of the interaction due to a decrease in the size of the layer of water of hydration which prevents the solute–gel interaction.

The most successful applications of the aromatic adsorption effect have probably been performed in the peptide separation field. It has been used both for the fractionation of protein hydrolysates and for the separation of naturally occurring peptides. By suitable choice of the medium it is possible to utilize both the molecular sieve effect and the aromatic adsorption effect of the Sephadex gel in the purification of a certain peptide containing one or more aromatic amino acids. It is ideally suited as a complement to ion-exchange chromatography because the two methods differentiate on the basis of different molecular properties.

EAKER[8] has studied the behaviour of tryptic digests of oxidized ribonuclease A in long narrow Sephadex columns. He found (Fig. 1) that the peptides of the hydrolysate grouped themselves into three sharply separated zones on Sephadex G-25 in 0.2 *M* acetic acid. The first zone contained peptides with 19–22 amino acid residues, the second zone contained tetra- and hexapeptides. The third zone contained, together with di- and tripeptides, one hepta- and one decapeptide, each of the two latter containing two tyrosine residues. The purification was further pursued by ion-exchange chromatography, but a partial purification could have been obtained by a rerun on the same Sephadex column in a medium which causes a complete depreciation of the aromatic adsorption, for example in the phenol–acetic acid–water solvent mixture introduced by SYNGE and collaborators[9,10] and used by CARNEGIE[11] in his work on estimation of molecular size of peptides by gel filtration on a micro scale. STEPANOV and collaborators[12] have used 8 *M* urea for the same purpose.

MACH AND TATUM[13] studying the environmental control of amino acid substitutions in the biosynthesis of the antibiotic cyclic decapeptides tyrocidine A, B and C, in a very elegant way utilized their different contents of the aromatic amino acid tryptophan for separation on a 200 cm long column of Sephadex G-25 equilibrated with 10 % acetic acid. The conditions were worked out by RUTTENBERG[14] from whose

doctoral dissertation Fig. 2 originates. The diagrams show the elution profiles obtained when intact tyrocidine and tyrocidine hydrolysate are run on the same column in three different concentrations of acetic acid; 1 %, 10 % and 50 %. In the hydrolysate run, in 1 % acetic acid, the aromatic amino acids are well separated from each other and from the neutral amino acids which are eluted together. Intact tyrosidine is strongly retarded. Tyrocidine A lacks tryptophan, tyrocidine B contains one and tyrocidine C contains two tryptophan residues. In 10 % acetic acid they are nicely separated according to their tryptophan content, but in 50 % acetic acid they are all eluted in one peak. In 10 % acetic acid phenylalanine and tyrosine in the tyrocidine hydrolysate are pushed into the neutral group of amino acids and in 50 % they are all eluted together.

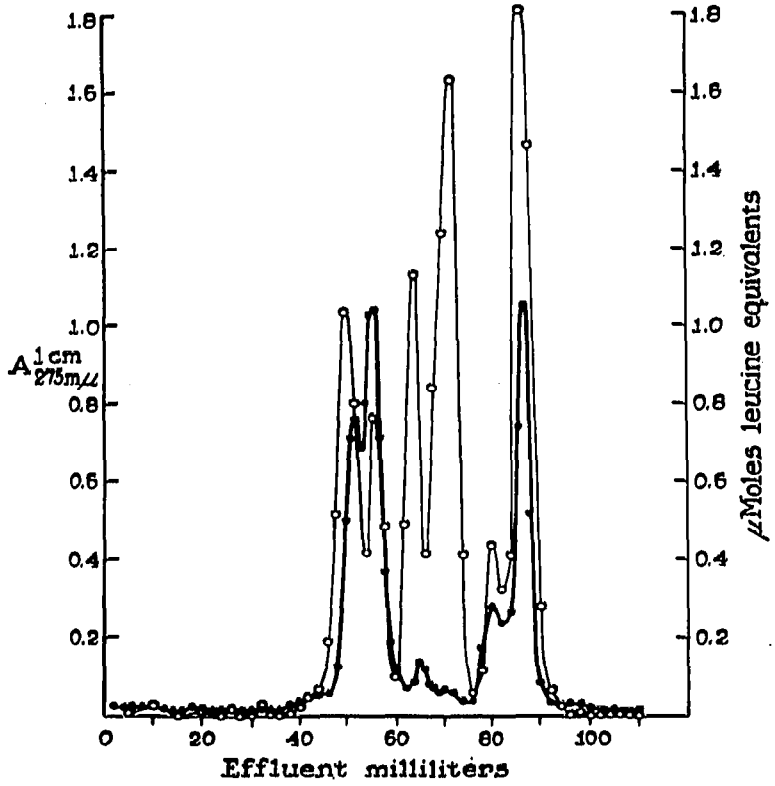

Fig. 1. Fractionation of tryptic digest of 22.6 mg of oxidized ribonuclease A on Sephadex G-25 in 0.2 *M* acetic acid. Column size: 0.9 × 150 cm; flow rate: 6.3 ml/h; fraction size: 1.0 ml. (–•–•–•) Absorption at 275 mμ; (O–O–O–O–O–) ninhydrin analysis. (After EAKER[8]; reproduced by permission of the author and Academic Press Inc., New York).

Many other applications of aromatic adsorption can be found in the literature. It has, for example, been used to examine the polyphenolic content of turf, soil, water, wine and beer, and for separation of the flavouring matter from the colour in coffee extracts.

It is often preferable to carry out the gel filtration in strongly alkaline solution when fractionation of aromatic substances is desired. For example the estrogenic isoflavones in red clover are effectively separated on Sephadex G-25 in 0.1 *M* ammonium hydroxide (Fig. 3)[15].

Very interesting results can be obtained when advantage is taken of the difference in adsorption strength, hence the difference in migration rate of certain compounds

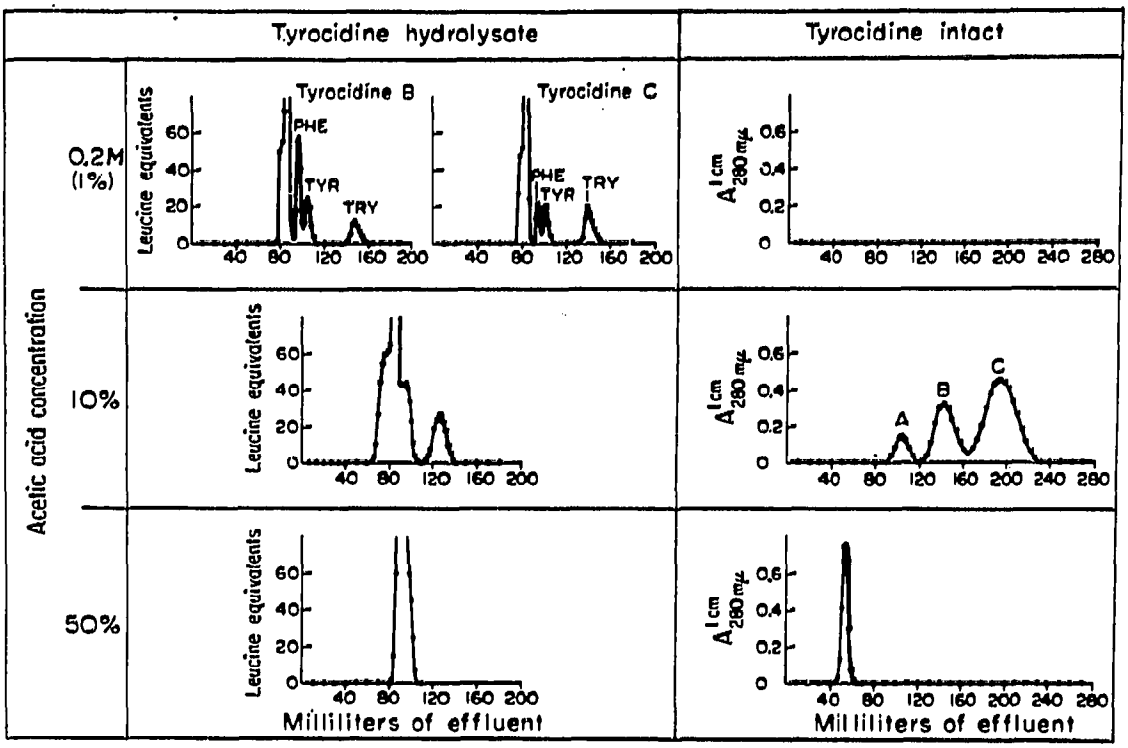

Fig. 2. Summary of results with chromatography on Sephadex G-25 in various concentrations of acetic acid of intact tyrocidine and tyrocidine hydrolysate. All experiments were performed on the same 0.9 × 150 cm column with loads from 1 to 4 μmoles. 2 ml fractions were collected at a flow rate of 8 ml/h. (After RUTTENBERG[14]; reproduced by permission of the author.)

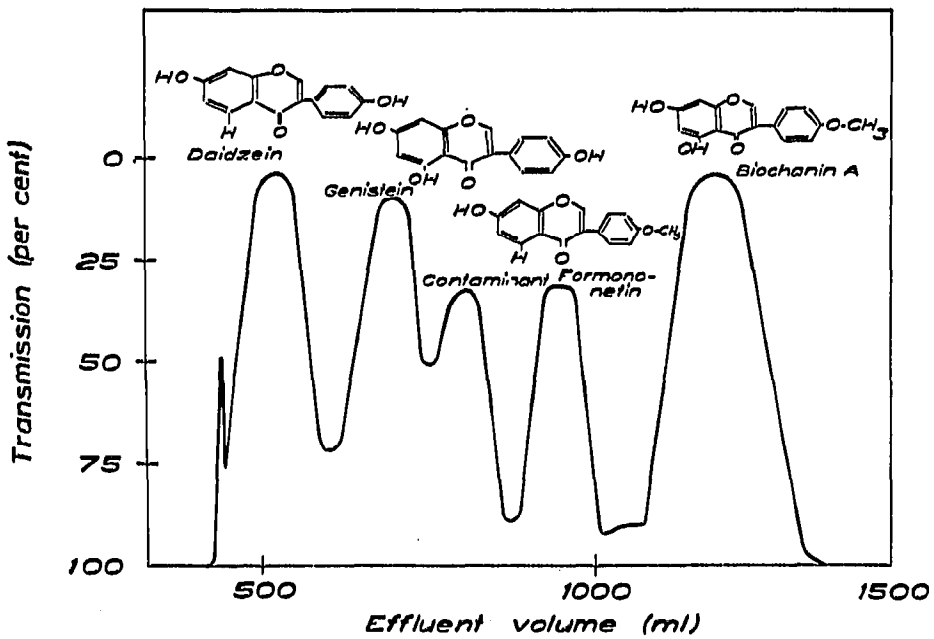

Fig. 3. Fractionation of a mixture of the estrogenic isoflavones genistein, biochanin A, formononetin and daidzein (5 mg of each) on Sephadex G-25 in 0.1 *M* ammonium hydroxide. Column size: 4.5 × 37 cm; flow rate: 1 ml per min. (After NILSSON[15]; reproduced by permission of the author.)

depending on whether they are in salt solution or in water. When a sample containing such a compound and sodium chloride is eluted with distilled water, the compound is concentrated during its passage through the column and eluted in a smaller volume than that of the original sample. This effect is only obtained if the actual compound is eluted faster than the salt in water, and is explained thus: When the compound is in the salt zone it is adsorbed and thus retarded. The salt zone moves downwards and is followed by water which accelerates the migration of the compound owing to the conditions mentioned. The final result is a concentration of the solute in the boundary between the salt and the water.

The mechanism described is probably responsible for the beautiful method for isolation of conjugated estrogens from urine described by BELING[16]. He used a column 1 cm by 50 cm with Sephadex G-25. Up to 20 ml of urine were added to the column and eluted with distilled water. The estrogens appeared in the effluent in only 3 ml and were recovered quantitatively. All the conjugated estrogens assayed for were present and most of the other materials in urine removed.

In a recent investigation, EAKER AND PORATH[17] analyzed very precisely and critically the behaviour of some low molecular weight solutes, mainly amino acids, on the very tightly cross-linked gel Sephadex G-10, under the influence of different solvent media. Their results have been presented in a large table containing more than 120 elution data tabulated in the form of K_d values.

With a higher matrix density, the low molecular weight solutes are influenced more by the gel chains both as far as steric hindrance and adsorption are concerned. Thus for charged solutes the ionic double layer, and for aromatic substances a stronger adsorption, plays a greater role in G-10 than in G-25.

Some of these effects, and some of those mentioned in the introduction, are illustrated by Fig. 4 (from EAKER AND PORATH), which shows the behaviour of some amino acids on a 1 cm × 142 cm column of Sephadex G-10 equilibrated with 1 % acetic acid, with 1 % acetic acid plus 0.5 *M* sodium chloride and with 1 % acetic acid plus 2 *M* sodium chloride.

Attention is drawn to three different types of compounds, *viz.* the basic amino acids, the aromatic amino acids and urea. The basic amino acids are highly charged in the acetic acid, their ionic double layer will be very large and they are therefore almost excluded. On the addition of salt, the hydration layer will decrease and the amino acids are eluted later. Arginine is most affected by the increase in salt concentration, which may be explained by the removal of part of the water of hydration which makes it possible for the π-electrons in the guanidino group to interact with the gel matrix. This last effect may also account for the stronger retardation of the aromatic amino acids at higher ionic strengths. Urea, whose π-electrons cover its planar structure and cause it to be retarded on almost everything, is neutral and thus not very sensitive to sodium chloride.

Fig. 5 shows the behaviour of some amino acids on the same column as in the preceding series of experiments, but now the effect of pH and the addition of pyridine will be discussed. In pattern A the eluent is 1 % acetic acid, pH 2.7, in B the eluent is 1 *M* pyridine–0.03 *M* acetic acid, pH 6.7 and in C it is 0.01 *M* NaOH, pH *ca.* 12. The ordinate (as in the preceding figure) is ninhydrin colour as recorded continuously with a standard Spinco Model 120 amino acid analyzer. The flow rate through the column was 10.0 ml/h in all cases.

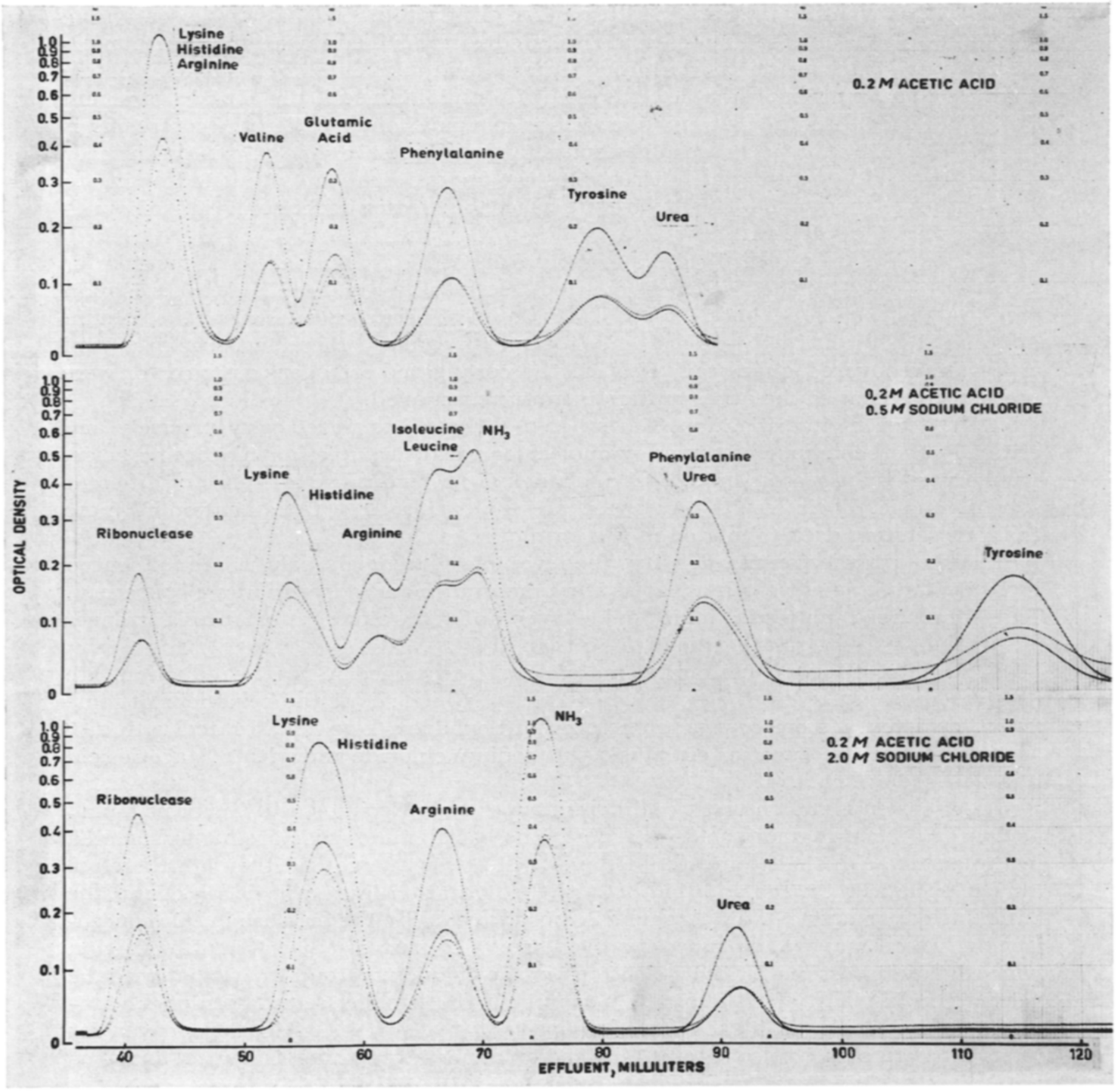

Fig. 4. Patterns showing the effect of salt on the elution behaviour of amino acids and other ninhydrin positive substances on a 1 × 142 cm column of Sephadex G-10. 0.5–2 μmoles of each solute (urea, 4–8 μmoles) in 1.0 ml. Tryptophan is out of the diagram in all cases. Tyrosine and phenylalanine are out of the diagram in the bottom chromatogram. The ordinate is ninhydrin colour as recorded continuously with a standard Spinco Model 120 amino acid analyzer. The flow rate through the column was 10.0 ml/h in all cases. (After EAKER AND PORATH[17]; reproduced by permisssion of the authors.)

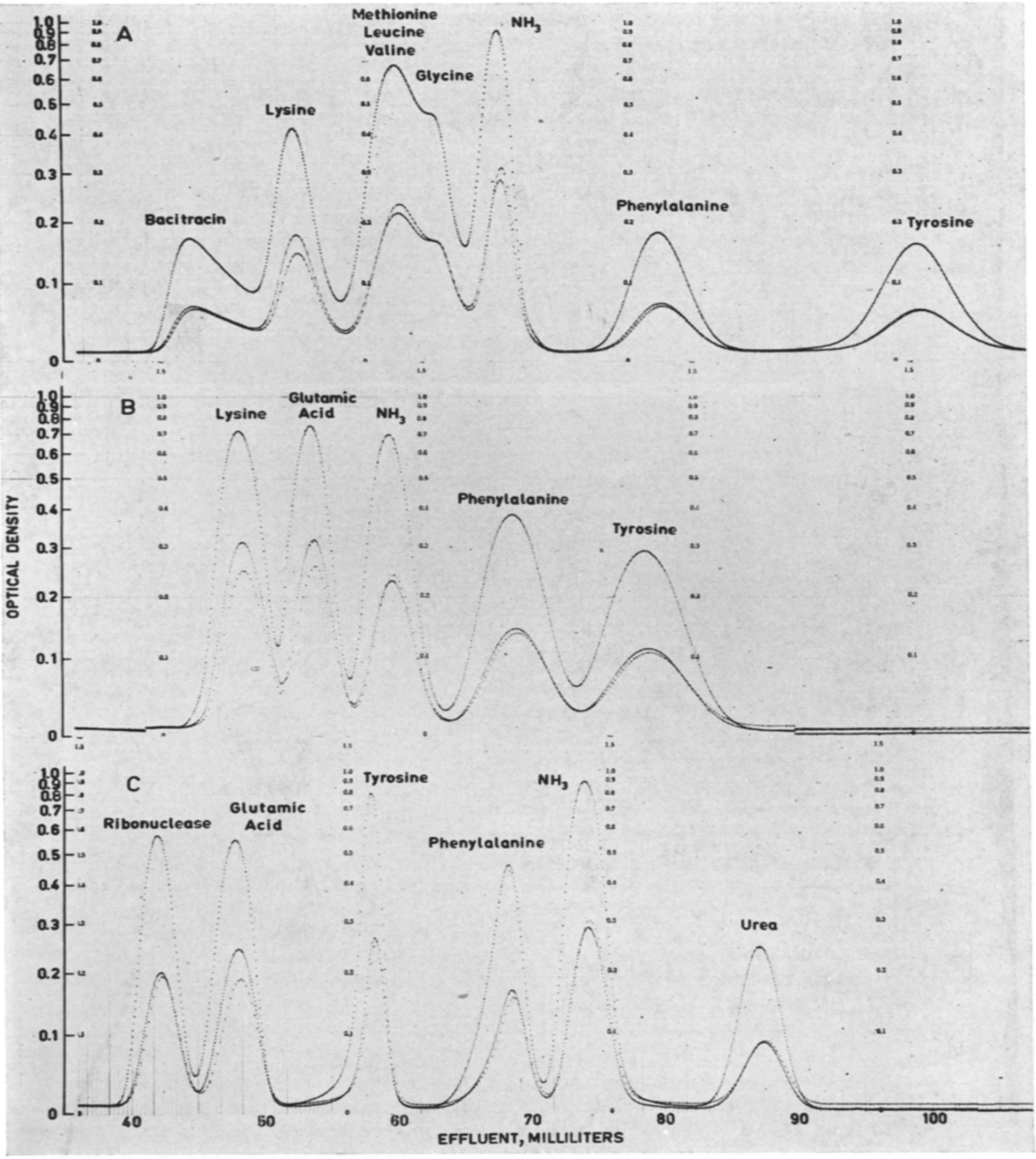

Fig. 5. Patterns obtained with amino acids and other ninhydrin positive substances on a 1 × 142 cm column of Sephadex G-10 in three different eluants. 0.5–2.0 μmoles of each solute (urea, 4–8 μmoles) in 1 ml. In pattern A the eluent is 0.2 *M* acetic acid, pH 2.7; in B the eluent is 1 *M* pyridine–0.03 *M* acetic acid, pH 6.7; and in C it is 0.01 *M* NaOH, pH *ca.* 12. Flow rate: 10.0 ml/h.

The effects one can observe can be summarized as follows: Highly charged substances tend to be excluded (for the reason just mentioned). Examples: The basic amino acids are excluded at acid pH (in the absence of salt) and the acidic amino acids are excluded at alkaline pH. Apparently this is quite a powerful effect, since tyrosine is eluted well in advance of phenylalanine at alkaline pH where the phenolic group is ionized.

On addition of 1 *M* pyridine the retardation of the aromatic amino acids (which here at pH 6.7 are neutral) is markedly decreased. The explanation may be that the pyridine competes for the interaction sites in the gel.

These experiments show very well the potentialities of the new, very tightly cross-linked gel Sephadex G-10 in the low molecular weight fractionation field. EAKER[18] stresses that it is possible to separate any pair of amino acids (excepting leucine–isoleucine) and almost any pair of smaller peptides on G-10, just by manipulating with the acetic acid concentration and the salt concentration, which are still rather mild conditions.

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SUMMARY

It is a well known fact that the more tightly cross-linked dextran gels of the Sephadex series interact with certain solutes, causing anomalously high elution volumes. In this paper the molecular basis for some of these interactions under the influence of different solvent media is discussed. A few examples from the literature are given, where advantage has been taken of these effects in certain separation problems.

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