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CHARGE-TRANSFER ADSORPTION IN AQUEOUS MEDIA

JERKER PORATH

Institute of Biochemistry, University of Uppsala, Box 576, S-751 23 Uppsala, Sweden

ABSTRACT

A brief review of charge-transfer (CT-) chromatography is presented. Hydrophilic gels such as Sephadex exhibit weak electron acceptor and donor properties due to the presence of matrix hydroxyls or matrix-bound water. Stronger CT-adsorbents for chromatography in aqueous solutions are obtained by introduction of electron acceptor or donor substituents. The adsorption properties of such gels are discussed with reference to results obtained with simple model substances such as amino acids and peptides for acceptor adsorbents and nucleotides for donor adsorbents. Metal chelate adsorption (ligand exchange), is considered as a special kind of CT-adsorption with great potential for group separation according to differential metal ion affinity of the solutes.

INTRODUCTION

I think it is appropriate to present a brief historical account on the uses of active charcoal, since this common material is in fact a special kind of 'impure' charge-transfer (CT-) adsorbent. Active charcoal was used independently by C.W. Scheele (1) and F. Fontana (2) to adsorb certain gases (in 1773 and 1777, respectively). A few years later J.T. Lowitz (3) used wood charcoal to remove colored contaminants from solutions, and in 1812 Derosne (4) used animal charcoal for decolorizing sugar beet extracts. It is therefore not surprising that charcoal was used as a general adsorbent during the early phases of the development of chromatographic methods. In the 1940ies Tiselius and his students used this adsorbent very often in their attempts to fractionate amino acids, peptides, carbohydrates and other substances. These studies were the foundation for the definition of displacement and frontal chromatography as well as gradient elution techniques (5).

It gradually became evident, however, that adsorption phenomena on active charcoal were too complicated to permit its successful use as a chromatographic medium for the fractionation of peptides or proteins. This is one of the reasons for the introduction and wide acceptance of simpler adsorbents, such as polymeric ion exchangers, molecular sieves and, more recently, even simple gel polymers with mixed hydrophobic-hydrophilic properties for the chromatographic fractionation of a wide variety of biological substances. All such adsorbents exhibit one or more of the adsorption effects seen with charcoal, but with the significant difference that their sorption behaviour is to some extent predictable and comparatively easier to control.

The fact that active charcoal shows a preference for aromatic substances is the main reason for its capacity to clarify colored extracts. This "aromatic adsorption" is determined most probably by a combination of hydrophobic interaction and electron-transfer leading to the formation of π,π-types of charge-transfer adsorbate complexes. The adsorption sites in charcoal may thus serve as electron donors and/or acceptors. But it has so far been difficult to *selectively* use only one or the other of the adsorption sites present on charcoal. It is both desirable and essential that such adsorption centers should be well-defined in order to permit study of the adsorption phenomena *in extenso* and thus to predict their effect in chromatographic experiments. This should be relatively easy to achieve if one could introduce well-defined groups into an inert polymer matrix to serve as the adsorption centers. The question is: can we prepare effective, non-ionic hydrophilic adsorbents that operate primarily by $\pi\pi$, $n\pi$ or $\sigma\pi$ interactions? But, before going deeper into this problem I would like to facilitate forthcoming discussions by classifying the various sorbents into the following main groups:

Class A: Sorbents without adsorption centers. Ex. Ideal molecular sieves.
Class B: Sorbents with unsupported adsorption centers. Ex. Inorganic salts, insoluble organic substances.
Class C: Sorbents of class B mixed with or coated on class A sorbents or inert solid supports.
Class D: Sorbents of class A with covalently attached substituents functioning as adsorption centers. Ex. Cross-linked polymer ion exchangers, bioaffinity adsorbents.
Class E: Sorbents of class D charged with bi- or multifunctional adsorbate molecules

or ions that in turn serve as specific adsorption centers. Ex. "Sandwich-type" immunosorbents.

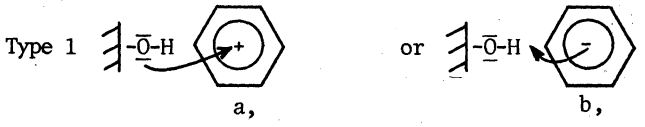
This classification is not exhaustive and there might occur overlapping so that extension might be necessary for other purposes.

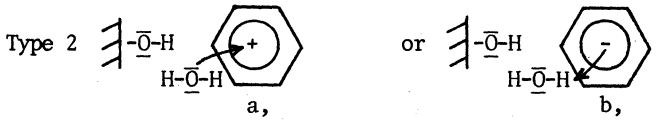
In order to synthesize a charge-transfer adsorbent we may select a sorbent which from practic al point of view belongs to class A, for example a hydrophilic gel substance such as Sephadex agarose, cross-linked polyacrylamide or hydroxyethyl polyacrylate. Adsorption sites may then be introduced in several ways. For example, a particulate adsorbent of class B can be enclose in a gel matrix such as agarose or polyacrylamide or it can be coated onto or precipitated on or within the support. Adsorbents may also be obtained by polymerisation or condensation of a suitable electron donor or acceptor monomer or by chemical coupling of the proper ligand substance to a preformed gel matrix.

Most charge-transfer adsorbents reported in the literature belong to class B or C. A good electron donor or acceptor has often been precipitated on silica gel. In what appears to be the first attempt to use charge-transfer complex formation for chromatographic separations Goldewicz in 1949 (6) impregnated silica gel with trinitrobenzene. Another example: silver ions immobilized in silica gel will form π-complexes with cis- and trans isomers of olefins (7,8). These pioneering studies were followed by others using also glass beads and Al_2O_3 and active charcoal as supports (9-13). Silica gel impregnated with benzoquinones (14) has been used for thin layer chromatography of aromatic hydrocarbons. Reviews have been published by Harvey and Halonen (15), Guha and Janak (16), Laub and Pecsok (17) and Schenk, Sullivan and Freyer (14). These studies, which chiefly deal with gas-liquid and thin layer chromatography in organic solvents, may be considered exploratory and have not as yet resulted in a breakthrough for CT-chromatography.

CT-CHROMATOGRAPHY IN AQUEOUS SYSTEMS

It is interesting to note that nobody before us seems to have tried to develop CT-chromatography in aqueous systems. This is certainly not due to lack of published examples in which the observed phenomena could be ascribed to electron transfer adsorption. The so-called "aromatic adsorption" on hydrophilic gels (18-21) may in part be due to some kind of hydrophobic interaction and also in part to charge-transfer, presumably of type 1 or/and 2:





Type 2 signifies water-mediated charge-transfer. Types 1b and 2b represent a particular type of hydrogen bonding.

Analogous charge-transfer interactions involving lone electron pairs explain the adsorption of amines, urea, thiourea, guanidine derivatives, etc., where also proton-electron bridges are involved to give a specialized form of CT-adsorption. I would here like to call attention to an example given about 10 years ago of n,π-interaction in a mixed solvent (22) of thiophen- and hydrated thiophen derivatives as revealed by chromtography on LH-Sephadex (Fig. 1).

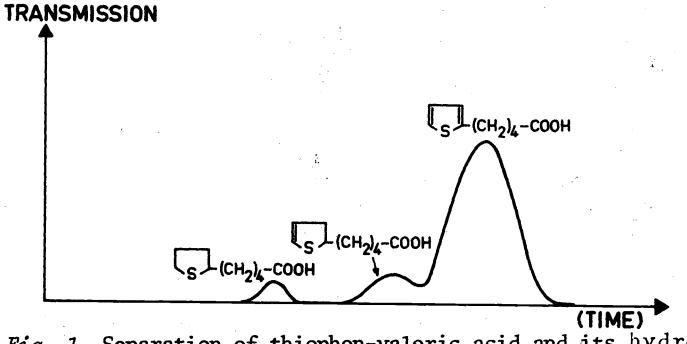


Fig. 1. Separation of thiophen-valeric acid and its hydrogenated derivatives on Sephadex LH-20 (4.5x51 cm columns) in 96 % ethanol (22).

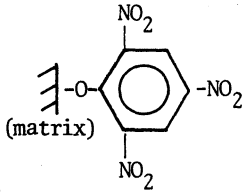
More then 10 years ago we also introduced aromatic substituents into Sephadex with the hope of increasing the affinity for aromatic solutes (23). The results were far from impressive, due to the apparently low degree of substitution and the limited electron affinity of the ligands. However, all hydrophilic gels containing aromatic ligands are likely to have π-acceptor or donor properties and are therefore potential CT-adsorbents - weak or strong. Likewise, gels with substituents containing free electron pairs, guanidine or imidazole groups for example, can serve as n-electron donor adsorbents, although ionic and other kind of interactions often prevail. Additional examples are Sephadex or Sepharose gels to which dyes have been coupled. Such colored gels usually contain sulfonic groups or other ionizable substituents, the adsorption effect of which can be suppressed by introducing high concentration of salt in the eluent often at the risk of increasing hydrophobic interaction.

An ideal acceptor or donor substituent should not be ionizable in the elution medium. Furthermore, it should not be hydrophobic. The latter condition can never be entirely fulfilled and it is therefore possible in practice to prepare CT-adsorbents that only approach the ideal.

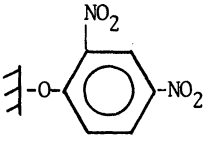
Electron-Acceptor Adsorbents

The choice of non-ionogenic acceptor ligand substances is easy, but their number is severely limited due to the further requirement that they should be soluble in the reaction medium and also yield gel products which are negligably hydrophobic. So far we have experience only with class D and E, hydrophilic CT-adsorbents (24-25).

The following nitroaromatic gels have been synthesized:



I



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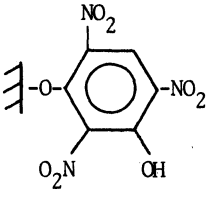

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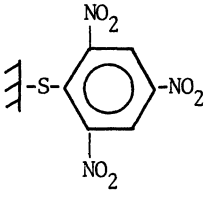

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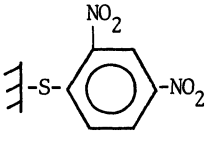

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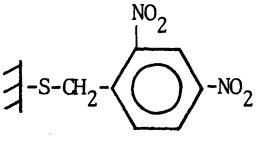
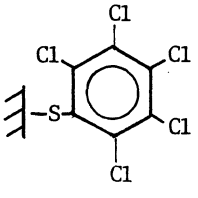


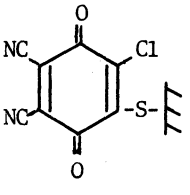
VI

Adsorbents of type I, III and IV are too lable to be useful and give considerable leakage already at pH 7. Type VI should be most stable, but in our hands type V adsorbents are by far the best.

We have also synthesized acceptor gels with pentachlorophenylsulphide (VII) and chlorodicyanobenzoquinone-sulphide (VIII) substituents:



VII



VIII

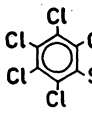
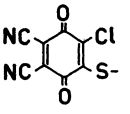
Some orienting experiments have also been made with riboflavin , luminol, and aminonaphtalamide substituted Sephadex.

For most of the exploratory adsorption studies simple model substances were used. Phenylalanine, tyrosine and tryptophan afford a convenient triplet of substances of increasing electron donor strength (24,25). Serotonin forms even stronger complex with riboflavin (and presumably also with other electron acceptors) than does tryptophan.

As seen in Table 1, the model substances are adsorbed more strongly from a salt solution of high molarity than from a buffer of low ionic strength. The adsorption is expressed in relative elution volumes, defined as V_E/V_T - a crude but convenient and sufficiently accurate measure that allows meaningful comparisons of chromatographic behaviour at the present stage of the development of CT-gels (V_E and V_T are elution volume and bed volume, respectively).

TABLE 1
SALT DEPENDENCE

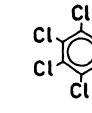
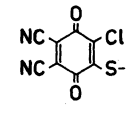
ADSORPTION (V_E/V_T-values) AT 20° IN Na-FORMATE, pH 3.2 WITH AND WITHOUT NaCl

LIGAND	PCP-S- 		DCQ-S- 	
LIGAND CONCENTRATION, µmol/g	~400		~70	
NaCl	0	4M	0	4M
TRP	2.8	7.9	3.3	6.4
TYR	1.1	2.3	1.4	2.0
PHE	1.0	1.7	1.3	1.3
SEROTONIN	3.7	14.0	9.8	12.0

The rather complicated picture which emerges from the table cannot be interpreted easily, but a stronger influence of hydrophobic interaction is indicated for the pentachlorophenyl-S-gel than for the quinone adsorbent. We might expect hydrophobic interaction to be stronger at pH 3.2 than in less acidic or alkaline solutions. However, the salt dependence is not a true measure of hydrophobic interaction: in fact a similar dependence may very well be consistent with the concept of CT-adsorption involving additional enthalpic gain of solvation and formation of ion pair adsorbates (25).

TABLE 2
TEMPERATURE DEPENDENCE

ADSORPTION (V_E/V_T) IN 0.1 M Na-FORMATE, pH 3.2 AND 0.1 M $(NH_4)_2CO_3$, pH 8.8

LIGAND	PCP-S- 				DCQ-S- 			
LIGAND CONCENTRATION, µmol/g	~400				~70			
pH	3.2		8.8		3.2		8.8	
TEMPERATURE	4°	20°	4°	20°	4°	20°	4°	20°
TRP	3.7	2.8	2.6	2.6	3.4	3.3	3.0	1.9
TYR	1.2	1.1	0.9	1.0	1.5	1.4	1.0	1.1
PHE	1.1	1.0	0.8	1.2	1.3	1.3	1.0	0.9
SEROTONIN	4.9	3.7	4.4	3.6	14.0	9.8	8.7	6.7

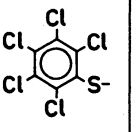
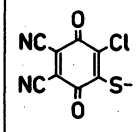
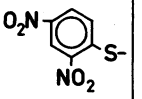
Adsorption as a function of temperature can give some insight into the nature of solute-sorbent interactions. Table 2 shows that adsorption is stronger in acid solution at 4° than at room temperature for PCP-Sephadex as well as for the quinone Sephadex. There are a few exceptions at pH 8.8, which in the case of phenylalanine is somewhat surprising and indicates that charge-transfer interaction is comparatively stronger in acidic milieu.

The adsorption power increases rapidly with the degree of substitution. The indoles are adsorbed very strongly on DCQ-Sephadex where the degree of substitution is about 200 µmol/g dry adsorbent.

We have so far discussed only the behaviour of simple model substances containing a single π electron-rich nucleus. The next step is to consider the adsorption of solutes having several donor centers. Table 3 shows that the cooperative effect results in very strong adsorption. The large increase in adsorption power observed even with a solute containing two indole residues might indicate a risk for irreversible adsorption of proteins containing many surface located donor substituents.

TABLE 3

COOPERATIVE ADSORPTION EFFECT IN 0.1 M FORMATE

LIGAND	PCP-S- 	DCQ-S- 	DNP-S- 
LIGAND CONCENTRATION, μmol/g	~400	~70	~200
TYR	1.1	1.4	1.1
TYR TYR	2.0	4.6	3.2
TYR TYR TYR	4.6	9.1	7.4
TRP	2.8	3.3	2.7
TRP TRP	19	>30	>30

We have already made some encouraging exploratory experiments with protein mixtures. In fact, in the literature I have found at least one article which has an optimistic view on the subject. Stepanov *et al.* (27) coupled mono DNP-hexamethylene diamine to Sepharose 4B with CNBr and obtained a useful adsorbent for many proteases, including pepsin, trypsin and chymotrypsin. They suggested that the adsorption was caused by hydrophobic interaction but made a careful interpretation. A Sepharose derivative of ε-aminocaproyl-D-alanine was found to be ineffective as an adsorbent for chymotrypsin which speaks in favour of another kind of mechanism.

An artificial mixture of all amino acids was chromatographed on DCQ-S-Sephadex. The basic amino acids migrated at different speeds (Fig. 2). Lysine is presumably adsorbed by n,π-electron interaction, while histidine and arginine are retarded as a result of the combined action of n,π and π,π-electron transfer and possibly also ion-dipole attraction. Aspartic and glutamic acids move ahead of the neutral non-aromatic amino acids, which indicates ion exclusion. If that is the case, it is rather surprising since there are no fixed ionic groups in the gel. Maybe the matrix-bound water is oriented such that the negative oxygen points toward the bulk water, thus forming a polarized layer that repels the negatively charged acidic ions. Admittedly, presence of such partially immobilized and oriented water layer is speculative but could explain some findings of linear adsorption with V_E/V_T >1 for basic peptides and small molecular size proteins on the unsubstituted matrix gel.

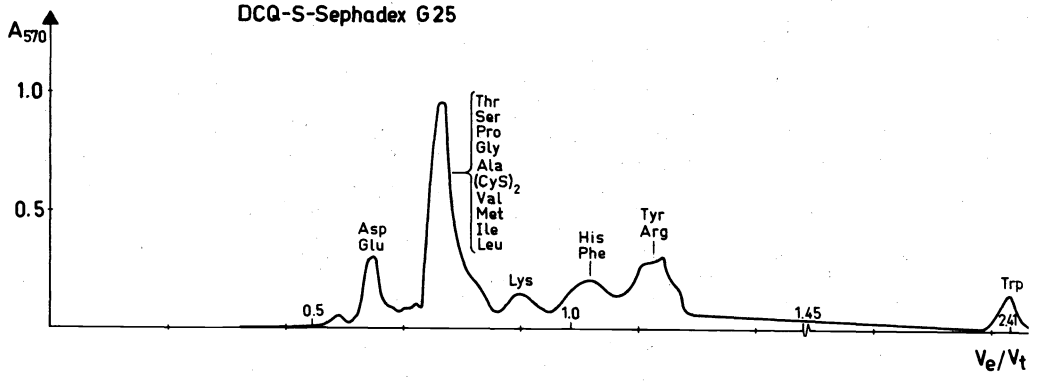


Fig. 2. Separation of an artificial amino acid mixture on the strong acceptor gel, DCQ-Sephadex G-25.

Water soluble vitamins comprise an other interesting group that can possibly be analyzed by CT-chromatography. Fig. 3 shows a chromatogram of an artificial mixture of some B-vitamins on DCQ-S-Sephadex. Riboflavin is a fairly strong electron acceptor, but it functions as a strong-

er donor than the other vitamins on the quinone ligand. A π-electron-rich substance may thus act as a donor as well as an acceptor. This accounts for self-complex formation with the consequence that un-reacted ligand substances are occasionally troublesome to remove from the CT-adsorbents.

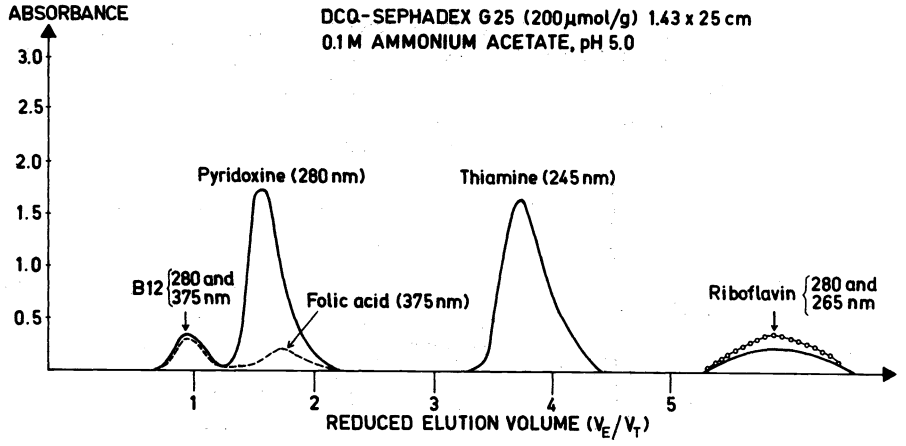
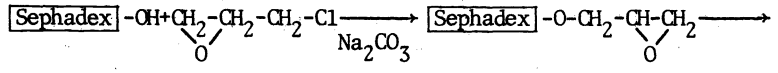
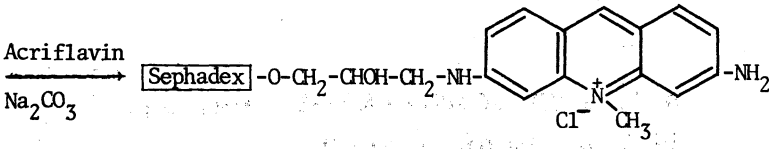


Fig. 3. Chromatogram of an artificial mixture of B-vitamins.

Electron-Donor Adsorbents

Riboflavin-Sephadex may thus serve as an ambivalent electron transfer gel. On the other hand acriflavin-gel is primarily an electron donor adsorbent in the presence of sufficient salt to suppress ionic interactions. An exploratory study of the adsorption properties of acriflavin-Sephadex has recently been made together with Jean-Marc Egly (26). The gel is preferably synthesized *via* oxirane-activated gel:





The aim was to develop suitable adsorbents and to find optimal conditions for charge-transfer adsorption chromatography of nucleotides. Some preliminary results are shown in Figs. 4-6. Purines have higher affinities toward the acriflavine ligand than do the pyrimidines. Phosphorylation of the nucleosides appreciably affects the adsorption by altering the electron distribution over the ring system and by lowering the position of the highest occupied molecular orbital to make the electron transfer less effective. It is interesting to note that the adsorption, as revealed by the V_E/V_T values, approaches a limiting value characteristic for the solute as the ionic strength or amine concentration increases. The adsorption increases as the temperature is lowered, indicating that hydrophobic interaction does not play a major role. Nucleoside mono-, di- and triphosphates can easily be separated (Fig. 7) without gradient elution as can also simple oligonucleotides (Fig. 8). It should be emphasized that the relative migration speed is not very sensitive to moderate alterations in the eluent buffer with respect to its pH, ionic strength, or the nature of the buffering ions.

It is interesting to note that whereas adsorption of aromatics on π-acceptor gels increases with salt concentration the adsorption of nucleotides to acriflavine gel decreases upon addition of salt to the buffer: presumably this reflects the suppression of intramolecular ionic interaction. Table 4 gives some further information on the behaviour of a model nucleoside and a nucleotide under different conditions. In no case is adsorption abolished, and the figures indicate the interplay of several factors the nature of which are difficult to explain. However, it seems likely that N-ethylmorpholine and urea might compete with the acriflavine by charge-transfer involving the lone pair in the nitrogen. The differential influence of amines has often been noticed, and at high concentration they seem to be efficient suppressors of n,π-interactions.

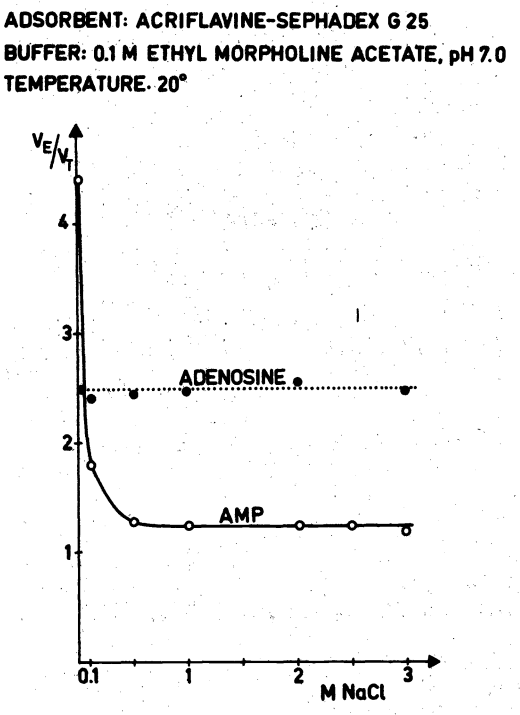


Fig. 4. Adsorption of adenosine and AMP as a function of salt concentration.

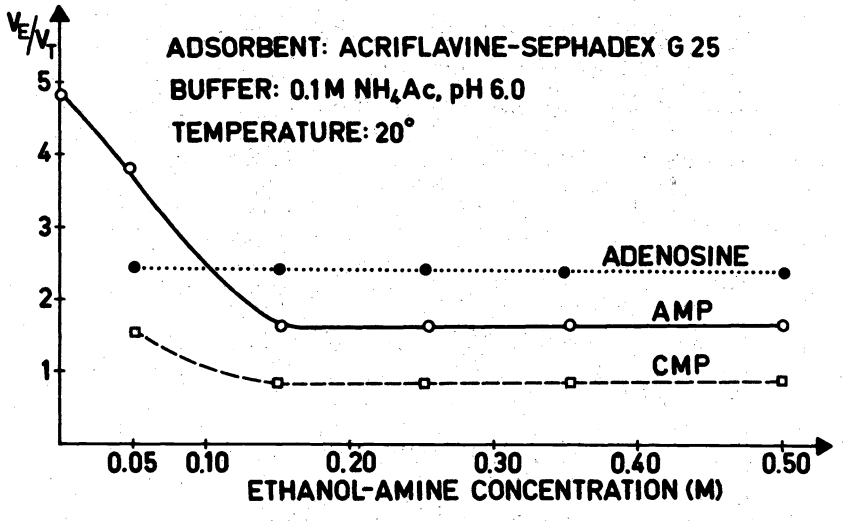


Fig. 5. Adsorption of adenosine, AMP and CMP as a function of ethanolamine concentration.

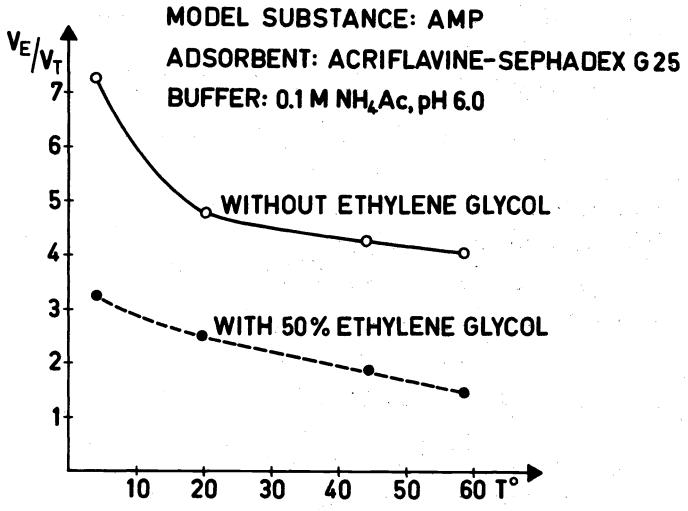


Fig. 6. Adsorption of AMP as a function of temperature.

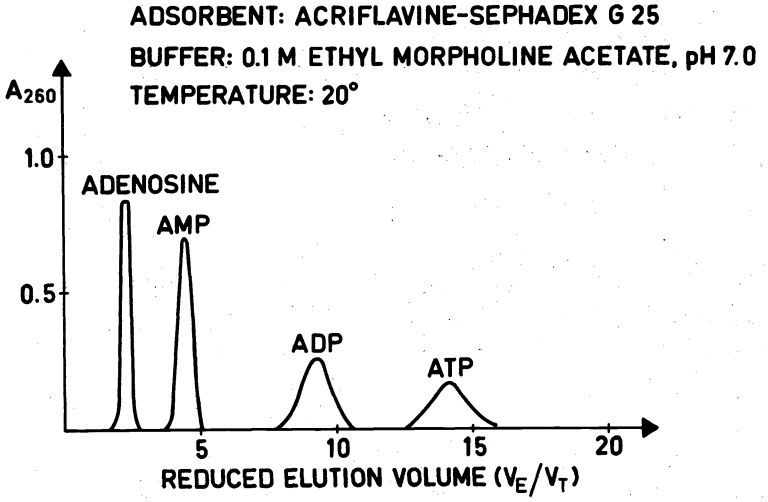


Fig. 7. Chromatogram of adenosine and adenosine phosphates on acriflavine-Sephadex in 0.1 M ethyl-morpholine acetate, pH 7.

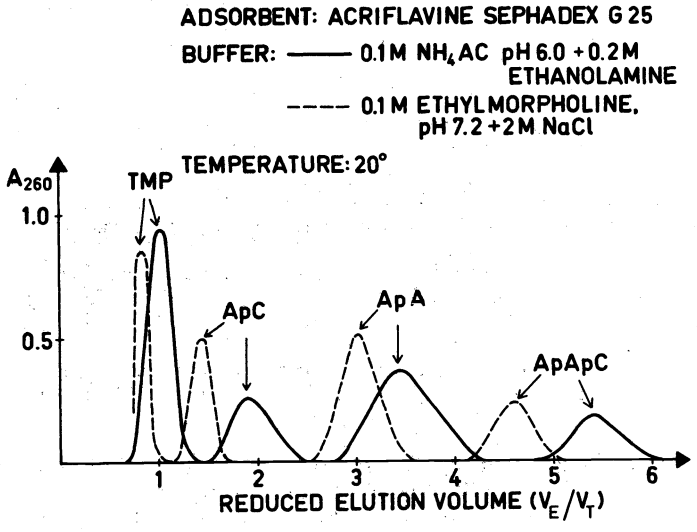


Fig. 8. Separation of oligonucleotides on acriflavine-Sephadex in two different buffer systems.

TABLE 4

ADSORPTION OF ADENOSINE AND AMP ON ACRIFLAVINE-SEPHADEX G25 IN SOME SELECTED ELUENTS

BUFFER COMPOSITION	ADENOSINE		AMP	
	4°	20°	4°	20°
0.1 M ETHYL MORPHOLINE-ACETATE, pH 7.0	3.24	2.40	7.22	4.80
-"- + 50 % ETHYLENE GLYCOL	1.56	1.71	4.07	2.78
-"- + 3 M UREA	1.98	1.75	3.47	2.23
-"- + 2 M NaCl	1.83	2.45	1.54	1.25

Metal Chelate Adsorbents

We shall also include coordinative binding between metal ions and molecules or ions with lone electron pairs or π electrons. Some old analytical methods for amino acids and proteins, now abandoned, did in fact depend on charge-transfer interactions. This is at least in part true for precipitation of proteins with heavy metal salts, tannins and many alkaloids. A heavy metal ion, tannic acid or an alkaloid such as morphine which is attached to a solid support should thus act as a charge-transfer adsorbent as well as an ion exchanger. The latter effect can easily be suppressed by high concentrations of salt.

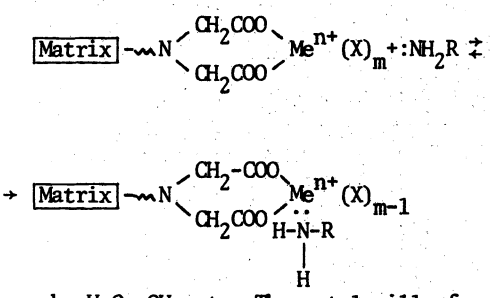
The metal ion may preferably be introduced into the gel matrix *via* a chelate-forming ligand.

$$\boxed{\text{Matrix}}\text{-chelator-Me}^{n+}$$

↑ primary adsorption center

↑ secondary adsorption center

Evidently such an adsorbent belongs to class E. If Me is a transition element it will still have capacity to coordinatively bind solutes with affinity for this metal. For example, if the ligand is the biscarboxymethylamino group a primary amine will be adsorbed by the reaction:



where X may be H_2O, OH, etc. The metal will of course not be so strongly bound as in EDTA solution, but many metal chelates are sufficiently stable adsorbents. The principle was first used under the name of ligand exchange chromatography by Helfferich (28) and later adopted by others. Cross-linked polystyrene is used as matrix for the chelate-forming polymer which is commercially available under the trade name Chelex.

The polystyrene matrix is swellable in organic solvents but not in water. A hydrophilic matrix is better suited for chromatography in aqueous systems and we have therefore chosen Sephadex and Sepharose.

Since such adsorbents have both primary and secondary adsorption centers desorption can be accomplished in two different ways: 1) The coordinate link to the adsorbate can be cleaved leaving the metal attached to the gel or 2) The metal can be displaced from the gel. In the former case the gel is still a metal chelate adsorbent and can be repeatedly used. In the latter case it must be recharged with the metal.

So far we have used Cu^{2+}, Zn^{2+} and Fe^{3+} most frequently (29). Studies of the behaviour of mixtures of all the common amino acids have shown that histidine and cysteine are preferentially adsorbed to zinc chelate gels in neutral solution whereas the Cu-chelate gels have a broader specificity. Fe^{3+}-gels adsorb tyrosine strongly.

Peptides with two tyrosine residues are more strongly retarded than peptides containing only one. This is illustrated in Fig. 9, which shows the separation of some enkephaline analogues on Fe^{3+}-chelate gel at pH 5.0 in ammonium acetate.

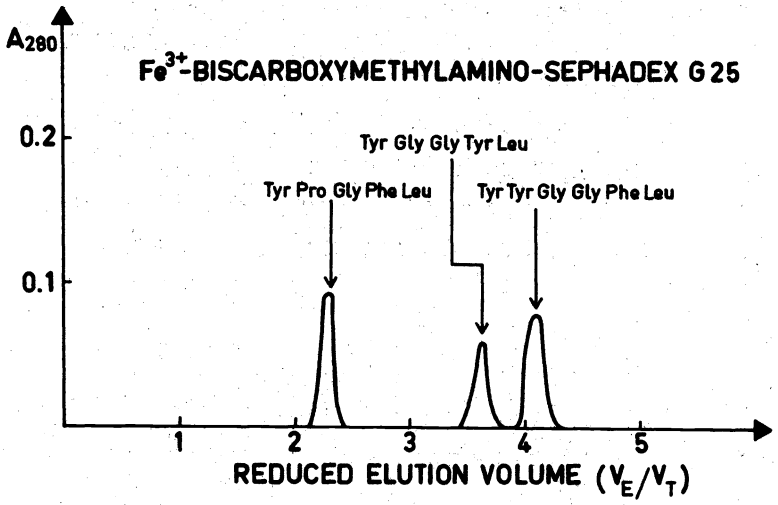


Fig. 9. Separation of some enkephaline analogues by metal-chelate adsorption chromatography. Eluent buffer: 0.1 M ammonium acetate, pH 4.5.

Metal chelate adsorption can be used for effective group fractionation of proteins (29,30), presumably according to their content of surface exposed electron donor groups. More fundamental studies are needed before the potential of this kind of protein chromatography can be fully exploited.

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