Journal of Chromatography, 159 (1978) 13–24
Chromatographic Reviews

CHREV. 110T5

EXPLORATIONS INTO THE FIELD OF CHARGE-TRANSFER ADSORPTION

JERKER PORATH
Institute of Biochemistry, University of Uppsala, Box 576, S-751 23 Uppsala (Sweden)

CONTENTS

1. INTRODUCTION

It has been known for more than a century that certain pairs of organic compounds interact to form more or less stable molecular complexes[1]. Such complexes are often less soluble than either partner alone and can be precipitated from complicated mixtures. The desired complex partner may subsequently be dissociated and recovered. Many organic compounds can also form complexes with inorganic salts, and this property can also be used for isolation and characterization purposes. In this paper are described some chromatographic methods that are under intense development in this laboratory which depend on molecular complex formation. I tentatively propose to call these methods electron donor–acceptor (EDA) or charge-transfer (CT) chromatography. A rational basis for the development of such methods is now available owing to recent advancements in the theoretical treatment of the formation and properties of charge-transfer compounds[2–7]. Equally important are such practical prerequisites as the proper gel materials[8–11] and methods for the synthesis of the appropriate adsorbents[12–15].

2. FREE AND IMMOBILIZED ELECTRON DONOR–ACCEPTOR COMPLEXES

2.1. General considerations

There is no general agreement regarding the definition of charge-transfer complexes. Following Mulliken and others, the dative compounds formed by the donor sharing a non-bonding lone electron pair (in an n-orbital) with an acceptor supplying a vacant (ν) orbital will be included. Such an $n\nu$ complex is rather strong. Halogen–benzene systems have been studied extensively but are of less importance in this con-

nection. However, other kinds of $\pi\sigma$ complexes formed from π-donors and σ-electron acceptors may take part in adsorption phenomena which are worth exploration in biochemistry and analytical or preparative organic chemistry. π,π complexes, which usually are weak, are more suitable as ligand–adsorbate pairs for chromatography.

The electronic structure of a complex AD is defined by the ground-state wave function:

$$\psi_N = a\psi_0\,(D,A) + b\psi_1\,(D^+\text{–}A^-),$$

where ψ_0 refers to the no-bond structure (including Van der Waals forces) and ψ_1 the dative form, and the coefficients a and b define the contributions of the species to the total wave function. The resonance provides the driving force for the formation of the complex.

In the ground state of the complex, A and D are kept together mainly by Van der Waals forces and the charge-transfer state accounts for only a small contribution to minimize the energy. According to Mulliken there is a high probability of a transition to an excited charge-transfer state described by the equation

$$\psi_v = -b^*\psi_0\,(D,A) + a^*\psi_1\,(D^+\text{–}A^-)$$

$a^* \approx a$; $b^* \approx b$ (if the overlap integral $\int\psi_0\psi dv = 0$, *i.e.*, a weak complex).

The charge transfer between the ground state and the excited state is associated with an absorption band approximately determined by the equation

$$h\nu \approx k\left(I_D - E_A - \frac{e^2}{r_{AD}} - \Delta\right)$$

where I_D is the ionization potential of the donor, E_A the electron affinity of the acceptor, r_{AD} the acceptor–donor distance within the complex, Δ is a small term involving other interactions and k is a constant.

A number of methods have been devised for approximate solutions of the Schrödinger equation even for complicated substances. In this context, the results are more interesting than the calculations. The roots of the equation define the energy levels of the molecular orbitals and those of the valence electrons determine the chemical properties.

The energies of the highest filled and the lowest vacant orbitals are of decisive importance for the manifestation of donor or acceptor properties by a substance. The smaller the energy difference between the highest occupied orbital of a presumptive donor and the lowest unfilled orbital of a presumptive acceptor, the greater is the chance for charge transfer to occur with the formation of a molecular complex and the stronger will be the attraction between the interacting partners.

Aromatic or heterocyclic compounds with strongly electron-attracting groups are good π-acceptors, whereas electron-releasing substituents enhance the donor properties. Pullman and Pullman[3] and others have published electron energy diagrams for hundreds of biologically important compounds. Such diagrams are valuable guides for the exploration of charge-transfer adsorption (Fig. 1).

Fig. 2 shows schematic representations of some types of complexes involving π-electrons. Figs. 2b and 2c depict a hypothetical charge transfer between hydroxyl

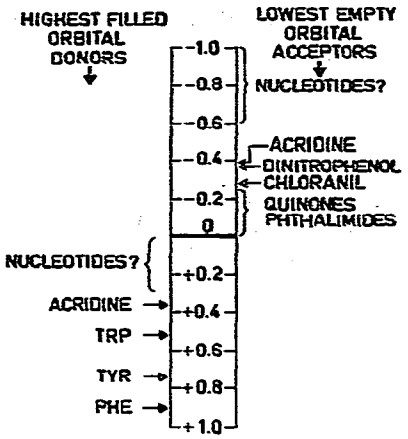


Fig. 1. Scale of energy eigen values (k_j values) obtained from approximate solution of the Schrödinger equation: $E_j = \alpha + k_j\beta$ where E_j is the electron energy in the j-orbital. α the coulombic integral and β the exchange integral. The scale has been drawn from data in ref. 3. A small k_j of the highest filled orbital indicates a good donor (high I_D) while a small k_j (low E_A) of the lowest vacant orbital characterizes a good acceptor.

groups in the matrix and aromatic adsorbates which may account for the well known adsorption of aromatic amino acids, aromatic nitro compounds and other π-electron-rich compounds on carbohydrate gels[16–18].

Charge-transfer complex formation has occasionally been used to accomplish chromatographic separations. Especially in gas chromatography donor or acceptor substances have been included in or adsorbed to a solid support such as silica gel[19–27]. There are also examples in the literature where donor or acceptor ligands have been introduced purposely into a polymer matrix such as polystyrene in order to obtain a charge-transfer adsorbent. However, we appear to have been the first to explore in aqueous systems the use of charge-transfer between solutes and hydrophilic adsor-

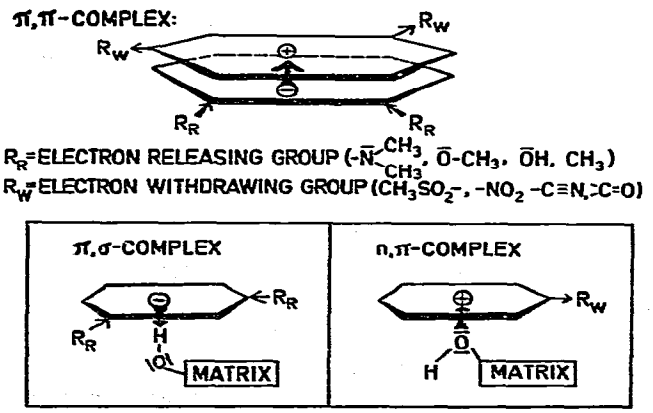


Fig. 2. Schematic representation of some types of complex that are involved in charge-transfer adsorption on polyhydroxylic gels.

bents[28]. The reason why this field has been neglected is probably that the results of extensive studies in organic systems do not appear very promising.

Charge-transfer adsorption can be described as follows:

$$\boxed{\text{Polymer}}\sim X_{(H_2O)_n} + Y_{(H_2O)_m} \rightleftharpoons \boxed{\text{Polymer}}\sim (XY)_{(H_2O)_v} + (n+m-v)\,H_2O$$

where X is acceptor and Y the donor or *vice versa*, and *n*, *m*, *v* signify the number of water molecules bound to X, Y and the adsorbed complex, respectively. The release and ordering of the bound water molecules can significally stabilize the complex, as indicated by the Born formula[29] (Fig. 3).

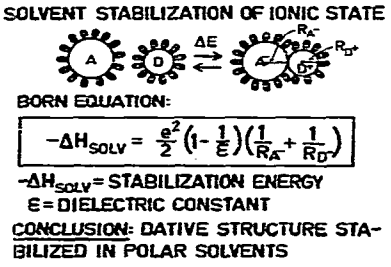


Fig. 3. Illustration of solvent interaction.

It is evident that there may be a larger energy and entropy gain as a consequence of the solvent effect in more polar media such as water compared with common organic solvents.

2.2. Support and mode of attachment

Any kind of sufficiently permeable, uncharged and rigid hydrophilic support can be used, but we have selected crosslinked dextran (Sephadex) and agarose (Sepharose) for reasons discussed elsewhere.

It is desirable to choose the method of ligand attachment such that the interfering effect of the connecting spacer bridge ("connector") will be negligible or as small as possible. We have found oxirane (epoxide) coupling to be especially useful[14]. This method can be used alone or together with some other reaction to effect stable attachment of the ligand.

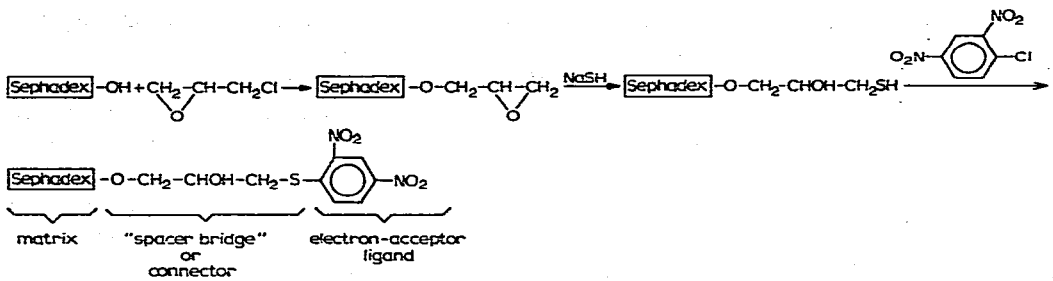
2.3. π,π complex-based adsorption

2.3.1. Acceptor gels

The solubility requirement limits the selection of ligand substances to comparatively simple aromatic compounds. After some exploratory experiments with riboflavin, we concentrated our work on some classes of substances that are known to be particularly strong acceptors, namely, nitrobenzenes, quinones and phthalazines. The last mentioned were soon abandoned because of their instability.

Aromatic nitro compounds have been extensively studied. Attempts over many years to use picric and styphnic acids and *p*-nitrophenyl ethers of Sephadex and Sepharose gave results of dubious value. Not until we coupled dinitrochloro(bromo-

or fluoro-)benzene with thiolated Sephadex did we obtain an adsorbent with satisfactory properties[28]. The preparation can be effected according to the following scheme:



Gels containing oxygen instead of sulphur seem to be much inferior as adsorbents. The non-bonding orbitals of the sulphur atom are higher in energy and are therefore less stable than those of the more electronegative oxygen atom in a corresponding ether derivative. One might therefore expect that the lone pair of electrons on the sulphur atom should decrease the electron density of the aromatic ring, thereby rendering the gel less effective as an acceptor adsorbent. The surprising acceptor efficiency might be due to a solvation effect (Fig. 4). The C–S link is less stable than the corresponding C–O bond, but the dinitrophenyl-S adsorbents can be used safely below pH 8 and can even withstand brief exposure to considerably more alkaline media. Trinitrophenyl thioether ligands are too unstable and mononitro derivatives are too weak acceptors to be useful for most purposes.

π-ELECTRON ACCEPTOR GEL

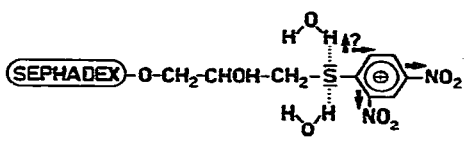
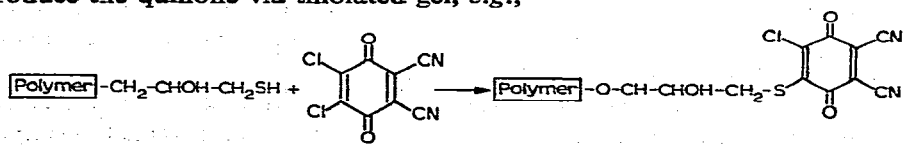


Fig. 4. Structure of DNP-S-Sephadex. Solvation is thought to explain the direction of electron withdrawal from the sulphur atom.

The quinones constitute a potentially very useful class of ligand molecules. They can be readily introduced into the Sephadex or Sepharose gel matrix either directly[30] or by the use of some suitable intermediate gel derivative[15]. The tendency of quinone ligands to couple covalently to solutes can be overcome by the use of suitably substituted derivatives.

Chloro- and cyano-group substituents further delocalize the π-electron cloud, and 2,3-dicyano-5,6-dichloroquinone is consequently one of the strongest acceptors known. To achieve a satisfactory degree of substitution we have found it necessary to introduce the quinone via thiolated gel, *e.g.*,



The dicyanoquinone gel does not seem to show any tendency to undergo covalent coupling reactions. Extensive treatment of the gel with glycine did not change the adsorption properties.

The potentialities of the acceptor gels can be illustrated by considering the results of some exploratory chromatographic experiments.

Columns of DNP-S-Sephadex G-25 were prepared[28] and a series of experiments was made to explore the influence of temperature, salt, pH and the presence of organic solvents in the buffer. Tryptamine, tryptophan, serotonin, tyrosine and derivatives were used as model solutes.

The temperature effect in pure aqueous buffer is shown in Fig. 5. Adsorption increases as the temperature is decreased and as the salt concentration is increased, whereas ethylene glycol decreases the solute–gel interaction. Similar (due to salt and ethylene glycol) effects characterize hydrophobic interaction. Hydrophobic interaction can play only a minor role, if any, as the donor–acceptor relationships between the solutes and the immobilized ligands are clearly demonstrated in these and numerous other examples. Estimation of thermodynamic parameters from curves such as those of Fig. 5 give values in the expected range for π,π complexes (*e.g.*, $\Delta H =$ 5–50 kJ/mole).

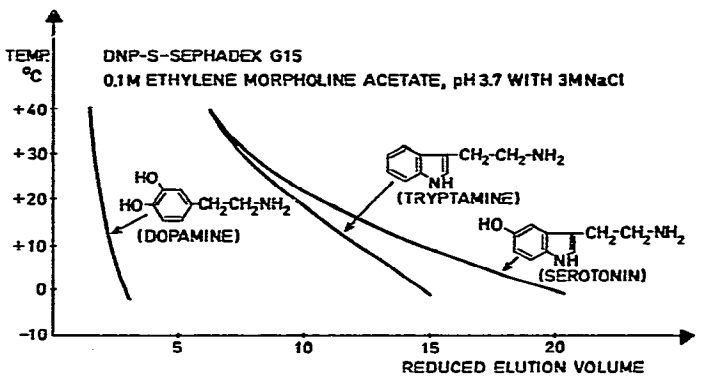


Fig. 5. Diagram showing how the adsorption of aromatics depends on the temperature. Separation is maximal at 0°.

Any doubt as to the type of interaction responsible for the adsorption is eliminated by experiments in which pyrogallol and tryptamine were introduced into a bed of DNP-S-Sephadex G-15 (with and without a high concentration of sodium chloride and other salts). The solutes moved on the column as separating red zones on the yellow background of the adsorbent. Upon leaving the column the solutes were recovered unchanged in colourless eluates. This is firm proof that the adsorption is due to the formation of a charge-transfer complex and not to hydrophobic interactions. The detection of the complex formation by the moving coloured zones facilitates the study of charge-transfer adsorption. It should be pointed out, however, that in most instances such coloured zones have not been observed, perhaps owing to the low concentrations of the solutes tested.

The adsorption isotherms are linear over a very wide range of concentration, which means that interacting substances move as discrete, compact, symmetrical

zones. The capacity is surprisingly high and seems to approach 1:1 molar relationships for the most strongly adsorbed species. About 1 g of pyrogallol can be adsorbed on a DNP-S-Sepharose 6B column with a bed volume of 50 ml (degree of substitution about 0.5 mmole of ligand per gram of dry gel). The acceptor gel can be used to concentrate and desalt solutions of aromatic compounds by taking advantage of the influence of ionic strength on the adsorption capacity.

In gel filtration we are limited to a V_E/V_T range* of about 0.4–0.9 whereas the corresponding range for gel chromatography on electron-donor and -acceptor adsorbents (EDA chromatography) is greater (about 1–15 V_T units). Problems will appear at the limits of this range. At and beyond the upper "limit" it might prove difficult to achieve displacement of the adsorbates. One possible solution to this problem is to select an adsorbent with less effective ligands. If instead certain desirable solutes are found to be eluted with insufficient retention, ligand substance should be reacted with the gel to give a more effective acceptor or donor with the highest possible degree of substitution. The chromatographic run should be performed at a temperature close to the freezing point, and it might be helpful to include a high concentration of salt in the buffer.

One of our objectives is to find suitable conditions for adsorption of proteins and peptides and our interest has therefore been concentrated on the amino acids that possess π-electron systems, namely, the aromatic amino acids phenylalanine, tyrosine and tryptophan, which should possess increasingly strong electron-releasing tendencies (see Fig. 1). In a set of experiments with a bed of particular DNP-S-Sephadex G-25 gel in ammonium formate at pH 3.2, tyrosine was only slightly retarded. The adsorption of tyrosyltyrosine was significantly stronger and trityrosine was adsorbed more strongly still (Table I). Evidently there is a strong cooperative adsorption effect which makes the method very promising. The incremental increase in V_E/V_T for tryptophan and tryptophyltryptophan is even more pronounced. It thus appears as if the cooperative adsorption effect opens up the field for polyaromatic polymers in general and peptides in particular. It may perhaps even be possible to synthesize charge-transfer adsorbents for proteins.

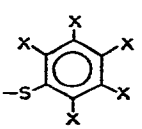
TABLE I

V_E/V_T VALUES FOR TYROSINE AND TYROSINE DERIVATIVES FROM CHROMATOGRAPHIC EXPERIMENTS ON DNP-S-SEPHAROSE 6B[28]

Test substance	V_E/V_T	*Test substance*	V_E/V_T
Tyr	1.11	3-Iodotyrosine	1.34
Tyr–Tyr	1.41	3,5-Diiodotyrosine	1.94
Tyr–Tyr–Tyr	2.08	3,5-Dinitrotyrosine	1.11

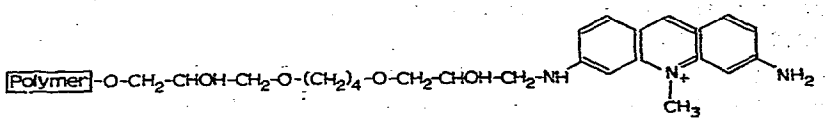
2.3.2. π-electron donor gels

An ideal donor ligand may have a structure such as



* V_E = Elution volume; V_T = total volume.

where X is a non-ionizable electron-releasing substituent such as CH_3 or Br. The necessary chemicals have not so far been available. Meanwhile, we have tried some other ligand substances that should be suitable for the synthesis of adsorbents for nucleic acids and their degradation products. For example, acriflavine has been chosen as it is both a donor and an acceptor (like acridine, see Fig. 1):



Dr. J.-M. Egly is presently studying the adsorption of nucleotides and mono- and oligonucleotides on such adsorbents based on Sephadex.

The adsorption of the nucleotides on the acriflavine gels displays a complicated pattern indicating ionic interaction and possibly hydrogen bonding in addition to charge transfer. The intense, dark brown colour of the gels makes detection of the complex formation difficult. However, Egly has found that at very high concentrations of adenosine monophosphate the gels turn darker. The influence of the phosphate group on the adsorption is surprisingly complex, as can be seen from Fig. 6, which demonstrates the variation of V_E/V_T.

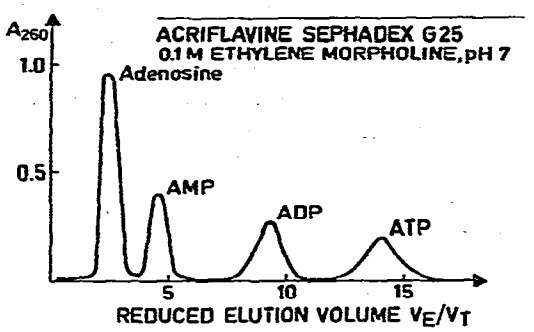


Fig. 6. Fractionation of adenosine and adenine ribonucleoside phosphates.

Even when ionic adsorption is depressed at high ionic strength there is still considerable retention of adenosine and cytosine monophosphates. Contrary to the situation at low ionic strength, nucleosides are more strongly adsorbed than are the

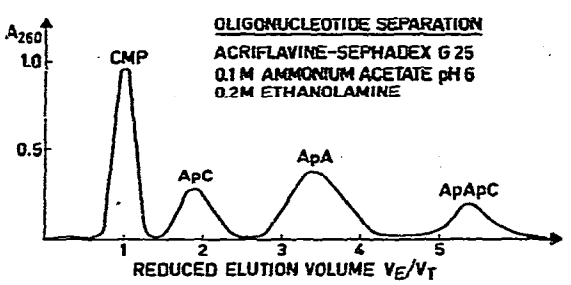
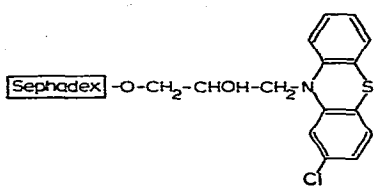


Fig. 7. Fractionation of some oligonucleotides on acriflavine-Sephadex under conditions where ionic adsorption ("ion exchange") is suppressed.

corresponding nucleotides. We ascribe the adsorption at high salt concentrations to charge-transfer dependent interaction (not necessarily caused only by π,π electron transfer). Preliminary results clearly show the cooperative interaction effects which make possible a separation of nucleotides according to size (Fig. 7).

We have reported recently[28] that phenothiazine derivatives are strongly adsorbed on DNP-Sepharose 6B. These compounds are thus very good π-electron donors. Chlorpromazine had a V_E/V_T of about 15 on a gel of much lower efficiency than those which have now been synthesized. A strong π-donor adsorbent should therefore be obtained by coupling phenothiazine to hydrophilic gels. Such a donor gel with the following structure has been synthesized:



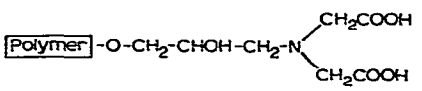
The coupling procedure has not yet been optimized, but the gels appear to be promising. They are only weakly coloured and show the expected affinities for nucleotides and other π-electron acceptor substances.

2.3.3. Metal chelate affinity gels

Hydrophilic gels substituted with chelating groups to which transition metal ions have been bound constitute a special type of charge-transfer adsorbent. The *d*-electron orbitals (or *d*-electron-containing hybridized orbitals) may overlap with the π-orbitals of aromatics or otherwise unsaturated solute species, including those which contain strong nucleophiles. In addition, n, π and n, σ complexes may be obtained.

Metal chelate adsorbents have been used in gas chromatography and in liquid chromatography[31–33], for instance, in the so-called ligand-exchange chromatography. The latter refers to an exchange of the ligands bound to the metal ion. The method that is discussed here is certainly related to but also differs from ligand-exchange chromatography in some respects. The metal ions can become so strongly fixed to the gel that they form permanent adsorption centres. Transition metal ions such as Cu^{2+}, Fe^{3+} and Zn^{2+} have excess affinity for thiol and amino groups and for phenolic hydroxyl-containing substances owing to the presence of weakly coordinated displaceable water, ammonia, counter ions, etc. These latter weakly bonded ligands can thus be exchanged for more strongly interacting nucleophiles or π-electron donors.

Most gels used so far contain biscarboxymethylamino groups ("half EDTA"), *e.g.*,



Preliminary studies on Cu^{2+} and Zn^{2+} gels have been published[34] and also an application to the isolation of lactoferrin from human milk[35]. In this paper some extensions of metal chelate affinity chromatography are mentioned.

The Cu^{2+} gels have a rather broad affinity, as demonstrated by the adsorption of all amino acids in the appropriate pH range. Zinc–chelate columns can be used to adsorb histidine and cysteine preferentially from protein hydrolyzates. Non-specific interaction can be suppressed by including a salt at a high concentration in the buffer.

The zinc–chelate gels show a weak affinity for some other amino acids, *e.g.*, tryptophan and tyrosine. Would it be possible to prepare an adsorbent especially suited for tyrosine and tyrosine-containing peptides?

Iron(III) ions are known to form complexes with phenolic compounds. We therefore studied the behaviour of tyrosine and some peptides on Fe^{3+}–chelate gels. Figs. 8 and 9 show diagrammatically the compilation of some chromatographic data obtained with iminodiacetic acid-substituted Sephadex G-25 charged with iron(III) ions. The order of increased adsorption is Tyr < Tyr–Tyr < Tyr–Tyr–Tyr, and the enkephalin analogues also separate according to their number of tyrosine residues.

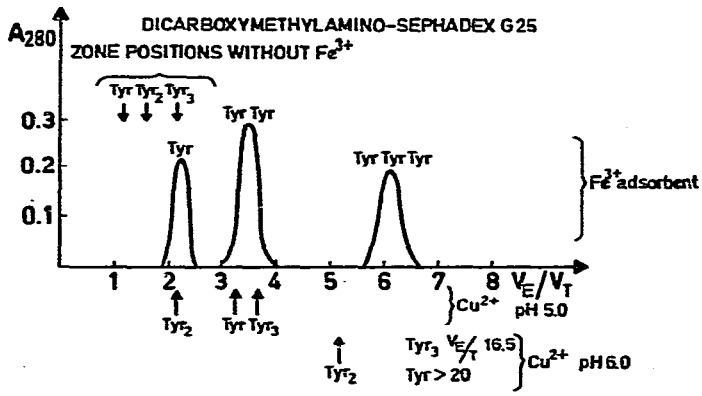


Fig. 8. Schematic diagram indicating the position in terms of V_E/V_T units of tyrosine and di- and trityrosines on Fe^{3+} and Cu^{2+} (lower part) loaded dicarboxymethylamino-Sephadex G-25. All chromatograms were run on the same column with and without the metal ion indicated. The chromatographic experiments using the Fe^{3+} adsorbent and the metal-free gel were run in 0.1 *M* ammonium acetate, pH 5.0.

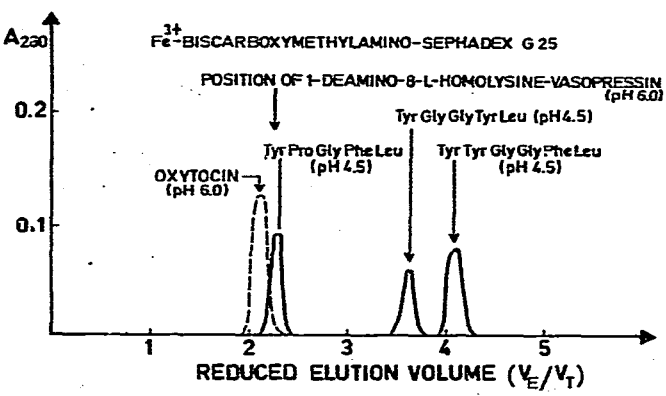
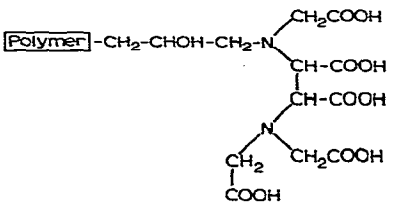


Fig. 9. Elution positions of some tyrosine-containing peptides on Fe^{3+}–chelate Sephadex G-25 in 0.1 *M* ammonium acetate, pH 5.0. Peptides containing a single tyrosine residue move faster than those containing two tyrosine residues per molecule.

Both the position and the number of tyrosyl residues in the peptides apparently affect the strength of adsorption.

As mentioned, in order to be particularly suitable as adsorbents, the chelated metal ions should be bound as strongly as possible to the matrix. In view of the fact that the association constant for the EDTA–Fe^{3+} complex is of the order of 10^{24}, whereas it is only about 10^{12} for iminodiacetic acid, one might expect that the binding of Fe^{3+} to the gel would not be strong enough. However, it has been proved experimentally that the gel matrix alters the chelating power considerably and it is also clear that adsorption of the metal ion cannot be described in terms of a well defined association constant. Instead, the strength of the metal binding depends on the ligand density, which seems to vary over the gel matrix. Presumably clusters of ligands are present in some regions while others are almost empty of iron(III) ions, because Fe^{3+} is not displaced even by strong EDTA solutions.

To increase the capacity further and improve the carboxymethylated amino gels, Dr. Viyajalakshmi has recently prepared very strong chelate adsorbents based on both crosslinked pectin and Sephadex. They contain carboxymethylated α,β-diaminosuccinic acid ligands:



These substituents should easily form strong complexes with ions capable of exhibiting high coordination numbers. The gels can still adsorb amino acids and other metal ion-binding solute species and have the advantage that they can be treated with chelating agents under drastic conditions to displace very strongly bound adsorbates.

3. CONCLUSIONS

Charge-transfer adsorption on hydrophilic gels seems to offer techniques for the isolation and characterization of many classes of synthetic and naturally occurring substances according to their aromaticity, heterocyclic character and nucleophilicity or electron-releasing power. The field has hardly been touched and its limits are unknown.

Chromatography on charge-transfer adsorbents seems to provide a new, sensitive technique for detection and for studies of molecular complex formation. It would be desirable to formulate theories for the quantitative interpretation of the charge-transfer adsorption in terms of thermodynamics and quantum chemistry.

4. SUMMARY

Theoretical conditions for charge-transfer adsorption are discussed briefly. The adsorbents are synthesized by covalently attaching electron donors or acceptors to suitable matrices such as agarose and Sephadex. Charge-transfer chromatography can be used for fractionation of aromatic substances or heterocyclic compounds such as nucleotides. Metal chelate gels are also described. These are selective adsorbents for peptides and proteins.

REFERENCES

1 P. Pfeiffer, *Organische Molekülverbindungen*, F. Enke, Stuttgart, 2nd ed., 1937.
2 A. Szent-Györgyi, *Introduction to Submolecular Biology*, Academic Press, New York, 1960.
3 B. Pullman and A. Pullman, *Rev. Mod. Phys.*, 32 (1960) 428.
4 S. P. McGlynn, *Radiat. Res.*, Suppl., 2 (1960) 300.
5 G. Briegleb, *Elektronen-Donator–Acceptor Komplexes*, Springer Verlag, Berlin, 1961.
6 R. S. Mulliken and W. B. Person, *Molecular Complexes, A Lecture and Reprint Volume*, Wiley-Interscience, New York, 1969.
7 M. A. Slifkin, *Charge Transfer Interactions of Biomolecules*, Academic Press, London, 1971.
8 J. Porath and P. Flodin, *Nature (London)* 183 (1959) 1657.
9 S. Hjertén, *Biochim. Biophys. Acta*, 79 (1964) 393.
10 J. Porath, J.-C. Janson and T. Låås, *J. Chromatogr.*, 60 (1975) 167.
11 J. Porath, T. Låås and J.-C. Janson, *J. Chromatogr.*, 103 (1975) 49.
12 R. Axén, J. Porath and S. Ernback, *Nature (London)*, 214 (1967) 1302.
13 J. Porath, *Methods Enzymol.*, 34B (1974) 13.
14 L. Sundberg and J. Porath, *J. Chromatogr.*, 90 (1974) 87.
15 J. Porath and R. Axén, *Methods Enzymol.*, 44 (1976) 19.
16 J. Porath, *Biochim. Biophys. Acta*, 39 (1960) 193.
17 B. Gelotte, *J. Chromatogr.*, 3 (1960) 330.
18 D. Eaker and J. Porath, *Separ. Sci.*, 2 (1967) 507.
19 L. J. Morris, *Chem. Ind. (London)*, (1962) 1238.
20 C. B. Barret, M. S. J. Dallas and F. B. Padley, *Chem. Ind. (London)*, (1962) 1050.
21 R. G. Harvey and M. Halonen, *J. Chromatogr.*, 25 (1968) 294.
22 A. Berg and J. Lam, *J. Chromatogr.*, 16 (1964) 157.
23 G. H. Schenk, G. L. Sullivan and P. A. Fryer, *J. Chromatogr.*, 89 (1974) 49.
24 B. L. Karger, M. Jartin, K. Loheac and G. Guichon, *Anal. Chem.*, 45 (1973) 496.
25 F. F. Yew and B. J. Mair, *Anal. Chem.*, 38 (1966) 231.
26 R. J. Laub and R. L. Pecsok, *J. Chromatogr.*, 113 (1975) 47.
27 D. M. Jewell, *Anal. Chem.*, 47 (1975) 2048.
28 J. Porath and K. Dahlgren Caldwell, *J. Chromatogr.*, 133 (1977) 180.
29 K. Tamary and M. Ishikawa, *Catalysis by Electron Donor–Acceptor Complexes*, Kodanska Ltd. and Wiley, New York, 1975, p. 25.
30 J. Brandt, L. O. Andersson and J. Porath, *Biochim. Biophys. Acta*, 386 (1975) 196.
31 F. Helfferich, *J. Amer. Chem. Soc.*, 84 (1962) 3242.
32 J. D. Navratil and H. F. Walton, *Anal. Chem.*, 47 (1975) 2443.
33 O. K. Guha and J. Janák, *J. Chromatogr.*, 68 (1972) 325.
34 J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature (London)*, 258 (1975) 598.
35 B. Lönnerdal, J. Carlsson and J. Porath, *FEBS Lett.*, 75 (1977) 89.